1 **Controlled Human Infection Models to Accelerate Vaccine Development**

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Summary

The timelines for developing vaccines against infectious diseases are lengthy, and often vaccines that reach the stage of large Phase 3 field trials fail to provide the desired level of protective efficacy. The application of controlled human challenge models of infection and disease at the appropriate stages of development could accelerate development of candidate vaccines, and in fact has done so successfully in some limited cases. Human challenge models could potentially be used to gather critical information on pathogenesis; inform strain selection for vaccines; explore cross-protective immunity; identify immune correlates of protection and mechanisms of protection induced by infection or evoked by candidate vaccines; guide decisions on appropriate trial endpoints; and evaluate vaccine efficacy.

We prepared this report to motivate fellow scientists to exploit the potential capacity of controlled human challenge experiments to advance vaccine development. In this review, we considered available challenge models for 17 infectious diseases in the context of the public health importance of each disease, the diversity and pathogenesis of the causative organisms, the vaccine candidates under development, and each model's capacity to evaluate them and identify correlates of protective immunity. Our broad assessment indicated that human challenge models have not yet reached their full potential to support the development of vaccines against infectious diseases. On the basis of our review, however, we believe that describing an ideal challenge model is possible, as is further developing existing and future challenge models.

Introduction

The physician-scientist Claude Bernard brought the discipline of experimental medicine to life in the mid-nineteenth century. With his series of groundbreaking discoveries in the field of human physiology, the idea that medicine in the service of human health should be firmly grounded in scientific knowledge gained through experimentation took hold and flourished. The iterative nature of experimental medicine was also a key tenet of Bernard's teaching. He viewed a scientific theory as the first important step away from the "groping and empiricism" that he eschewed, but he also emphasized that each scientific theory must be further tested, and either accepted or discarded as a result of new data (1).

Physicians and scientists early in the twenty-first century still struggle with the role of experimental medicine in the development of vaccines. The history of vaccine development is largely one of the "groping and empiricism" that Bernard sought to overcome. Edward Jenner developed the smallpox vaccine based on observation (the protected milkmaids), confirmed by experimental medicine in human volunteers (vaccination of children in the community). He had no knowledge of the underlying mechanisms of protective immunity. He did not recommend mass vaccination against smallpox based on a p-value from a Phase 3 clinical trial, but rather on his own careful observations. Nevertheless, the smallpox vaccine that Jenner first developed remains the sole example of the eradication of a disease through vaccination. The polio vaccines developed by Jonas Salk and Albert Sabin and the measles vaccine developed by Maurice Hilleman may also achieve eradications, but these goals have proved more elusive than the world first expected. For many, if not most, of the vaccines in use today, a clear

83 understanding of the mechanism(s) of protection has not been available to guide new vaccine
84 development or improve on vaccine availability.

85 Today, vaccines are lifesaving tools that underpin improved global health, yet morbidity and mortality
86 from infectious diseases is still unacceptably high. The reasons for this are many. First, information is
87 incomplete on the fraction of global morbidity and mortality attributable to many of the key pathogens
88 that are potential, or actual, targets of vaccine development, which makes prioritizing overall vaccine
89 development efforts difficult. Second, vaccine development is still largely an empirical process, usually
90 conducted without the guidance of a correlate of protective immunity with which to optimize a vaccine.
91 Vaccines are most often optimized for the strongest measurable immune responses, without fore
92 knowledge of whether a given response or combination of responses will afford protection. The
93 probability of a vaccine development program's success is therefore difficult to predict prior to the
94 conduct of large, costly, and time-consuming field efficacy trials. Third, even when vaccines exist that
95 can prevent infectious diseases, multiple factors can prevent realization of their full impact, including
96 barriers to access, such as high costs; constraints on supply chain and distribution, such as delivery
97 difficulty to remote areas and limited cold chain capacity; and chronic conditions that diminish
98 immunogenicity, such as immunodeficiency syndromes and environmental enteric dysfunction. Fourth, a
99 legitimate difference of opinion exists on how to make the preclinical and early clinical phases of vaccine
100 development more grounded in scientific data. To date, the scientific community has heavily relied on *in*
101 *vitro* and animal model data to guide vaccine development, following a long tradition in the development
102 of new medicines and vaccines. Indeed, Claude Bernard enjoyed the success that he did largely because
103 the aspects of physiology that he studied were sufficiently conserved between animals and humans to
104 permit direct extrapolation. The protective immunity provided by vaccines often seems to be the
105 exception to the rule. Even the best animal models of disease may poorly predict protective immune
106 responses.

107 "Clinical data trumps all" is a common saying among medical researchers when they become frustrated
108 by the limitations of translating results obtained from animal experiments that are not reproduced when
109 tested in humans. Another phrase heard over and over is that "mice lie and monkeys exaggerate". Hence
110 the continuous stride to learn directly from humans what we need to know for the advancement of
111 medicine or understanding of human physiology. However, direct research in humans is plagued by
112 multiple challenges, including the obvious ethical concerns of subjecting humans to tests that may
113 endanger their health. Additional challenges to address include the enormous genetic, environmental,
114 nutritional, gender, age-related, and other variables among potential research subjects. Some of the
115 mantras in experimental research are the use of a control group and the isolation of the variable studied,
116 so that the results can be interpreted with the least amount of noise. This is usually feasible in animal
117 models, but often impossible to achieve in humans. Indeed, even once robust efficacy data is obtained
118 from a clinical study, results from real-world implementation sometimes fail to match those from
119 carefully controlled trials. To mitigate these risks, the use of a parallel, concurrent control group and the
120 approach to blinding investigators and subjects to avoid bias is mandatory. Challenging humans with
121 pathogenic microorganisms to test new prevention or treatment modalities is a promising approach, as
122 long as the model resembles the disease, the selected participants are as uniform as possible, the control
123 group is appropriate, and the sample size sufficient to satisfy the hypothesis and other caveats. More often
124 than not the human challenge field has to be satisfied with generating data progressively to address
125 uncertain findings in the initial attempts to develop the model and expect to accumulate information in a

stepwise fashion. With that caveat, human challenge models may indeed be the most promising approach. We acknowledge that while no model is perfect, each can provide unique insights that are otherwise unattainable.

The limitations of animal models for supporting vaccine development have long been recognized. For the last 70 years, human challenge studies with many important pathogens have been conducted to gain more relevant data on pathogenesis, immunity, and the protective efficacy of candidate vaccines. The exact role of human challenge studies in vaccine development, however, is the subject of ongoing debate.

Some of the human challenge models in current use face intrinsic limitations with respect to the selection and availability of challenge strains, their routes of administration, and the capacity to evaluate the full spectrum of clinical disease. Human challenge models may achieve greater impact in their support of vaccine development by increasing focus on those models with the fewest intrinsic limitations and a robust candidate vaccine pipeline. Looking across the many challenge models, we recognize common obstacles to be overcome. Coordination of experimental human challenge studies that support vaccine development could accelerate progress, foster collaboration and knowledge sharing, and encourage study consolidation to address problems that are common to broad categories of diseases. Such an approach could also provide broader access to the most advanced technologies that have the potential to accelerate candidate antigen selection, improve the evaluation of immune responses generated by candidate vaccines, and enable the discovery of new mechanisms of resistance to infection.

The goal of this report is to describe the background, context, and present experience with human challenge studies, along with a critical analysis of their role and limitations in support of vaccine development. The framework in which human challenge studies are being conducted is also examined, with the hope of building a comprehensive case that the expanded use of human challenge studies could increase the speed of vaccine development and probability of vaccine success. At the conclusion of this report, we will return to this proposition and provide summary recommendations from the research conducted to date.

Approach

We focused our analysis on 17 diseases. The diseases reviewed include the vector-borne diseases malaria and dengue; the enteric diseases cholera, enterotoxigenic *Escherichia coli*, *Shigella*, *Campylobacter*, typhoid fevers (*Salmonella*), norovirus, *Cryptosporidium*, rotavirus, and poliovirus; and the respiratory diseases influenza, respiratory syncytial virus, pneumococcus, tuberculosis, pertussis (whooping cough), and severe acute respiratory syndrome coronavirus 2 (the virus responsible for COVID-19). These diseases were selected to cover a range of levels of development of their corresponding human challenge models, from well-developed (malaria, cholera, influenza, etc.) to speculative (COVID-19). The diseases were also selected on the basis of those for which the models have a strong potential for impact on vaccine development.

In most cases, the challenge models involve fully virulent wild-type pathogens. However, in a few cases in which this is neither ethical nor practical, “pseudo-challenge” studies with live-attenuated organisms such as vaccine strains have been used as alternatives. These include dengue, rotavirus, poliovirus, and

164 influenza. Challenges with attenuated dengue and influenza viruses are addressed in their respective
165 sections of vector-borne and respiratory diseases, whereas rotavirus and poliovirus are covered together in
166 a dedicated pseudo-challenge section under enteric diseases.

167 The literature searches on each of the diseases were conducted mostly through PubMed
168 (https://pubmed.ncbi.nlm.nih.gov/) at the National Center for Biotechnology Information, US National
169 Library of Medicine, US National Institutes of Health; and through ClinicalTrials.gov. Some of the
170 information available on the websites of the World Health Organization, the US Centers for Disease
171 Control and Prevention, and other online sources was also included.

172 For each disease, the following four topics were researched: (1) epidemiology and public health impact of
173 the disease, (2) diversity and pathogenesis of the causative organisms, (3) current vaccine development,
174 and (4) human challenge models of the disease and their utilization.

175 The review for each disease area includes figures and tables. Most of the tables in this report represent
176 compilations of available sources. The reference list contains original articles or other sources from which
177 the information that is presented was compiled. Many recent review articles were used for this report, as
178 well as current articles on specific studies or topics that were deemed important to include. Google
179 searches and ClinicalTrials.gov were used to capture unpublished or ongoing studies. It was not feasible
180 in the time available to comprehensively review the literature for any of the diseases included in this
181 report. For each disease area, we include a bibliography of the articles and other sources used to direct the
182 reader to more detailed information for further reading.

183 This report also includes a section that provides a comprehensive review of the regulatory and ethical
184 considerations related to human challenge studies. The section describes the different types of human
185 challenge studies and compares the regulatory requirements for each type of study in the United States
186 with those in the United Kingdom. The regulatory agencies and advisory groups with jurisdiction over
187 human challenge studies in the United States and the United Kingdom are also described in this section.
188 The ethics section includes considerations for studies conducted with residents of endemic regions.

189 The final section of this report presents our conclusions from the review and analysis we conducted, along
190 with summary recommendations. We also offer specific guidance on the future development and
191 utilization of each of the human challenge models reviewed.

192

193 # Literature review on the role of human challenge models in
194 # the development of vaccines

195 ## Vector-borne diseases

196 ## Malaria

197 More than 3 billion people live in malaria-endemic areas of the world. Despite significant advances in
198 vector control and treatment, there were an estimated 229 million malaria cases and 409,000 deaths
199 attributable to malaria in 87 malaria endemic countries in 2019 with 95 percent of the malaria cases
200 concentrated in 29 countries, most of which are in sub-Saharan Africa (2).

201 The human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent and
202 clinically significant of the human malaria parasites. *Plasmodium ovale*, *Plasmodium malariae*, and
203 *Plasmodium knowlesi* cause a smaller fraction of infections worldwide.

204 ### The malaria parasite life cycle

205 The life cycle of *P. falciparum* is shown in Figure 1, which also indicates the parasite life cycle
206 developmental stages at which interventions that include vaccines, small-molecule therapeutics, and
207 biologicals are applied in human challenge models.

208 **Figure 1. *Plasmodium* life cycle and malaria challenge platforms.**

209 Briefly, when female malaria-infected *Anopheles* mosquitoes seeking a blood meal bite the skin of a
210 human, approximately 15 to 100 infectious-stage sporozoites are injected into the skin. The sporozoites
211 migrate to the liver either through the lymphatics or direct blood circulation. Sporozoites traverse the cell
212 membranes of endothelial cells, Kupffer cells, and even several liver hepatocytes before invading into a
213 single hepatocyte to develop into exo-erythrocytic-stage parasites. This stage of the life cycle is known as
214 the pre-erythrocytic stage, the primary target of vaccines that target the sporozoite and developing liver-
215 stage parasite. Sporozoites develop within hepatocytes, where they multiply many thousand-fold, then
216 enter blood circulation and invade human erythrocytes. The majority of blood-stage parasites replicate
217 asexually in red blood cells, with cycles of amplification every 48 to 72 hours depending on the malaria
218 species. The blood stage of infection is the major cause of malaria symptoms, morbidity, and mortality. A
219 small proportion of blood-stage parasites develop into male and female gametocytes, the sexual stage of
220 the parasite's life cycle. The gametocytes are the only forms that can re-infect *Anopheles* mosquitoes
221 when the mosquito bites an infected host to obtain a blood meal. In the mosquito, gametocytes form a
222 zygote, traverse the mosquito's midgut, and develop into oocyst stages from which infective sporozoites
223 are formed. Sporozoites find their way to the mosquito's salivary glands, where they can initiate a new
224 infection upon feeding on a susceptible host.

225 *P. vivax* differs from *P. falciparum* in important features. Following invasion into hepatocytes, some
226 sporozoites enter a dormant period of quiescence (termed hypnozoite-stage parasites), which may last
227 from several weeks to more than a year. At defined intervals ranging from a few weeks to a year,

228 hypnozoite-stage parasites may resume development within the hepatocyte, multiply, and emerge into the
229 blood stream to initiate repeated cycles of blood-stage infection. Additionally, *P. vivax* gametocytes
230 emerge and develop simultaneously with the asexual-stage parasites and are susceptible to antimalarial
231 drugs used to treat asexual blood-stage parasites.

232 **Controlled human malaria infection models**

233 Human challenge models have been developed for all three phases of parasite development: the pre-
234 erythrocytic stage, the blood stage, and the transmission stage. Reviews of challenge models and their
235 citations have recently been published (3–6). The sections that follow describe in detail how these
236 challenge platforms have been developed for *P. falciparum*, *P. vivax*, and *P. malariae*, and we describe
237 how the challenge platforms are used to study the natural history of disease, how they contribute to
238 vaccine development and inform our knowledge of immunologic correlates of protection, and how such
239 challenge models are currently being used in drug development of new antimalarials. Importantly, we
240 comment on the appropriate strengths and limitations of human challenge models in malaria research and
241 development.

242 The history of challenging humans with malaria parasites is instructive on how society, medicine, and
243 product development have evolved over the last 100 years with respect to the ethical conduct of such
244 trials, the quality management systems of the challenge platforms, the reproducibility of the challenge
245 methodology, the worldwide and country-specific regulatory environment, and the knowledge gained
246 from such studies. Judging the present by the standards of the past should cause one to pause and consider
247 the risk-benefit analysis of utilizing human malaria challenge models to achieve particular outcomes,
248 notably accelerating vaccine and drug development in a safe and reproducible manner.

249 Human malaria challenge was extensively used in the early 1900s as a mechanism to treat the ravages of
250 neurosyphilis, for which no alternative therapeutic options were available. An early publication described
251 how malaria sporozoites from infected anopheline mosquitoes were used to initiate infection in patients
252 suffering from neurosyphilis (7), and provides the contextual background, despite its limitations in
253 methodology and deficiencies in obtaining informed consent, for the adaptation and development of the
254 safe and reproducible challenge models utilized presently. The knowledge gained from human malaria
255 challenge studies has increased our understanding of the pathogenesis of the disease and the successes
256 and failures related to product development.

257 **Parasite and mosquito vectors in human malaria challenge models**

258 In the modern era, the first demonstration that protection against malaria challenge in a volunteer
259 previously immunized with a live-attenuated parasite took place at the University of Maryland, where
260 human volunteers were challenged with the bite of infected mosquitoes that had previously fed on
261 gametocytemic *P. falciparum*-infected persons (8). Despite the success of this proof-of-concept trial, it
262 soon became apparent that it was not feasible to rely solely on naturally infected gametocyte-positive
263 human subjects to serve as the source for a mosquito blood meal in order to produce viable infectious
264 sporozoites used to challenge volunteers with the bites of infected *Anopheles* mosquitoes. The
265 development of a *P. falciparum* (NF54 strain) asexual- and sexual-stage continuous culture system
266 produced limitless quantities of gametocyte-stage parasites that were placed in membrane feeders where
267 batches of either *Anopheles stephensi* or *Anopheles freeborni* mosquitoes could feed on a mixture of

268 *P. falciparum* and fresh normal blood. After seven to ten days, the mosquito infection rate with oocysts
269 exceeded 90 percent and salivary gland sporozoites were detected 14 to 17 days post-feeding (9).
270 Volunteers either previously immunized with an experimental vaccine candidate and infectivity controls
271 that did not receive any experimental vaccine were challenged by the bites of five infected mosquitoes,
272 which were permitted to bite and obtain a blood meal for five minutes (9). Volunteers were hospitalized
273 or otherwise congregated together in a hotel and closely monitored daily for the emergence of ring-stage
274 asexual parasites and symptoms. The NF54 strain (and the 3D7 parasite clone of NF54) is sensitive to
275 multiple antimalarials, including chloroquine, atovaquone/proguanil, and artemether/lumefantrine, thus
276 ensuring that infections can reliably be cleared in volunteers. To date, three additional *P. falciparum*
277 parasite strains have been maintained in continuous culture, producing infectious gametocytes to
278 *An. stephensi* mosquitoes used in human challenge studies (Figure 2) (10–12) that differ in genome
279 structure, sequence, and immunogenic potential (13).

280 **Figure 2. Parasite strains and mosquito vectors commonly used in challenge models.**

281 Compared to *P. falciparum* parasites, *P. vivax* parasites cannot be continuously cultured *in vitro* to
282 produce infectious gametocytes; therefore, the challenge model is limited to using naturally acquired
283 parasites obtained from donor volunteers that present to a health treatment facility with clinical vivax
284 malaria infection (14–17). Small aliquots of blood (approximately 10 ml) obtained from individuals with
285 *P. vivax* parasitemia must immediately be transferred to membrane feeders where *Anopheles dirus* or
286 *Anopheles albimanus* mosquitoes are permitted to feed (Figure 2).

287 A third *Plasmodium* species, *P. malariae*, obtained from a naturally infected patient, has been used to
288 develop a controlled human malaria challenge model using small aliquots of asexual blood-stage parasites
289 to infect healthy volunteers (18).

290 A well-developed quality management system is invaluable with regard to the rigorous screening and
291 testing procedures on all aspects of the challenge model. This includes testing for ABO/Rh blood group
292 and screening the infected human donor blood from whom the parasite is isolated for transmissible
293 agents, including HIV, hepatitis B and C viruses, human T-cell leukemia virus, syphilis, and other
294 pathogens, depending on the specific requirements of the regulatory agencies. The insectary and mosquito
295 colony are critical aspects to ensure a safe and reproducible quality management system.

296 A convening of experts in both pre-erythrocytic- and blood-stage challenge models was held in 2009 in
297 which general principles were proposed regarding the standardization of the challenge models across
298 centers (19, 20). In addition to procedures to safely protect volunteers from harm, increasingly the
299 application of such challenge models includes both ethical considerations (21, 22) and regulatory
300 oversight (23), which differ depending on the country.

301 **Pre-erythrocytic-stage challenge model development**

302 Presently, there are two distinct types of controlled human malaria infection (CHMI) that initiate malaria
303 infection in the host. First, sporozoite infection is initiated by the bites of volunteers by *Plasmodium*-
304 infected female *Anopheles* mosquitoes that release sporozoites from the salivary gland while the
305 mosquitoes feed for a blood meal on the skin of the volunteers. We commonly refer to this model as the
306 mosquito bite challenge model. The second method, recently developed, is direct venous inoculation of

307 cryopreserved sporozoites into the host by needle and syringe. Figure 3 illustrates the two challenge
308 methods that use infectious sporozoites to initiate infection.

309 **Figure 3. Pre-erythrocytic human challenge models using mosquito bite delivery or direct venous**
310 **inoculation of infectious sporozoites.**

311 We highlight four factors that impact the operational feasibility and interpretation of the outcomes from
312 each of these two delivery modalities. Any challenge model, independent of pathogen under
313 consideration, should be standardized to ensure the methodology results in uniform infectivity of
314 volunteers that are challenged. The product, defined as the *Plasmodium* species and/or parasite strain, is
315 generally produced under Good Manufacturing Practice (GMP), since different strains have varying
316 susceptibility to different antimalarial medications to clear blood-stage parasites and differences in
317 potency that affect the pre-patent period (time to detection of blood-stage parasitemia). Understanding the
318 "force of infection," namely the minimal inoculum required to initiate infection in "control" (no
319 intervention) subjects, is crucial in weighing whether to continue or abandon an intervention such as a
320 vaccine candidate or new antimalarial compound.

321 Regardless of the particular challenge model, such malaria challenge studies are not statistically powered
322 to detect true differences between those that are protected and those that are not, due to the logistical
323 constraints on the number of subjects that can safely be followed during these Phase 2 challenge trials.
324 Typically, the number of human subjects per group has varied from as few as five to ten persons to
325 approximately 30. These numbers are modest, but can provide a measure of confidence that the outcome
326 of the challenge (presence or absence of blood-stage parasitemia) provides Go/No-Go criteria for further
327 testing in the field under natural transmission. In the sections that follow, we highlight the performance
328 characteristics of each challenge model and provide an exhaustive list of the types of malaria vaccine
329 candidates that have been tested under each model. We also provide a perspective that includes the
330 advantages and limitations of each challenge model.

331 **Mosquito bite challenge model**

332 In 1986, Chulay and colleagues reported on how the bites of five *An. stephensi* mosquitoes infected with
333 the NF54 strain of *P. falciparum* could successfully infect adult volunteers using a methodology in which
334 the emergence of parasites into the peripheral blood circulation was closely monitored by detecting ring-
335 stage parasites on thick blood smears (9). This first practical challenge model has been the workhorse
336 over the last 34 years, and has been successfully used to rapidly and safely evaluate vaccine efficacy for
337 pre-erythrocytic vaccine candidates and increasingly to evaluate the new antimalarial therapeutics.

338 The salient features of the *P. falciparum* sporozoite challenge model include the continuous use of the
339 model for nearly 30 years to support malaria vaccine and drug development, in which more than 2000
340 human volunteers have participated in challenge studies. Human challenge studies have been performed
341 at multiple sites in the United States, Europe, Australia, South America, and Africa. Importantly, World
342 Health Organization consensus guidelines for conducting challenge studies have been established to
343 harmonize across sites (19).

344 Briefly, frozen blood-stage parasites produced under GMP are thawed and expanded in human
345 erythrocytes in culture. Laboratory-reared *An. stephensi* mosquitoes are infected by feeding through a thin
346 membrane on cultures containing gametocytes. The presence of sporozoites in the mosquitoes is

347 confirmed two weeks later by dissecting salivary glands, and typically five infected mosquitoes are placed
348 in a mesh-covered carton. Volunteers place their arm over the carton and allow mosquitoes to feed for
349 five minutes. After the feeding opportunity, the researchers verify that the mosquitoes have ingested
350 blood and contain sporozoites.

351 Volunteer subjects are healthy individuals 18 to 50 years of age selected from the community. The studies
352 are conducted under an institutional review board-approved protocol and informed consent is obtained.
353 Starting five days after challenge, the volunteers are closely followed for signs and symptoms of malaria
354 and evaluated using thick blood smears. Blood smears permit the counting of parasites under a
355 microscope. During days 9 through 19, when malaria parasites are expected in blood, volunteers are
356 housed at a single hotel with clinical and laboratory staff continuously available. As soon as two or more
357 parasites are detected in thick blood smears, treatment with a standard antimalarial drug is initiated. Blood
358 smears are obtained daily following antimalarial treatment until three consecutive negative smears
359 indicate it is safe to release the volunteer from the study. Volunteers that do not develop malaria by day
360 28 (well beyond the pre-patency period of seven to 25 days for malaria infection) do not receive malaria
361 treatment and are considered "protected."

362 A critical component of any challenge model is the force of infection of the inoculum that can achieve a
363 successful infection in the host. Too low of an inoculum will result in too few infected individuals in a
364 "control" group that do not receive a vaccine, making efficacy comparisons to the experimental vaccine
365 group difficult to interpret. Too high an infectious inoculum will overcome any vaccine-induced
366 protection.

367 It is therefore imperative that the force of infection is sufficiently potent to ensure that every individual in
368 the control group becomes infected after challenge. This requirement poses a risk to the interpretation of
369 efficacy outcomes because of the force of infection (number of infectious mosquito bites that may
370 overcome vaccine-induced immunity). If the challenge model is insufficiently reproducible (between
371 subjects within a trial or variation between trials), the variability can undermine the interpretation of
372 endpoints, resulting in wasted effort, expense, and premature termination of otherwise promising vaccine
373 candidates. We provide an example that illustrates this point. The authors of a vaccine trial rationalized
374 the use of fewer infectious mosquito bites to try to mimic natural infection (24). The authors noted that
375 "because of the concern that previous experimental challenges conducted with the bites of five infected
376 mosquitoes were unrealistically severe and may have overwhelmed any vaccine-induced immunity, the
377 first four vaccinated volunteers and three nonimmunized control volunteers were challenged with two
378 infected mosquito bites each. The failure of two of three control volunteers to develop patent malaria
379 infections led to a decision to challenge the two remaining groups of volunteers with five infected
380 mosquito bites" (24).

381 The force of infection that ensures uniformity of infection also depends on the *Plasmodium* species and
382 strain. The NF54 strain of *P. falciparum* and a cloned derivative of NF54, called 3D7 and produced under
383 GMP, have been used in most mosquito bite challenge models. Graded numbers of NF54-infected
384 mosquito bites (one to five) using aseptic-produced infected mosquitoes or different *P. falciparum* strains
385 have been used in CHMI, but it is not universally accepted that a lower number of infectious mosquito
386 bites (one to three) provides any advantage over the five-bite model. *P. vivax* challenge models using
387 different numbers of infectious mosquito bites have been developed. Described in a following section in

this chapter, the models rely exclusively on natural infection, and thus such natural strains of *P. vivax* used in challenges are likely to differ with respect to potency and infectiousness. Therefore, whether fewer than five infected bites can consistently result in uniform infection (detection of blood-stage parasitemia) in a subject remains to be confirmed.

Challenging a human volunteer with *P. falciparum* malaria parasites has intrinsic risks. Malaria can be a life-threatening illness and every challenge has the potential to produce serious adverse events (SAEs). Through the course of more than 1,000 malaria challenges at US Army and US Navy sites, no SAEs or cases of severe malaria have been reported; however, some variation in the conduct of human challenge studies with malaria across challenge centers where such studies are performed does occur. There are no symptoms associated with the liver stage of infection. However, shortly after liver-stage merozoites emerge into the peripheral blood circulation, the accumulation of asexual parasites reaches a threshold whereupon symptoms associated with classical malaria disease, such as fever, myalgias, headaches, nausea, and vomiting, and laboratory abnormalities such as mild thrombocytopenia, emerge (25, 26). As challenge models have been refined over the past two decades with broader international participation, there is not a consensus among the experts on whether or not volunteers participating under informed consent need to be treated immediately upon detection of any parasites in the peripheral circulation in order to protect them from any adverse event. There is a cushion window of several days coincident with a few replication cycles of parasites before treatment is initiated that would allow for scientific inquiry related to whether an intervention would prevent the emergence of clinical symptomatology associated with uncomplicated malaria in addition to the typical endpoints that measure prevention of infection. Untreated subjects will progress to severe disease; and if left untreated, this may result in death. Therefore, it is required that any malaria challenge use parasites (*P. falciparum* or *P. vivax* strains) that are susceptible to antimalarial medications. Detecting the presence of asexual blood-stage parasites early in infection is dependent upon highly sensitive and specific diagnostic assays. The preparation and interpretation of thick and thin blood smears by microscopy is the "gold standard" diagnostic assay. Until recently, CHMI studies have relied exclusively on detecting ring-stage parasites on a thick blood smear. The minimal level of detection by microscopy is ten to 50 parasites per microliter of blood, which is significantly lower than antigen rapid detection tests. However, the recent development of the molecular detection of parasites using polymerase chain reaction (PCR) has emerged and has become the go-to standard operating procedure at several CHMI test centers (27–29). The US Food and Drug Administration has qualified one such molecular PCR assay, "*Plasmodium falciparum* 18S rRNA/rDNA (copies/ml) measured in blood samples by a nucleic acid amplification test assay" for "a monitoring biomarker, that when positive, informs initiation of treatment with an anti-malarial drug >6 days following CHMI with *P. falciparum* sporozoites in healthy subjects (18-50 years old) from non-endemic areas enrolled in clinical studies for vaccine and/or drug development" (30).

The complexities of the mosquito bite challenge model have limited the number of test centers that can safely conduct CHMI using *Anopheles*-infected mosquitoes to bite volunteers. The establishment of such challenge centers requires a secure biocontainment insectary with trained entomologists and parasitologists, individuals with training in diagnostics (microscopy and molecular PCR detection), clinicians, and a clinical trial facility. The US military Walter Reed Army Institute of Research (WRAIR) has supported malaria challenge trials using the bites of infectious mosquitoes since the 1980s and has safely conducted dozens of such trials without any study-related SAEs. Using the same exact challenge model standard operating procedures, the CHMI model developed by WRAIR has been transferred to the

Center for Infectious Disease Research in Seattle, Washington; the University of Maryland; and the University of Oxford, where the challenge of human volunteers with *P. falciparum* 3D7 parasites was successfully achieved (31).

Vaccine efficacy assessed using the mosquito bite challenge model

One of the key applications of the human challenge model for malaria is to assess the potential for vaccine efficacy in small numbers of volunteers. This is particularly relevant for pre-erythrocytic malaria vaccines that prevent infection. The primary endpoint in CHMI vaccine trials is the presence or absence of blood-stage parasites occurring in a person immunized with an experimental vaccine candidate. Since the 1980s, dozens of malaria vaccine trials have been conducted around the world that use the bites from falciparum-infected anopheline mosquitoes to deliver sporozoites.

The concept that human malaria challenge trials could be exploited to assess vaccine efficacy was first demonstrated by David Clyde and colleagues at the University of Maryland in the early 1970s. Volunteers were immunized with bites from hundreds of irradiated *Anopheles* mosquitoes infected with *P. falciparum* and were then challenged with wild-type *P. falciparum*-infected mosquitoes (8). Subsequently, investigators tested subunit recombinant protein (32) or peptide vaccines (33) based on the circumsporozoite amino acid repeat units and who were challenged. Small numbers of vaccinated subjects did not develop detectable parasitemia and were classified as "protected" compared to those who developed malaria in the control group, which did not receive the vaccine. These first demonstrations initiated the testing of various sporozoite and/or liver-stage antigens either singly or in combination with various expression platforms over the ensuing 30 years. By far the most extensively investigated malaria vaccine that progressed from CHMI clinical trials to field studies is the RTS,S vaccine (34). The first demonstration that RTS,S could protect a person in a CHMI was in a person immunized with RTS,S formulated with aluminum hydroxide adjuvant. This was followed by the seminal trial that catapulted RTS,S development and subsequent field testing, which demonstrated high-level protective efficacy when RTS,S was formulated with AS01 adjuvants (comprised of monophosphoryl lipid A, QS21, and an oil-in-water formulation). Several RTS,S vaccine CHMI trials followed that improved on the formulation by testing various dosages, number of doses, and schedule. Additional iterations ensued that included testing of a construct that lacked the amino acid repeat units; the combination of additional malaria antigens such as MSP-1 and TRAP with RTS,S (35); and prime-boost combinations using viral vectors expressing malaria genes to prime followed by RTS,S boost (36–39).

In addition to the circumsporozoite protein (CSP) antigen, other *P. falciparum* antigens expressed as recombinant proteins have been tested in CHMI trials, including PfCS102 (40); LSA1 (41); CelTOS (42, 43); blood-stage vaccine candidates that used mosquito bite challenge, GMZ2 (44) and AMA1 (45); and an epitope virus-like particle vaccine containing a single B-cell and two T-cell epitopes from CSP antigen (46).

Vaccine development platforms including DNA and viral vectors used singly or in prime-boost configurations have been tested extensively in CHMI using mosquito bite challenge and reviews have been excellent. Viral vectors include vaccinia containing seven different malaria antigens from all stages of the life cycle (47), adenovirus 5 (Ad5) vectors (48), and chimpanzee adenovirus 63 (ChAd63) (49). However, most CHMI trials of viral-vectored vaccines were prime-boost combinations that included

471 combinations of DNA/Ad5 (50), DNA/MVA (modified vaccinia virus Ankara) (51), Pox/MVA (52), and
472 ChAd63/MVA (49).

473 Whole-parasite vaccine constructs have received much attention in recent years and such vaccines are
474 based on the manufacture of aseptic, metabolically active, cryopreserved sporozoite vaccines (53, 54).
475 The evaluation of such whole-parasite vaccines includes genetically modified parasites that prevent
476 development of the parasite in the liver (55) and experimental medicine studies that deliver infectious
477 sporozoites under chemoprophylaxis with different antimalarials given before immunization with the bite
478 of live wild-type infected mosquitoes (56–58).

479 **Vaccine efficacy assessed by the injection of purified cryopreserved sporozoites**

480 Because mosquito bite challenge is laborious, requiring extensive insectary facilities that are not
481 amenable to transfer across clinical trial sites, a developmental program was initiated more than a decade
482 ago to assess whether sporozoites harvested from the mosquito under GMP could be cryopreserved,
483 thawed, and injected into human volunteers to establish a liver-stage infection with subsequent emergence
484 of blood-stage parasites into the peripheral circulation. It is crucial to note that an infection in a human
485 requires as few as 15 to 100 sporozoites delivered with the bite of a single infected mosquito. Critically,
486 this cannot be achieved with cryopreserved sporozoites, probably because the processes required to
487 harvest, freeze, thaw, and administer sporozoites with a needle and syringe take a toll on parasite viability
488 and infectiousness. Nevertheless, many studies have been conducted to assess whether such
489 cryopreserved sporozoites could be administered in a similar manner to delivery by an infectious
490 mosquito bite (Figure 3 above). This includes several studies that assessed intradermal (59–61),
491 subcutaneous (62), intramuscular (63, 64), and intravenous administration by a procedure called direct
492 venous inoculation (63, 65). Vaccine efficacy using PfSPZ cryopreserved sporozoites has been studied in
493 CHMI challenge trials, resulting in varying protection related to dosage and number of immunizations
494 and primarily using direct venous inoculation as a method to introduce infectious sporozoites in the
495 challenge model.

496 There is controversy within the field of malaria vaccinology regarding whether using such cryopreserved
497 sporozoites as a challenge method is a suitable surrogate of natural infection. As stated previously,
498 sporozoites delivered by the bite of infectious mosquitoes are deposited directly into the skin, and traverse
499 through the epithelial, endothelial, and Kupffer cells, the lymphatic system, and venous circulation to
500 arrive in the final destination, a hepatocyte. Vaccines that target sporozoites may elicit antibody-mediated
501 immune responses that neutralize and or kill sporozoites in the skin (and in the peripheral circulation)
502 before entry into hepatocytes. Cryopreserved sporozoites delivered by direct venous inoculation, but not
503 sporozoites delivered by mosquito bite, bypass the skin; and thus, this delivery mode would exclude any
504 mechanism of action that relies on activity within the skin. Therefore, one must exert caution when
505 interpreting CHMI vaccine trials that rely on direct venous inoculation. Nevertheless, there are some
506 advantages, including easier implementation, lower cost, no requirement for an insectary, and easier
507 transference of the challenge model to international testing centers.

508 **Pathogenesis, immunologic, and transcriptional profiling in controlled human malaria infection**
509 **clinical trials**

Several studies have advanced our knowledge of pathogenic processes that take place during malaria infection following CHMI, such as alterations in blood coagulation (66) and the impact of sickle cell trait on time to blood-stage infection after challenge (67).

Significant advances in our understanding of the immunologic mechanisms related to protection obtained from serum and peripheral blood mononuclear cells from CHMI vaccine trials have been realized and have been nicely reviewed in several publications (68–70). Nevertheless, protection against malaria infection is complex and there are many redundant escape routes the parasite uses to circumvent the immunologic pressures placed on it. This section of the chapter provides some examples of the variety of methods and tools that have been used to evaluate innate, humoral, and cellular immune responses to various malaria vaccines.

Innate responses following CHMI can be gleaned from interventions that potentiate the innate immune responses, such as prior bacillus Calmette-Guérin vaccination (71). Both humoral (antibody) and cell-mediated immune responses in CHMI trials from semi-immune persons previously exposed to malaria may be different from those observed in persons with no prior malaria exposure or infection (72, 73).

Humoral immune responses to both pre-erythrocytic- and blood-stage vaccines have increased our knowledge and understanding of how immunization impacts protection against challenge, but no single correlate of protection has been found. One of the more exciting developments coming from CHMI vaccine trials and specifically from individuals protected against malaria challenge is the identification and isolation of monoclonal antibodies derived from plasmablast or memory B-cell populations in immunized persons. These antibodies reveal important insights related to protection against the circumsporozoite antigen on sporozoites, and that could form the basis for a new malaria control tool in the form of a prophylaxis against malaria infection (74, 75).

Likewise, multiple immunologic investigations that have examined T-cell responses to viral-vectored malaria vaccines have indicated that antigen-specific $CD4^+$ and $CD8^+$ T cells are induced in subjects protected from malaria infection compared to those not protected; however, no absolute threshold indicative of a correlate of protection has been found (76–79).

Gene expression methodologies using high-resolution transcriptomics have been used to understand the molecular dynamics that occur before, during, and after CHMI (80) in both the parasite (81) and the human host (82–86). Early changes in host transcriptional profiles occur prior to the onset of clinical symptoms in hundreds of genes, uniting pathways from the cell nucleus, intracellular compartments, cell membranes, and extracellular space, providing a glimpse into how parasite infection precipitates a coordinated host response. Of particular interest, in an area that is emerging, is to further understand the transcriptional changes in gene expression that are induced by immunization with whole parasites, RTS,S, or viral vectors and that influence whether a person becomes infected or is protected following CHMI.

Blood-stage challenge model

A blood-stage challenge model has been developed for both *P. falciparum* (87, 88) and *P. vivax* (89, 90) parasites that can be used to understand specifically the onset of pathogenesis, to evaluate whether new antimalarial drugs can kill parasites, and to evaluate vaccine efficacy that targets post-pre-erythrocytic stages. This model is illustrated in Figure 4.

549 **Figure 4. Blood-stage controlled human malaria infection.**

550 The salient features of this model are similar to the challenge model developed for pre-erythrocytic
551 malaria interventions. This includes both mosquito bite (Figure 4A) and the direct venous inoculation
552 (Figure 4B) of GMP ring-stage parasites into the host, which bypasses the liver stage of infection.
553 Parasite cell banks for intravenous inoculation are prepared according to GMP and cryopreserved in small
554 aliquots that can be readily sent worldwide to clinical trial test centers. Extremely low numbers of ring-
555 stage parasites, ranging from a few hundred to 2,500, can be directly introduced into the host, establishing
556 rounds of blood-stage parasite multiplication every 48 hours that are detected by sensitive PCR (Figure
557 4C). Both the multiplication and killing of the parasite following administration of experimental or
558 licensed antimalarial drugs can be closely monitored in the host even before the onset of clinical
559 symptoms associated with malaria illness (Figure 4D). Changes in the host (91) and the parasite (92)
560 occur after blood-stage challenge.

561 The practical application of the blood-stage challenge model is to evaluate blood-stage vaccines. Both
562 whole organisms and subunit blood-stage vaccines have been evaluated with this challenge model, and
563 immunologic profiling responses, including antibodies that inhibit the growth of the parasite and cell-
564 mediated responses elicited by the vaccine, have been studied (93–95).

565 **Transmission-stage challenge model**

566 Recently, there have been efforts to develop a human challenge model that can measure the transmission
567 of malaria parasite gametocyte stages from an infected human volunteer to anopheline mosquitoes (96–
568 100). The rationale behind the development of such a model is to assess new antimalarial drugs,
569 monoclonal antibody biologics, and vaccines able to interrupt the transmission of the parasite to the
570 mosquito, thereby leading to eventual malaria elimination. The development of such a model is
571 technically challenging since the sexual-stage forms (male and female gametocytes) of the parasite
572 primarily in *P. falciparum* infection are quite low in density. Briefly, due to factors still not well
573 understood, at some point in the blood-stage life cycle there is a transcriptionally related commitment of a
574 few asexual-stage parasites to become sexual-stage parasites, which results in two forms: male and female
575 gametocytes. Upon blood feeding by a mosquito, both a female and a male gametocyte are taken up,
576 fertilize, and develop into a zygote in the mosquito gut; undergo further differentiation into ookinetes that
577 traverse the midgut epithelium; and develop into oocyst-stage parasites. This initial process of
578 development to the oocyst stage occurs over seven to nine days, at which time the oocysts undergo further
579 development into infectious sporozoites (14 to 21 days since the first blood feed), which eventually find
580 their way to the mosquito salivary glands to transmit sporozoites to a new human host. There are several
581 choke points in this sexual-stage part of the life cycle that a challenge model needs to overcome. We
582 illustrate the development of this challenge model in Figure 5.

583 **Figure 5. Transmission-stage challenge model.**

584 The first step (step 1) is to establish an infection in the volunteer. This may be accomplished either by the
585 bite of five infectious *Anopheles* mosquitoes, or by the direct venous inoculation of blood-stage parasites
586 into the host. In Phase 2 (Figure 5), asexual-stage blood parasites begin to multiply in the blood. It is
587 critical to not permit the onset of clinical symptomatology in the volunteer, which would necessitate
588 treatment. In step 2, the appearance of sexual-stage parasites is different between *P. vivax* infections and

589 *P. falciparum* infections. In *P. vivax* malaria, sexual-stage parasites develop early in infection, coincident
590 with the appearance of asexual-stage parasites, albeit in extremely low numbers due to their confinement
591 to early reticulocytes and not in mature red blood cells. This early commitment enabled development of a
592 transmission challenge model for *P. vivax* malaria, which was limited by the parasite-vector
593 compatibility, but proof of concept was demonstrated for the first time. In *P. falciparum* malaria infection
594 in challenge volunteers, the appearance of gametocytes in the circulation would not normally occur until
595 the parasite density was sufficiently high enough to start the commitment process from the asexual stage
596 to the sexual stage of development, because adverse events such as fever would preclude waiting until
597 gametocytes would appear. Therefore, it is essential to stimulate gametocytogenesis early, before
598 symptoms appear. One strategy adopted is the use of subcurative doses of antimalarials, which results in
599 the appearance of gametocytes in the peripheral circulation. This is illustrated in Figure 5, Phase 2.
600 Critical to this approach is the fine-tuning of antimalarial dosing at concentrations that suppress but do
601 not kill asexual-stage parasites, and still induce gametocytogenesis (with drugs such as sulphadoxine-
602 pyremethamine and piperaquine); however, refinement of the approach will require further development.

603 As the asymptomatic (but infected) volunteer develops gametocytes, there are two methods to determine
604 transmissibility (Figure 5, Phase 3). First, blood is taken and placed into a membrane device in which 20
605 to 30 uninfected mosquitoes are allowed to feed for five to ten minutes. This method is referred to as the
606 direct membrane feeding assay. After seven to nine days, the mosquitoes are dissected and the number of
607 oocyst-stage parasites quantified. The second method, the direct skin feed, allows uninfected *Anopheles*
608 mosquitoes to feed directly on the arms of volunteers infected with gametocytes, and oocyst stages are
609 detected in dissected mosquitoes seven to nine days after feeding on the subjects. Proof of concept for
610 both methodologies has been established (99, 100). We are in the early days of the refinement of the
611 transmission-stage model, but it should be amenable to testing many different new drugs and vaccines
612 that conceptually could interrupt transmission.

613 **Specific considerations for *Plasmodium vivax* challenge models**

614 The development of a challenge model for *P. vivax* malaria is unique. The asexual stage of the parasite
615 cannot be cultured *in vitro*, which precludes the standard methodology for obtaining gametocytes to feed
616 to mosquitoes, which then could produce infectious sporozoites for challenge. Therefore, a predefined
617 strain used in challenge studies cannot be obtained, instead necessitating reliance on naturally occurring
618 vivax strain parasites, which only develop in Duffy-positive reticulocyte blood cells circulating in the
619 population. The parasites that infect the mosquitoes are thus field isolates that vary from experiment to
620 experiment. The requirement that parasites be fully susceptible to antimalarial drugs is more difficult to
621 verify with the field isolates used in the *P. vivax* model as compared to cloned parasites used in the
622 *P. falciparum* model. The second challenge is the parasite-vector competence, which limits the spectrum
623 of *Anopheles* mosquito species that can transmit the parasite, such as *An. albimanus* in South America
624 and *An. dirus* in Southeast Asia.

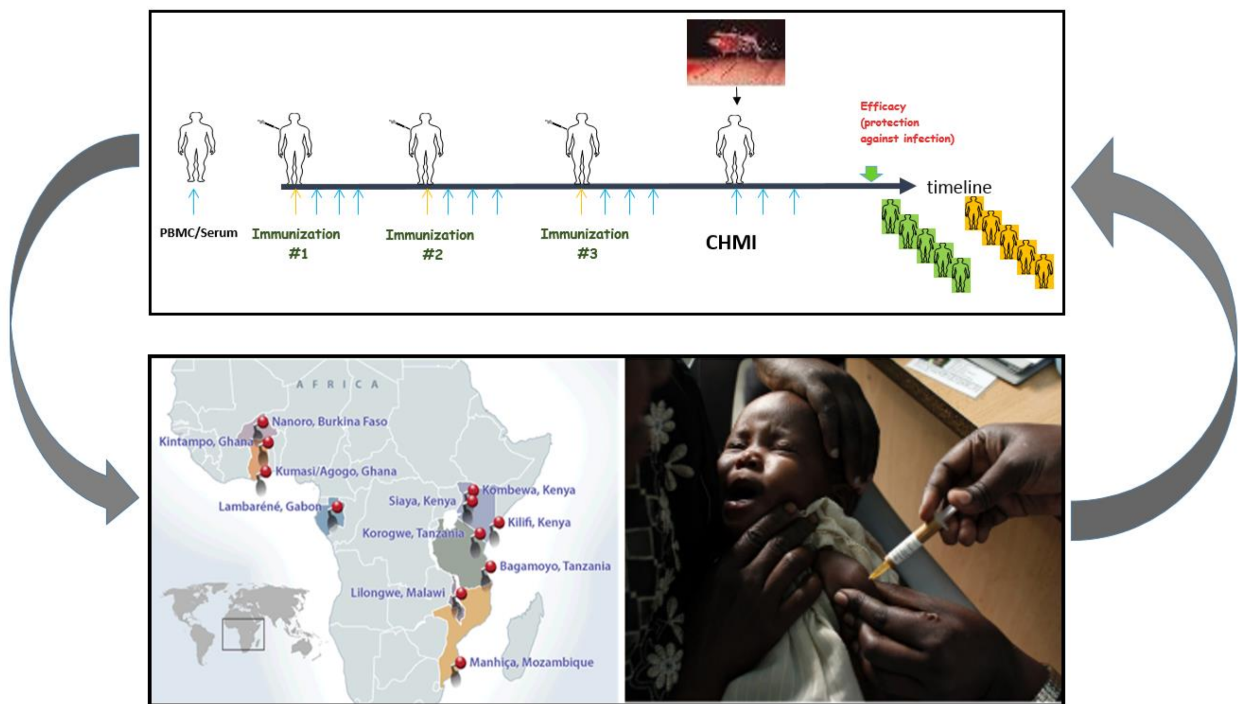
625 The human challenge model for *P. vivax* also faces an additional safety concern compared to the
626 *P. falciparum* model. After *P. vivax* infection, a portion of the parasites lie dormant in the liver as
627 hypnozoites that can cause recurring episodes of malaria if not eliminated. In the current *P. vivax*
628 challenge model, the antimalarial drug primaquine is prescribed to clear all liver-stage parasites from
629 study volunteers, but long-term follow-up would be required to verify that the clearance of liver stages

630 has occurred. In one such challenge trial, two subjects failed to be cleared with a combination of
631 chloroquine plus primaquine and relapsed multiple times, leading to the discovery that a polymorphism in
632 the cytochrome P450 2D6 allele precludes the metabolism of primaquine to its active component (101).

633 **Summary**

634 The human challenge models for malaria can be major accelerators for malaria vaccine development.
635 When more of the models become fully developed, they could provide the capacity to evaluate vaccines
636 against *P. falciparum* and *P. vivax* malaria, and against the pre-erythrocytic and blood stages of the
637 parasite life cycle. A wide range of vaccine types targeting different candidate antigens for each of these
638 parasites, and for different stages of infection, could then be systematically compared. Ideally, vaccine
639 development is an iterative process, as illustrated in Figure 6.

640 **Figure 6. Malaria vaccine development from controlled human malaria infection to the field and back.**



641

642 Nevertheless, one would be remiss in relying exclusively on a challenge model without validating a
643 candidate's vaccine efficacy in the field. As stated previously, the upside is to de-risk clinical
644 development in terms of both time and cost. It is equally important to appreciate the limitations of a
645 human challenge model. Such limitations include whether a person has previously been exposed to
646 naturally acquired malaria infection, or whether a person being immunized has concurrent subclinical
647 malaria infection. This cannot be accommodated in challenge models currently used to assess vaccine
648 efficacy. As discussed previously, the force of infection is critical, since only a single mosquito bite is
649 normally sufficient to establish infection. If on the other hand a challenge model requires the bite from
650 five infectious mosquitoes, there is the possibility that one prematurely discards a promising vaccine
651 candidate because insufficient protection is observed after five infected mosquito bites. Other
652 considerations include both host and parasite genetic diversity. Most malaria vaccines using CHMI are
653 tested primarily in volunteers in the United States and Europe. The target for such vaccines is persons

654 residing in sub-Saharan Africa, where the human leukocyte antigen class 1 and class 2 alleles differ
655 across populations, rendering interpretation of studies from the "North" to the "South" challenging. In
656 addition, there are potentially vastly different outcomes of challenge studies among previously infected or
657 chronically infected persons. Malaria parasites are extremely genetically diverse, resulting in differences
658 in fitness, invasion potency, and vaccine escape mutations. Relying on a single strain of *P. falciparum*
659 malaria to adequately predict protection in the field is risky; therefore, quickly transitioning to trials in
660 naturally exposed populations of all ages after having achieved a measure of efficacy after challenge will
661 enable rapid and thoughtful decisions around advancement of promising vaccine candidates.

662 Dengue

663 Epidemiology, pathogenesis, diversity, and public health impact of dengue virus

664 According to the World Health Organization (WHO) (102), roughly 40 percent of the world's population
665 are at risk of infection with dengue virus. The US Centers for Disease Control and Prevention (CDC) and
666 WHO websites report that approximately 400 million infections with dengue occur annually, with 100
667 million symptomatic infections, 500,000 cases of dengue hemorrhagic fever, and 22,000 deaths, mostly in
668 children. It is clear that the number of dengue cases has been rapidly expanding (103, 104). No specific
669 drugs exist to treat dengue infection and only a single vaccine, Dengvaxia® (chimeric yellow fever
670 dengue-tetravalent dengue vaccine, CYD-TDV, Sanofi Pasteur), has been licensed, although several are
671 in late-stage clinical development.

672 Dengue is a single-stranded RNA, enveloped flavivirus whose close relatives are yellow fever, Japanese
673 encephalitis, and West Nile viruses. There are four serotypes of dengue virus (abbreviated DENV):
674 DENV-1, DENV-2, DENV-3, and DENV-4. The E glycoprotein mediates virus attachment and entry into
675 cells and is the target of virus-neutralizing antibodies. Dengue virus has two target cells with different
676 receptors that mediate virus entry. In dendritic cells, DC-SIGN is the receptor (105) and in macrophages
677 the mannose receptor serves this function (106).

678 Dengue viruses require passage through one of two mosquito species, *Aedes aegypti* or *Aedes albopictus*,
679 to complete their life cycle. When these mosquitoes bite a viremic human, the virus is taken up, replicates
680 in the mosquito gut, and spreads to the salivary glands from which the mosquito transmits the virus to
681 humans through bites. In principle, vector control efforts could play a key role in reducing dengue disease
682 burden, but historically these efforts have had mixed results (107–109). Novel approaches such as release
683 of *Wolbachia*-infected mosquitos hold some promise (110).

684 While most infections with dengue viruses are asymptomatic, up to 10 percent of individuals develop
685 dengue fever (DF), characterized by high fever, severe headache, joint pain, rash, and mild bleeding. A
686 small proportion of those with DF progress to dengue hemorrhagic fever and dengue shock syndrome,
687 which can lead to failure of the circulatory system, shock, and death.

688 The main risk factor for severe dengue disease is infection with a second dengue serotype following
689 primary infection (111). The estimated increase in risk for severe disease with a heterologous secondary
690 infection is 15- to 80-fold.

691 Understanding the underlying mechanisms of disease enhancement upon secondary heterologous
692 infection is critically important for the evaluation and utilization of dengue vaccines and for human
693 challenge models of dengue. In a primary infection, serotype-specific B and T cells successfully curtail
694 the infection (112). However, in a secondary heterologous infection, cross-reactive antibodies not only
695 fail to neutralize the virus, but instead help it infect target cells leading to massive virus production and
696 over-production of cytokines which increase blood vessel permeability, resulting in dengue hemorrhagic
697 fever and risk of death. These two mechanisms are presented in Figure 7.

698 **Figure 7. Potential mechanisms for immune-mediated enhancement of dengue virus infection.**

699 WHO organized a technical consultation in 2013 among experts on the long-term safety assessment of
700 live-attenuated dengue vaccines to assess the theoretical risks of enhanced disease during clinical trials
701 (113). Long-term follow-up analyses of Dengvaxia (CYD-TDV) Phase 3 trials unfortunately indicated
702 that dengue-naïve individuals receiving this live-attenuated vaccine were at greater risk of subsequent
703 severe disease (114). In light of these findings, a second WHO guidance on evaluating safety and efficacy
704 of subsequent dengue vaccines, including the importance of long-term follow-up, was released (115).

705 **The dengue human challenge model**

706 Experimental human infection with dengue viruses has a history of over a century, as described in recent
707 reviews (116, 117). Early work identified durable homotypic protection and short-term heterotypic
708 protection among different dengue serotypes—key results that have both informed and challenged
709 vaccine development against dengue to the present day. In the last few years, a focused effort has come
710 underway to re-develop human dengue infection models that can support vaccine and drug development
711 while meeting current regulatory requirements (118–121).

712 The first requirement for the re-developed human dengue infection models is to produce suitably
713 attenuated challenge strains of dengue virus using Good Manufacturing Practice. Researchers at the US
714 military Walter Reed Army Institute of Research (WRAIR) took advantage of their previous work on
715 dengue virus attenuation in an effort to develop new challenge strains. Over the years, dengue virus
716 strains have been passaged through a number of cell types in culture (117). Mammen et al. evaluated
717 seven of these attenuated strains by subcutaneous inoculation in flavivirus-naïve human volunteers in an
718 inpatient setting. DF was defined as sustained fever for 48 hours or more, and by concurrent viremia as
719 defined by virus culture *in vitro* (122).

720 Dengue fever has a typical incubation period of seven days or more. Physicians conducted assessments
721 every day for 14 days and five outpatient visits took place over a period of six weeks. Volunteers also
722 underwent chest X-ray, abdominal ultrasound, and punch biopsy of any rash that developed while an
723 inpatient. At study conclusion, volunteers were provided with a medical statement enabling them to notify
724 future medical providers of their potential to develop dengue hemorrhagic fever or dengue shock
725 syndrome upon re-exposure to dengue virus (122).

726 Table 1 details the results of the challenge study. The volunteers challenged with the DENV-2 and
727 DENV-4 strains did not meet the case definition of DF, but those challenged with the DENV-4 strains
728 were infected and had mild perihepatic effusions that were only detectable by ultrasonography. The
729 volunteers challenged with the DENV-1 and DENV-3 strains developed DF, and in some cases, other

730 clinical manifestations of dengue virus infection. The DENV-1 and DENV-3 challenge strains were
731 selected for further use in the challenge model and were manufactured under Good Manufacturing
732 Practice.

Table 1. Clinical manifestations of dengue fever in human volunteers challenged with attenuated strains of four dengue serotypes.

Strain	Passage history (number)	Clinical manifestations[a]
DENV-1 45AZ5	FRhL (20)	Two volunteers were challenged. Both developed DF and one also developed mild effusions.
DENV-2 S16803	Mosquito (1), PGMK (4), PDK (10), FRhL (3)	Three volunteers were challenged with one of these two strains. None developed DF.
DENV-2 PR159	PGMK (6), FRhL (1)	
DENV-3 CH53489 cl24/28	PGMK (4), C6/36 (5), FRhL (1)	Three volunteers were challenged. All developed DF and two developed elevated levels of liver enzymes.
DENV-4 341750	Mosquito (1), PDK (6), FRhL (3)	Two volunteers were challenged. Neither developed DF.
DENV-4 341750	Mosquito (1), PGMK (5), FRhL (4)	Two volunteers were challenged. Neither met the case definition of DF, but both became viremic and developed mild effusions.
DENV-4 H-241	Mosquito (1), C6/36 (2), FRhL (1)	

Abbreviations: C6/36, culture-adapted *Aedes albopictus* larval cells; DENV, dengue virus; DF, dengue fever; FRhL, fetal Rhesus macaque lung cells; Mosquito, *Toxorhynchites amboinensis*; PDK, primary dog kidney cells; PGMK, primary green monkey kidney cells.

[a] Effusions are accumulations of fluid that has leaked from small capillaries. Elevated liver enzymes in the blood is evidence of liver damage.

733 The dengue challenge model described above was also used to study volunteers that had previously
734 received candidate dengue vaccines (116, 123). Ten subjects previously vaccinated with the live-
735 attenuated, tetravalent candidate TDEN under development by WRAIR and GlaxoSmithKline (GSK)
736 were challenged 12 to 24 months after vaccination with the live-attenuated DENV-1 or DENV-3 strains
737 described in Table 2. Four unvaccinated, dengue-naïve subjects were challenged as controls, two with
738 each challenge strain.

Table 2. Attenuated dengue virus challenge strains.

Strain	Method of production and administration	Stock producer
DENV-1 45AZ5	Isolated from a patient infected with dengue in Micronesia in 1974. Challenge doses are reconstituted from freeze-dried stocks in sterile water and administered to healthy adult volunteers by subcutaneous injection.	Walter Reed Army Institute of Research
DENV-3 CH53489 cl24/28	Isolated from a patient infected with dengue in Thailand in 1974. Challenge doses are reconstituted from freeze-dried stocks in sterile water and administered to healthy adult volunteers by subcutaneous injection.	Walter Reed Army Institute of Research

Table 2. Attenuated dengue virus challenge strains.

Strain	Method of production and administration	Stock producer
rDEN2Δ30	Recombinant virus derived from DENV-2 Tonga/74, isolated from a patient infected with dengue in the Kingdom of Tonga in 1974, with a 31-nucleotide deletion from the 3' untranslated region. Challenge doses are thawed from stocks frozen at -80 °C and administered to healthy adult volunteers by subcutaneous injection.	US National Institute of Allergy and Infectious Diseases

Abbreviation: DENV, dengue virus.

739 In this study, many of the volunteers developed DF symptoms without meeting the formal case definition.
740 The main reason was because many were still viremic from their prior vaccination. Four of five
741 vaccinated and one of two control subjects developed elevated liver enzymes, some at high levels. All
742 liver enzymes returned to normal by day 30.

743 Gunther et al. studied serum levels of soluble cytokine receptors and the levels of IFN-γ and other
744 cytokines in peripheral blood mononuclear cells re-stimulated with laboratory strains of four dengue
745 serotypes in the 14 subjects from the human challenge study of Sun et al. described above (116). This
746 study suggested dengue virus may suppress cellular immunity during peak viral replication by a
747 mechanism that inhibits production of IFN-γ.

748 The DENV-1 strain was recently re-derived by transfection into FRhL cells and formulated for use in
749 human challenge studies (124). A small Phase 1 study successfully demonstrated this strain could infect
750 and cause moderate disease without long-term sequelae.

751 A second challenge model for DENV serotypes, developed by the US National Institutes of Health, Johns
752 Hopkins University, and the University of Vermont using the genetically attenuated strain rDEN2Δ30
753 (125), is derived from an isolate from a dengue outbreak in the Kingdom of Tonga in 1974. This strain
754 causes relatively mild disease, and is more highly attenuated than the serially passaged strains developed
755 at WRAIR. It was initially developed as a vaccine, but found to be insufficiently attenuated for this
756 purpose (118, 126). Administration of 10^3 plaque forming units (PFU) as a single subcutaneous dose did
757 not induce fever, but resulted in notable viremia: rDEN2Δ30 was isolated from all ten subjects at a mean
758 peak titer of 2.5 $\log_{10}$ PFU/ml (range 1.5–3.3 $\log_{10}$ PFU/ml). Furthermore, 80 percent of subjects
759 developed a maculo-papular rash that was graded at moderate severity in 50 percent of cases. However,
760 these characteristics suggested it could still be useful as a challenge strain. The fact that rDEN2Δ30 is
761 produced by recombinant DNA technology is a distinct advantage with respect to production and
762 characterization of this strain. A further analysis of T-cell responses in subjects challenged with
763 rDEN2Δ30 indicated that responses were generally similar to those induced by natural infection,
764 particularly with respect to non-structural proteins NS1, NS3, and NS5 (127). These results provided
765 additional support for the suitability of rDEN2Δ30 as a challenge strain in a dengue human infection
766 model.

767 **Dengue vaccine development**

768 Several candidate dengue vaccines are in early or advanced clinical development and a number of
769 "second generation" candidate vaccines are in the preclinical stage (128–130). Table 3 summarizes the
770 clinical pipeline for candidate dengue vaccines.

Table 3. Dengue vaccines licensed or in clinical development.

Vaccine type	Approach	Stage of development	Developer(s)
Live-attenuated			
Dengvaxia (CYD-TDV)	Genes encoding prM and E proteins of each of the four dengue serotypes were cloned into the backbone of the yellow fever vaccine YFV 17D.	Licensed	Sanofi Pasteur (licensed from Acambis)
TDEN (aka TDV)	Strains of four dengue serotypes attenuated through serial passage in culture were combined for a tetravalent vaccine. The initial vaccine was reformulated after additional passages in serum-free media, and was stabilized with carbohydrate rather than serum albumin.	Phase 2	WRAIR and GSK
Butantan-DV (aka Tetra-Vax-DV, TV003)	Attenuation through introduction of a 30-nucleotide deletion (Δ30) in the 3-prime untranslated region of the genome of a DENV-4 cDNA clone. The prM and E genes and flanking genome sequences of the three other dengue serotypes were cloned into the DENV-4 backbone.	Phase 3	US National Institutes of Health and Johns Hopkins University, University of Vermont, and Instituto Butantan
TAK-003 (aka DENVax, TDV)	A cDNA clone of the strain DENV-2 PDK-53 attenuated through serial passage in cell culture in Thailand was used as the backbone for insertion of prM and E genes of the other three serotypes.	Phase 3	CDC and Takeda Vaccines
Whole-inactivated			
DPIV	Dengue viruses have now been grown to high enough titers in cell culture to permit purification and inactivation. A formalin-inactivated tetravalent vaccine has been produced.	Phase 2	WRAIR, GSK, and Fiocruz
Prime-boost	Priming with live-attenuated TDEN followed by boosting with DPIV is being explored in two trials.	Phase 1	WRAIR and NMRC
Recombinant subunit			
V180	Stably transformed *Drosophila* S2 cells produce high levels of a truncated form of the dengue E protein, called 80E. Tetravalent formulations of 80E proteins have been produced with Alhydrogel® or ISCOMATRIX® adjuvant.	Phase 1	Merck
TVDV	DNA plasmids expressing prM and E genes of four dengue serotypes have been	Phase 1	NMRC

Table 3. Dengue vaccines licensed or in clinical development.

Vaccine type	Approach	Stage of development	Developer(s)
	constructed and combined for a tetravalent vaccine. Various delivery methods including needle-free injection systems and intradermal electroporation are being explored.		

Abbreviations: aka, also known as; CDC, US Centers for Disease Control and Prevention; DENV, dengue virus; DPIV, dengue purified inactivated vaccine; E, envelope; Fiocruz, Fundação Oswaldo Cruz; GSK, GlaxoSmithKline; NMRC, US Naval Medical Research Center; PDK, primary dog kidney cells; prM, precursor membrane protein; TVDV, tetravalent dengue DNA vaccine; WRAIR, Walter Reed Army Institute of Research.

771 Dengvaxia, developed by Sanofi Pasteur, is the only one licensed, and the entire development program
772 and deployment experience thus far has been reviewed (131). In a Phase 2 trial in Thai school children
773 (132), the overall protection against dengue infection with CYD-TDV was 30.2 percent. Although some
774 protection was observed with three of the four DENV serotypes, no protection was seen against infection
775 with DENV-2. Phase 3 trials with CYD-TDV were conducted in children in five countries in the Asia-
776 Pacific region (133) and in children in five Latin American countries (134). Phase 3 trials showed 56.5
777 percent efficacy and 60.8 percent efficacy in the Asia-Pacific region and Latin America, respectively. In
778 all three of the trials described above, the level of protection against DENV-3 and DENV-4 was higher
779 than against DENV-1 and DENV-2. These efficacy results were disappointing in light of evidence for
780 induction of neutralizing antibodies, particularly against DENV-2. More concerning was the evidence for
781 increased risk of severe disease in dengue-naïve individuals that received Dengvaxia (114), particularly in
782 younger children. These findings have restricted the broad deployment of Dengvaxia and led WHO to
783 recommend its use only in dengue-endemic countries with a high burden of disease (at least 70 percent
784 seropositive) and in individuals with laboratory-confirmed previous exposure to dengue. WHO has also
785 recommended against use of Dengvaxia in children younger than 9 years old, which includes those most
786 vulnerable to morbidity and mortality caused by dengue. A comparison of B and T-cell epitopes targeted
787 by Dengvaxia in contrast to two other vaccine candidates (Butantan-DV and TAK-003, described below)
788 suggested lack of targeting appropriate T cell epitopes could account for the limitations observed with
789 Dengvaxia (135).

790 Another tetravalent live-attenuated dengue vaccine candidate, TDEN (previously known as TDV), is
791 under development by WRAIR and GSK. This vaccine is comprised of strains of four dengue serotypes
792 attenuated through serial passage in dog kidney cells and then in fetal Rhesus lung cells, and is in Phase 2
793 (see Table 3 for details). TDEN was initially evaluated in infants, children, and adults in Phase 1 and 2
794 trials (136). Since then, the vaccine has been reformulated with carbohydrate stabilizer rather than serum
795 albumin and lyophilized as a tetravalent product. The reformulated product was taken forward into two
796 Phase 2 studies in dengue-endemic regions. The first was conducted in Thailand and enrolled 120 mostly
797 primed (i.e., dengue seropositive) adults (137). Two doses were administered six months apart. Nearly all
798 vaccinees had a tetravalent response three months following the second dose, as defined by at least a 10-
799 fold increase in reciprocal titer of neutralizing antibodies against all four serotypes. In this study, TDEN
800 was generally safe and well tolerated: there were no vaccine-related serious adverse events or cases of
801 dengue fever and only five subjects receiving TDEN had low-level viremia.

802 The second Phase 2 endemic-setting study with TDEN was conducted in Puerto Rico and enrolled 636
803 individuals from ages 1 to 50 years (138). Approximately half of these individuals were seropositive at
804 baseline, although this was highly age related: in the youngest age stratum, children under 2 years old,
805 fewer than 10 percent were seropositive, whereas among adults (21 to 50 years old), 93 percent were
806 seropositive. As in the Thailand study, subjects received two doses six months apart. Among seronegative
807 and seropositive individuals, the tetravalent response rates were 81 and 99 percent, respectively, at one
808 month after the second dose. The safety profiles between vaccine and placebo groups were similar among
809 both seropositive and seronegative individuals; for example, moderate severity fever was reported by
810 approximately 5 percent of subjects across all groups. There were no vaccine-related serious adverse
811 events and no confirmed cases of dengue fever. In a follow-on study to examine cell-mediated immune
812 responses, a representative subset of 244 participants was selected and $CD4^+$ T-cell, $CD8^+$ T-cell, and
813 memory B-cell responses were characterized (139). TDEN was found to be poorly to moderately
814 immunogenic by these criteria, regardless of setting or whether subjects were previously exposed to
815 dengue.

816 A third live-attenuated tetravalent dengue vaccine candidate (Butantan-DV, previously known as TV003
817 or Tetra-Vax-DV) is under development by the US National Institutes of Health (NIH), the Instituto
818 Butantan (Brazil), and Johns Hopkins University. This vaccine was attenuated through a 30-nucleotide
819 deletion in the 3-prime UTR, and by additional mutations of a cloned DENV-4 strain. The prM and E
820 genes of the three other serotypes, together with some genome regions flanking the prM and E genes,
821 were then cloned into the attenuated DENV-4 backbone (140). One unique feature of this vaccine is that it
822 is not transmissible to mosquitoes. During development, many different constructs were evaluated alone
823 or in combination (112, 141, 142). Thirteen different Phase 1 trials separately evaluated eight different
824 monovalent vaccine constructs. Four monovalent constructs were selected and evaluated in different
825 admixtures in a Phase 1 trial. Admixture TV003 produced serum antibody responses against DENV-1 and
826 DENV-4 in 100 percent of vaccinees, against DENV-3 in 85 percent of vaccinees, and against DENV-2
827 in 50 percent of vaccinees. This vaccine was then advanced to a Phase 2 trial in a dengue-endemic
828 country, Brazil, in collaboration with the Instituto Butantan (143). This study tested a lyophilized
829 formulation of TV003 designated Butantan-DV and found 64 percent of dengue-naïve and 55 percent of
830 dengue-exposed individuals had a tetravalent neutralizing response, a difference that was not statistically
831 significant. The safety profile for Butantan-DV was acceptable and consistent with Phase 1 trials with
832 TV003 conducted in the United States; therefore, Butantan-DV was advanced to a randomized, placebo-
833 controlled Phase 3 trial in Brazil, which is ongoing (ClinicalTrials.gov Identifier NCT02406729) (144).
834 This trial plans to enroll approximately 17,000 participants from 2 to 59 years of age. Efficacy evaluation
835 of the Phase 2 study subjects is also ongoing.

836 In parallel to the Phase 2 trial in Brazil, TV003 was also tested in a dengue controlled human infection
837 model in volunteers in the United States (125). Volunteers received a single dose of either TV003 or
838 placebo, and then six months later were challenged with 10^3 PFU of rDEN2Δ30. All 21 subjects that
839 received TV003 were completely protected from the challenge and did not experience any viremia or
840 rash. In contrast, all 20 subjects in the placebo group experienced viremia with a mean peak titer of 2.3 ±
841 0.1 $\log_{10}$ PFU/ml, and 80 percent experienced a rash. This study demonstrated the potential value of the
842 dengue controlled human infection model in generating proof of concept for a vaccine candidate such as
843 TV003 before investing in a larger, more expensive Phase 3 trial. A series of follow-on studies on the
844 immunological responses of participants from this challenge study further demonstrated the utility of the

845 model in conducting detailed analyses that would be more difficult with patients infected in an endemic
846 setting. Nivarthi et al. characterized targets of memory B-cell responses (145) and Graham et al.
847 characterized the quality of vaccine-induced $CD4^+$ and $CD8^+$ T cells specific to the various DENV
848 serotypes (146). In light of the modest efficacy of Dengvaxia, a series of studies was undertaken to
849 compare the immune response of TV003 (147–150). Taken together, these studies suggested that in
850 contrast to Dengvaxia, TV003 induces a robust and balanced immune response against all four DENV
851 serotypes after a single dose, regardless of prior dengue exposure, thus increasing the likelihood that it
852 will confer strong immunity without increasing risk of more severe disease from subsequent infections.

853 A fourth live-attenuated tetravalent vaccine candidate called TAK-003 (previously known as TDV and
854 DENVax) is under development by the CDC and Takeda Vaccines. It is constructed from a cDNA clone
855 of a DENV-2 strain attenuated through serial passage in dog kidney cells in culture, into which prM and E
856 genes of the other three dengue serotypes were cloned. TAK-003 (and various precursor formulations,
857 admixtures of the four-component attenuated DENV strains, and dose regimens) were tested in at least
858 nine studies in healthy, dengue-naïve adult and adolescent volunteers in the United States, Mexico, and
859 Colombia as well as in both adults and children in dengue-endemic countries in Asia and Latin America
860 (151–161). Collectively, these studies found that one or two doses induced a tetravalent neutralizing
861 antibody response ranging from 60 to 97 percent of subjects, depending on previous dengue exposure.
862 The vaccine was well tolerated at multiple dose levels and not associated with serious adverse events.
863 Taken together, these studies supported the safety and efficacy of this vaccine candidate that justified
864 progressing it to a Phase 3 study of more than 20,000 children aged 4 to 16 years at 28 study sites in eight
865 dengue-endemic countries, including five in Latin America and three in Asia (ClinicalTrials.gov Identifier
866 NCT02747927) (162). These subjects received two doses subcutaneously of a dose-optimized,
867 lyophilized, and reconstituted formulation or placebo given three months apart. It was recently reported
868 that TAK-003 achieved its primary efficacy endpoint in this study of 80.2 percent overall efficacy in
869 reduction of virologically confirmed dengue (VCD) (163). It was also recently reported that TAK-003
870 achieved secondary efficacy endpoints including 76 percent efficacy in individuals previously exposed to
871 dengue, 66 percent efficacy in dengue-naïve individuals, 90 percent efficacy against hospitalization due to
872 dengue, and 86 percent efficacy against dengue hemorrhagic fever (164). Rates of serious adverse events
873 were similar between the vaccine and placebo groups, and the overall safety profile importantly did not
874 indicate a similar risk as was previously observed with Dengvaxia in dengue-naïve vaccinees. In a follow-
875 on report after two years, TAK-003 was still 56 percent efficacious in preventing any VCD and 76
876 percent efficacious against hospitalization (165). However, the efficacy in preventing any VCD among 4-
877 and 5-year-olds dropped substantially from 73 percent in the year 1 to only 25 percent in year 2. No
878 additional safety risks were noted in year 2. Long-term follow-up of this study to continue to track the
879 safety and efficacy of TAK-003 is still in progress and is expected to be completed at the end of 2021.
880 Ancillary studies have further analyzed the immune response from subjects in some of the studies
881 described above and characterized in detail the humoral and cellular responses associated with immunity
882 induced by TAK-003. In particular, like Butantan-DV (aka TV-003) described above, TAK-003
883 stimulates a relatively balanced profile of neutralizing antibodies (166) and memory B cells (167) against
884 the four DENV serotypes, and also stimulates a $CD8^+$ T-cell response against DENV non-structural
885 proteins NS1, NS3, and NS5, which are associated with a protective immune response (168–170). In
886 contrast, a recent publication highlighted the difference between type-specific neutralizing antibodies

887 against DENV-2 as compared to cross-reactive DENV-1, DENV-3, and DENV-4 neutralizing antibodies
888 (171).

889 An adjuvanted tetravalent dengue purified inactivated vaccine (DPIV) is under development by WRAIR,
890 the Oswaldo Cruz Foundation, and GSK. Three clinical studies comparing various dose levels, adjuvants,
891 and schedules have been completed. These included Phase 1 (172) and Phase 1/2 (173) studies in dengue-
892 naïve adults, and a Phase 1 study in Puerto Rican adults (174, 175), most of whom were previously
893 exposed to dengue. In the dengue-naïve populations, two doses of DPIV adjuvanted with GSK's
894 proprietary $AS03_B$ adjuvant given intramuscularly one month apart induced a tetravalent neutralizing
895 antibody response in 100 percent of subjects at one month after the second dose. These responses waned
896 somewhat over the following year, but a subset of individuals boosted again at 12 months demonstrated a
897 robust anamnestic response. Among previously dengue-exposed individuals, DPIV stimulated
898 neutralizing antibody titers that generally persisted for at least three years. In both populations, DPIV was
899 safe and well tolerated, supporting further development of this candidate. A prime-boost strategy using
900 the live-attenuated TDEN candidate (described above) as a prime followed by boosting with DPIV has
901 been explored in two Phase 1 clinical trials conducted by WRAIR and the US Naval Medical Research
902 Center (NMRC) (ClinicalTrials.gov Identifiers NCT03141138 and NCT02239614) (176, 177). In the first
903 of these two to report results, it was found that priming with DPIV and boosting with TDEN yielded the
904 higher neutralizing titers and rate of tetravalent seroconversion as opposed to the opposite sequence (178).

905 Recombinant subunit and DNA vaccine candidates are also in clinical trials (see Table 3) (128). A
906 tetravalent recombinant subunit vaccine candidate composed of truncations of the E proteins from all four
907 serotypes designated V180 is being developed by Merck. A previous monovalent version composed of
908 only the DENV-1 80E protein was developed by Hawaii Biotech and tested in a Phase 1 study (179).
909 V180 contains 10 µg each of DENV-1, DENV-2, and DENV-3 80E and 20 µg of DENV-4 80E. V180 has
910 been tested in a Phase 1 trial in healthy, dengue-naïve Australian adults comparing various adjuvants and
911 dose levels (180). Tetravalent responses were 71 to 88 percent after two months but declined to 0 to 43
912 percent after one year. To investigate responses in the context of prior dengue exposure, V180 was also
913 tested in individuals that had previously received a live-attenuated tetravalent dengue vaccine (TV003 or
914 a related formulation, TV005, described above) and found to be similarly immunogenic (181). NMRC is
915 pursuing a DNA vaccine approach for dengue. A prototype plasmid encoding DENV-1 prM and E
916 delivered by needle-free Biojector® intramuscular injection was previously tested in a Phase 1 study and
917 found to be safe, but only mildly immunogenic (182). A tetravalent dengue DNA vaccine (TVDV)
918 composed of plasmids expressing prM and E proteins from all four serotypes with a lipid adjuvant
919 (Vaxfectin®) was then tested in a Phase 1 study (183). TVDV was delivered by standard needle-and-
920 syringe intramuscular injection and stimulated minimal neutralizing antibodies, but a notable T-cell IFN-γ
921 response. Alternative delivery approaches for TVDV that may increase immunogenicity, such as
922 intradermal electroporation, have been explored using a nonhuman primate model and may be applied in
923 future clinical studies (184).

924 An even greater number of candidate dengue vaccines are in preclinical development. Table 4
925 summarizes the number and types of these vaccine candidates, as reviewed by Schmitz et al. (185) and
926 Redoni et al. (128). The recombinant subunit vaccines are mostly expressed in *Escherichia coli* or yeast
927 cells. Each of the five virus-vectored vaccines uses a different virus vector. Finally, several candidate
928 vaccines incorporating non-structural proteins (mainly NS1) are being evaluated in mouse models (186).

Table 4. Candidate dengue vaccines in preclinical development.

Vaccine type	Number of candidates	Developer(s)
Live-attenuated	3	Chiang Mai University, Arbovax, Beijing Institute of Microbiology and Epidemiology
Inactivated	1	NMRC
Recombinant subunit	4	ICGEB-India, National Health Research Institutes-Taiwan
DNA	4	Inovio, Kobe University, CDC, NMRC, Fiocruz
Virus-like particles	4	Cytos, ICGEB-India, Kobe University, NCGEB-Thailand
Virus-vectored	5	ICGEB-India, GenPhar/NMRC, University of North Carolina, University of Texas, Themis Bioscience/Institut Pasteur

Abbreviations: CDC, US Centers for Disease Control and Prevention; Fiocruz, Fundação Oswaldo Cruz; ICGEB-India, International Center for Genetic Engineering and Biotechnology; NCGEB-Thailand, National Center for Genetic Engineering and Biotechnology; NMRC, US Naval Medical Research Center.

929 **Summary**

930 The importance of dengue virus as a threat to international public health has incrementally increased in
931 the last 30 years; yet, large areas of the world (including most of Africa) are at present spared because of
932 the absence of the insect vectors. The intermixing of DENV serotypes and disease enhancement upon
933 heterologous secondary infection could possibly be the engine that has driven the rapid spread and
934 increased morbidity and mortality from dengue virus infection in the last century.

935 The very nature of the disease makes developing a vaccine against DENV a formidable task (187). The
936 ideal dengue vaccine would provide a similar level of protection against each of the four dengue
937 serotypes. The single live-attenuated tetravalent vaccine licensed to date has generated imbalanced
938 immunity, which has been highlighted as an explanation for its limited efficacy and safety concerns,
939 particularly in dengue-naïve children. However, the two live-attenuated candidates that followed into
940 Phase 3 trials both induce a more balanced response and seem extremely promising. Alternative
941 approaches including whole-inactivated and subunit vaccines are advancing through early clinical
942 development and may augment or complement the live-attenuated approach, for example as part of a
943 prime-boost strategy.

944 The development of a human challenge model for dengue has been correspondingly complex (118). A
945 significant limitation, however, is the fact that attenuated challenge strains of DENV-2 and DENV-4 have
946 not been fully characterized in human challenge studies. Furthermore, even the current DENV-1 and
947 DENV-3 challenge strains may be introducing added variability into the model because of their long and
948 often complex passage histories. The newly developed human challenge models for dengue viruses have
949 sparked considerable interest and comment in the community of dengue researchers and vaccine
950 developers (117, 119, 188), along with some valuable suggestions for continued development of the
951 models (189). The newly developed models using attenuated strains of DENV may become important
952 contributors to the overall landscape of dengue vaccine development and introduction.

953

954 # Enteric diseases

955 ## Cholera

956 **Epidemiology, public health impact, and pathogenesis of *Vibrio cholerae***

957 Cholera is a diarrheal disease caused by the bacterium *Vibrio cholerae.* According to recent estimates,
958 cholera results in 2.8 million cases and nearly 100,000 deaths annually (190). Until the 1970s, the major
959 burden of cholera infection was in South and Southeast Asia, but outbreaks are now frequent in Africa
960 and have also occurred in countries such as Haiti (191) and Yemen (192).

961 Access to clean water, good sanitation, and proper hygiene will halt the spread of cholera, but these
962 measures are unlikely to be universally available in the near term. Five cholera vaccines are licensed in
963 multiple countries, but most are used in reactive vaccination campaigns in response to outbreaks. To
964 make the best use of vaccine supply, the World Health Organization (WHO) recommends a cholera
965 vaccine stockpile to contain outbreaks (193).

966 Long-term vaccination policies may be considered as stakeholders assess the introduction of cholera
967 vaccines into national policies. Bangladesh has taken the lead in this area with several Phase 4 trials to
968 assess a cholera vaccine's feasibility for use and delivery as part of a national vaccine program, and have
969 evaluated parameters such as single-dose regimens, limited-duration storage without refrigeration, and
970 safety in pregnancy (194–197).

971 More than 200 serogroups of *V. cholerae* are found in aquatic environments, but only two serogroups
972 (O1 and O139) are associated with cholera outbreaks in humans (198). The O139 serogroup was first
973 identified in India and Bangladesh in 1992, but has since declined. The O1 serogroup has been
974 responsible for the seven global cholera pandemics that have occurred since 1817 (198). The O1
975 serogroup has two biotypes, called El Tor and classical, which are further divided into serotypes Ogawa
976 and Inaba. Infections with O1 and O139 serogroups are not protective against subsequent infection with
977 the heterologous serogroup (190). Cross-protective immunity between different biotypes and serotypes
978 was evaluated in a human challenge study described below.

979 *V. cholerae* are non-invasive, but produce an enterotoxin called cholera toxin which binds to ganglioside
980 receptors, and causes a massive egress of water and electrolytes and a severe watery diarrhea ensues
981 (198). Treatment consists of oral rehydration and antibiotics which dramatically reduce mortality from
982 approximately 15 percent to about 1 percent.

983 **Cholera vaccine development**

984 Five whole-inactivated oral cholera vaccines are currently available—Dukoral®, Shanchol, Euvichol®,
985 mORC-Vax™, and OraVacs®—as well as one live-attenuated vaccine, Vaxchora® (see Table 5 for more
986 details). Dukoral, Shanchol, and Euvichol are licensed for international use. Dukoral consists of formalin
987 and heat-inactivated *V. cholerae* bacteria (O1 serogroup, classical and El Tor biotypes, Inaba and Ogawa
988 serotypes), plus recombinant cholera toxin B-subunit (rCTB) which lacks toxigenic activity. Dukoral is
989 administered in three doses, at least one week apart, in children younger than 5 years of age. For other age
990 groups, it is given in two doses at least one week apart. This vaccine has a requirement for reconstitution

991 in buffer at the time of use in order to protect the rCTB from stomach acid. Dukoral was evaluated in
992 Phase 3 efficacy trials in Bangladesh and Peru and in a Phase 4 effectiveness trial in Mozambique. In
993 Bangladesh, a double-blind, placebo-controlled trial in 90,000 volunteers showed a cumulative vaccine
994 efficacy over three years of 50 percent in all age groups. The vaccine efficacy was 19 percent in children
995 less than 5 years of age and 65 percent in children older than 5 years (199, 200). In a trial of 1,400 adult
996 male recruits from the Peruvian military, vaccine efficacy was 86 percent after five months (201). The
997 Mozambique trial studied the vaccine in 22,000 volunteers (30 percent of whom were HIV infected) and
998 showed 85 percent protection after one year of follow-up (202). Dukoral has a minimum age requirement
999 of 2 years.

Table 5. Currently available cholera vaccines.

Trade name	Producer	Location(s) of licensure	Cost per dose (USD)
Dukoral	Crucell Sweden AB, acquired by Valneva (Sweden) in 2015	International	$4.70
Shanchol	Shantha Biotechnics Ltd. (India), acquired by Sanofi Pasteur in 2009	South and Southeast Asia	$1.84
Euvichol	EuBiologics (South Korea)	International	$1.20
mORC-Vax	Vabiotech (Vietnam)	Vietnam	N/A
OraVacs	Shanghai United Cell Biotechnology (China)	China and the Philippines	N/A
Vaxchora	PaxVax, acquired by Emergent BioSolutions (United States) in 2018	United States	$270

Abbreviation: N/A, information not available.

1000 Shanchol is a vaccine similar to Dukoral, licensed for use in South and Southeast Asia. It contains the
1001 same inactivated *V. cholerae* as Dukoral, but also contains *V. cholerae* of the O139 serogroup. Shanchol
1002 does not contain rCTB. It is administered in two doses at least one week apart. It underwent an efficacy
1003 trial in India with 67,000 volunteers, which showed 68 percent cumulative efficacy over three years in all
1004 age groups. The vaccine efficacy was 43 percent over three years in children less than 5 years of age
1005 (203). Shanchol has a minimum age requirement for administration of 1 year. A version of this vaccine,
1006 mORC-Vax, is manufactured in Vietnam for in-country use.

1007 OraVacs is manufactured in China and licensed for use in China and the Philippines. Like Dukoral, this
1008 vaccine contains *V. cholerae* of the O1 serotype, El Tor, and classical biotypes, but it also contains rCTB.
1009 OraVacs is administered in enteric-coated capsules in two doses at least one week apart. Efficacy data for
1010 this vaccine seem to be unavailable in the literature.

1011 Euvichol, developed by EuBiologics, was first prequalified by WHO in 2015 (204). Euvichol is an
1012 inactivated whole-cell vaccine that has a similar composition as Shanchol: it contains the O1 and O139
1013 serogroups, Inaba and Ogawa serotypes, and the El Tor and classical biotypes. Like Shanchol, it does not
1014 contain rCTB. Euvichol was approved by the South Korea Ministry of Food and Drug Safety on the basis
1015 of a non-inferiority trial in comparison with Shanchol in the Philippines (205). This Phase 3 study of
1016 1,263 adults and children found that vibriocidal responses after two doses of each vaccine were very
1017 similar: 75 to 80 percent in adults and roughly 90 percent in children. A recently developed presentation,

1018 Euvichol-Plus®, is packaged in plastic tubes rather than glass vials. This presentation reduces product
1019 volume and weight, thus facilitating distribution in low-resource settings.

1020 Hillchol™ (Hilleman Laboratories, India), another killed whole-cell vaccine candidate, is currently
1021 progressing through development and represents an important novel approach. Hillchol replaces the four
1022 O1 Ogawa/Inaba strains in Dukoral, Shanchol, and Euvichol with a single El Tor Hikojima strain that
1023 expresses both Ogawa and Inaba lipopolysaccharide (LPS) (206). In a recent Phase 1/2 study in
1024 Bangladesh, Hillchol demonstrated a safety and immunogenicity profile that was non-inferior to Shanchol
1025 (207). Hillchol-B contains the antitoxin component rCTB, which is produced by a novel process that
1026 reduces cost relative to previous methods. Hillchol-B was recently licensed to Bharat Biotech
1027 International, which will conduct a Phase 3 study to support licensure. Hillchol-B is intended for use in
1028 low- and middle-income countries (LMICs) at an estimated cost of less than US$1 per dose.

1029 A live-attenuated cholera vaccine previously known as CVD 103-HgR, Orochol or Mutachol, is now
1030 marketed by Emergent BioSolutions as Vaxchora. This vaccine did not provide significant protection with
1031 one dose in a trial involving 67,000 children and adults in Indonesia (208). The vaccine was used
1032 effectively in Micronesia to help control a cholera epidemic. In a retrospective analysis, the single-dose
1033 vaccine was 79 percent effective in the target population (209). An early precursor of this vaccine was
1034 highly protective in a human challenge model after only a single dose (210). The newer Vaxchora
1035 formulation of this vaccine, originally developed by PaxVax and then acquired by Emergent
1036 BioSolutions, was recently shown in a Phase 3 trial of 197 American adults to be 90 percent effective
1037 against moderate-to-severe disease caused by experimental infection with *V. cholerae* ten days after a
1038 single dose, and 80 percent protective after three months (211). PaxVax received US Food and Drug
1039 Administration approval for Vaxchora in 2016 for use by adult travelers to cholera-endemic countries.
1040 The cost per dose is approximately $270 (212), which may be acceptable for travelers from high-income
1041 countries, but is obviously not compatible with introduction in LMICs. Additional studies are needed to
1042 determine if Vaxchora, or an optimized formulation, could play a role in reducing cholera disease burden
1043 in LMICs, particularly among children. A recent Phase 4 study demonstrated the safety and
1044 immunogenicity of Vaxchora among American children and adolescents 2–17 years of age is non-inferior
1045 to adults (213–215).

1046 There has been significant progress in the establishment and deployment of a WHO-coordinated oral
1047 cholera vaccine stockpile over the past several years (193). There are now processes in place to receive
1048 and evaluate requests for oral cholera vaccine usage in both outbreak and endemic scenarios. As
1049 mentioned above, Bangladesh was the first country to actively investigate this option, and other countries,
1050 including Mozambique, South Sudan, Cameroon, Uganda, Nigeria, Somalia, and Malawi, have also
1051 implemented mass vaccination campaigns (216–222). Further success with this approach will require
1052 sustained efforts and commitments from numerous humanitarian organizations, including WHO, Gavi,
1053 the Vaccine Alliance, local ministries of health, and others.

1054 The development of live-attenuated vaccines, which could have potential for greater vaccine efficacy with
1055 one dose, has also lagged behind the whole-inactivated vaccine approach. Interest in this area may be
1056 renewed with the recent success of Vaxchora described previously. A conjugate vaccine composed of
1057 *V. cholerae* O1 Inaba O-specific polysaccharide and recombinant tetanus toxin heavy chain is
1058 approaching Phase 1 clinical testing (223–225).

1059 The reluctance to use licensed cholera vaccines for mass vaccination campaigns is based partly on the
1060 vaccines' limited efficacy in clinical trials. When a vaccine of limited efficacy is administered to a
1061 substantial fraction of a population, however, the whole population sees the benefit through herd
1062 immunity—an effect whereby a critical mass of vaccinated people in a community can break the chain of
1063 transmission and actually protect unvaccinated people. Longini et al. have developed a mathematical
1064 model to estimate the combined effects of vaccination and herd immunity, and applied their model to oral
1065 cholera vaccination in Bangladesh (226). The model suggested 58 percent vaccine coverage could reduce
1066 cases by nearly 20-fold in vaccinated individuals, but also 6-fold in unvaccinated people. These
1067 reductions could protect children under 2 years of age, who are ineligible for the most widely licensed
1068 cholera vaccines.

1069 **Human challenge studies with *Vibrio cholerae***

1070 Human challenge studies with *V. cholerae* have been conducted to study disease pathogenesis, natural
1071 history, and immunity, including cross-protection, in addition to the assessment of cholera vaccines (227–
1072 232). The challenge strains have been attenuated or wild-type *V. cholerae* of different serogroups,
1073 biotypes, and serotypes grown in the laboratory and administered orally to adult human volunteers.
1074 Hospital isolation wards were used to care for and monitor volunteers during these studies and to prevent
1075 the spread of cholera in the community. Volunteers were evaluated for the onset, duration, and severity of
1076 the watery diarrhea caused by infection with *V. cholerae*.

1077 One of the earliest human challenge studies with *V. cholerae* was designed to measure cross-protective
1078 immunity across different *V. cholerae* biotypes and serotypes (227). Infection with either of the classical
1079 serotypes provided complete homologous and heterologous protection from disease, and no shedding of
1080 the challenge strains by the human volunteers was detected upon re-challenge. Infection with either of the
1081 El Tor serotypes provided 90 percent homologous and 90 percent heterologous protection from disease,
1082 but shedding of the challenge strain did occur in about 30 percent of re-challenged volunteers. The
1083 finding that infection with the El Tor biotype does not fully protect against shedding after re-exposure is
1084 of critical importance for the epidemiology of cholera infection, since shedding is a major factor in the
1085 onward transmission of *V. cholerae*.

1086 The epidemiology of *V. cholerae* supports the idea that infections with the El Tor biotype strains are a
1087 more serious concern than those with the classical biotype. Strains of the El Tor biotype have largely
1088 replaced the classical biotype strains of *V. cholerae* worldwide (198). Moreover, the circulating strains are
1089 actually a hybrid between the classical and El Tor biotypes; they are phenotypically El Tor but they
1090 produce the cholera toxin of the classical biotype (233). It is unclear when this hybrid strain arose, but it
1091 is clearly circulating in human populations and causing new outbreaks. The recent protracted cholera
1092 epidemic in Haiti is caused by this hybrid strain.

1093 A number of human challenge studies with *V. cholerae* have been conducted for evaluating candidate
1094 vaccines (see Table 6). One whole, killed vaccine and five live-attenuated vaccines have been evaluated
1095 for protection against disease in human challenge studies. Nearly 400 human volunteers participated in
1096 these studies, in which challenge strains of the O1 and O139 serogroups and of the O1 El Tor Ogawa and
1097 Inaba serotypes were used. The overall experience has been that oral challenges with wild-type
1098 *V. cholerae* have been safe in healthy adult volunteers. A three-site cholera human challenge study was
1099 conducted with CVD 103-HgR/Vaxchora (211). As noted above, Vaxchora was found to be 80 to 90

1100 percent protective against moderate-to-severe diarrhea caused by challenge with 1×10^5 colony forming
1101 units of *V. cholerae* O1 El Tor Inaba N16961. This study was the first use of the cholera human challenge
1102 model in a Phase 3 study that served as a basis for licensure without additional field studies.

Table 6. Human challenge studies with *Vibrio cholerae* after administration of candidate vaccines.

Vaccine	Number of volunteers	Challenge strain	Reference
Dukoral with or without cholera toxin B-subunit	35	O1 El Tor Inaba	Black, 1987 (228)
Live-attenuated vaccine CVD 112, based on a serogroup O139 strain of *V. cholerae*	23	O139	Tacket, 1995 (229)
Live-attenuated vaccine CVD 111, based on an O1 El Tor Ogawa strain of *V. cholerae*	26	O1 El Tor Ogawa	Tacket, 1997 (230)
Live-attenuated vaccine CVD 103-HgR, based on an O1 classical Inaba strain of *V. cholerae*	51	O1 El Tor Inaba	Tacket, 1999 (210)
Peru-15, a live-attenuated vaccine based on an O1 El Tor Inaba strain of *V. cholerae*	36	O1 El Tor Inaba	Cohen, 2002 (231)
V. cholerae 638, a live-attenuated vaccine based on an O1 El Tor Ogawa strain	21	O1 El Tor Ogawa	Garcia, 2005 (232)
Live-attenuated vaccine CVD 103-HgR (Vaxchora formulation)	197	O1 El Tor Inaba	Chen, 2016 (211)

1103 Correlates of protective immunity against cholera have not been conclusively established. In the field,
1104 higher vibriocidal antibody titers have been associated with a reduced risk of illness (234), but have not
1105 predicted protection in challenge studies (210). Recent field studies have also been used to investigate
1106 correlates of protective immunity. These studies have indicated that O1 LPS-specific memory B-cell
1107 levels and levels of serum immunoglobulin A against CTB, O1 LPS, and toxin-coregulated pilus A
1108 (TcpA) may also be markers for reduced risk of cholera (235, 236). One of these findings was supported
1109 by the recent report that CVD 103-HgR (Vaxchora) primed response to TcpA in a human challenge study
1110 (237). Gut microbiome characteristics have also been associated with cholera vaccine responses (238).

1111 Human challenge studies have provided some evidence of protective immunity. Vaccinated individuals
1112 that are re-challenged show a marked reduction of shedding of the bacteria, which is taken as evidence of
1113 antibacterial immunity interfering with survival and growth of vibrios in the intestine. During vaccine
1114 development, mucosal sampling is not routinely performed and evaluation has relied on serum vibriocidal
1115 antibody titers. A high baseline in individuals residing in cholera-endemic areas, however, complicates
1116 the use of this marker as a surrogate for cholera immunity.

1117 **Summary**

1118 *Vibrio cholerae* is a continuing source of morbidity and mortality in humans living in cholera-endemic
1119 regions of the world. Outbreaks of cholera have continued to occur, and most of the recent outbreaks have
1120 been in Africa. The outbreaks in Haiti and Yemen affected 5 percent of each country's population. Three
1121 internationally licensed cholera vaccines and three other cholera vaccines licensed for a limited
1122 geographic region or for use in a single country are currently available, and have been used in several
1123 mass vaccination campaigns in Haiti, Bangladesh, Iraq, and at least nine countries in sub-Saharan Africa
1124 (216, 239). Epidemiological modeling suggests that even modest coverage with vaccines akin to those

1125 already available, combined with herd immunity, could lead to major reductions in the numbers of
1126 cholera cases in communities where vaccination is provided, including among young children who are
1127 ineligible for vaccination.

1128 Human challenge studies have played a significant role in the development of cholera vaccines, especially
1129 those studies that demonstrated cross-protection among biotypes and serotypes, and the study of Dukoral
1130 with and without CTB. Vaxchora is the first vaccine licensed for travelers solely on the basis of a human
1131 challenge study (i.e., in the absence of additional field trials), a significant milestone for the controlled
1132 human infection model field. However, as this vaccine is only approved for use by adult travelers, it
1133 remains to be seen what impact it will have on reducing burden in LMICs. Human challenge studies could
1134 be further applied to evaluate new candidate vaccines for evidence on correlates of protective immunity,
1135 especially if safe and well tolerated, and procedures for direct or indirect mucosal sampling could be
1136 applied. Challenge studies could also be helpful toward down-selecting candidate live-attenuated vaccines
1137 by studying how the immune responses in the field trial of CVD 103-HgR differed from those seen in the
1138 challenge model with a precursor of this vaccine.

1139

Enterotoxigenic *Escherichia coli*

Epidemiology and public health impact of enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) remains among the most common bacterial causes of diarrhea-associated morbidity and mortality (240–246). ETEC is often the first bacterial illness that children experience in endemic areas, with infants and young children experiencing two to five diarrhea episodes due to ETEC during their first three years of life (247–249). Recent studies in sub-Saharan Africa and South Asia conducted under the Global Enteric Multicenter Study (GEMS and GEM-1A) have reaffirmed the continuing importance of ETEC as one of the top four causes of moderate-to-severe diarrhea among children less than 5 years of age in both regions (243, 250). In a prospective community-based assessment of the role of ETEC in morbidity and mortality among infants and young children in low-resource settings, ETEC was also found to be associated with persistent diarrhea, which is consistent with its role in contributing to stunting in these settings (244, 251–256).

It is estimated that ETEC causes about 220 million diarrhea episodes globally, with approximately 75 million episodes in children under 5 years of age and between 19,000 and 42,000 yearly deaths in this age group (240, 256–260). Because ETEC infection is associated with persistent diarrhea, which can lead to stunting early in life, infants and young children are at a higher risk of death due to other infectious disease causes, which may contribute an additional 34,000 deaths annually to the global ETEC mortality burden among infants and young children in low-resource settings. In addition, environmental enteric dysfunction and stunting associated with ETEC infection is thought to contribute to chronic poor health outcomes later in life, such as diabetes, obesity, and hypertension (259, 261), thus further highlighting the importance of developing effective vaccine interventions that induce long-lasting immunity against the most common ETEC strains causing life-threatening illness in infants and young children. ETEC is also the most frequent bacterial cause of diarrhea among travelers to Africa, Asia, and Latin America, including military personnel deployed to these areas. ETEC, in both travel and endemic settings, is becoming increasingly refractory to antibiotic treatment and is now considered a growing antimicrobial resistance threat (262). Wellcome Trust recently recommended that vaccine development for enteric *E. coli*-associated illness, including ETEC, be accelerated (262).

ETEC strains have a wide geographic distribution. They are endemic in most of Africa, Asia, and Latin America. Systematic estimates of ETEC prevalence in cases of diarrhea by country or region have been difficult because of complex diagnostics. An improved laboratory assay using real-time quantitative PCR to concurrently detect the presence and relative level of 19 enteropathogens including ETEC has been described (263). Despite improvements in ETEC detection, the true global burden of ETEC-associated morbidity and mortality remains under debate.

Pathogenesis and diversity of enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* is usually acquired by ingesting contaminated food or water. ETEC colonizes the small intestine by binding to epithelial cells using hair-like structures on the surface of ETEC bacteria termed fimbriae, which promote adhesion and the intracellular delivery of enterotoxins. The enterotoxins disrupt enzymes in cellular metabolic pathways that control the transport of electrolytes (such as chloride, sodium, potassium, and calcium) in and out of cells. Toxin delivery results in moderate

to massive release of fluid and electrolytes into the lumen of the small bowel, resulting in a life-threatening watery diarrhea.

Two ETEC toxins exist: heat-labile toxin (LT) and heat-stable toxin (ST). LT is a protein that has a similar structure and 80 percent amino acid homology to cholera toxin. ST is a small peptide consisting of 18 to 20 amino acids that is unique to ETEC. LT and ST interfere with separate metabolic pathways leading to the same effects. Epidemiological studies have shown that strains expressing ST, either alone or in combination with LT, are associated with the most severe episodes of ETEC diarrhea in young children (264). LT-only expressing ETEC strains have tended to be discounted as human pathogens because of their lack of association with moderate-to-severe diarrhea in young children in studies such as GEMS (243, 265). However, it is clear from other epidemiological studies and controlled human infection model (CHIM) trials that subpopulations of LT-only ETEC strains expressing colonization factors, like CS17, can be human pathogens and should be considered in disease burden estimates (248, 249, 266–274). In the community-based MAL-ED study, LT-only expressing ETEC strains were shown to be associated with persistent diarrhea, thus further supporting the concept that at least some LT-only strains can be human pathogens (252). New animal model data as well as *in vitro* studies also support the pathogenicity of LT-only strains and the potential contribution of LT-producing ETEC strains to not only acute and persistent diarrhea but long-term intestinal sequelae as well, such as environmental enteric dysfunction (EED) and loss of gut barrier function (272, 273). More recent CHIM data also indicates that even asymptomatic ETEC infection can induce significant intestinal inflammation which provided additional evidence that ETEC can contribute to stunting, EED and malnutrition (275, 276).

ETEC encodes proteins termed colonization factor (CF) antigens, by which ETEC are characterized. ETEC strains are also characterized into serogroups using O (lipopolysaccharide) and H (flagella) antigens. Considerable effort has been made to find associations between O, H, CF, and toxins that would identify common constellations of antigens useful for inclusion in vaccines, but when the complexity of this approach was recognized, it was acknowledged that these antigens are “too diverse to be practical unless common epitopes can be identified and exploited” (277).

A subsequent review of the published literature on CF antigen and toxin expression (278) also found that 27 percent of isolates express LT alone and another 33 percent of isolates express LT in combination with ST. The most frequently expressed CFs were CFA/I (17 percent), CFA/II (9 percent), and CFA/IV (18 percent). Isidean et al. found marked variation across regions and populations (278). The most widely distributed and commonly found ETEC phenotype was O6:H16 CFA/II LTST, which accounted for 11 percent of isolates (277).

The CFs were subsequently renamed *E. coli* surface (CS) antigens in the mid-1990s due to a better understanding of the range of structures the CFs represented (264). In this review, we include reports that were published before and after the CS nomenclature was adopted. Wherever possible, the term “colonization factor” is used to include CF and CS designations.

Enterotoxigenic *Escherichia coli* vaccine development

Prevention and treatment options to address diarrheal illness from ETEC exist and are important for reducing the health impact of the high burden of infection. However, they are not always practical to implement and sustain in many low-resource settings due to compliance issues or decreasing

1219 effectiveness (i.e., antibiotics). As such, these interventions have limitations with respect to achieving
1220 equitable and sustainable coverage. An ETEC vaccine could play a critical and complementary role in the
1221 most resource-constrained and highly impacted parts of the world.

1222 The development of an effective ETEC vaccine is an important goal for public health in low- and middle-
1223 income countries (LMICs) and would also be of benefit to international travelers to endemic areas (279–
1224 281). ETEC vaccine development has also been a World Health Organization (WHO) priority for the last
1225 20 years and a guidance document published in 2006 has helped guide development efforts (282). In
1226 addition to potential direct effects on morbidity, mortality, and other ETEC-associated longer-term
1227 negative health outcomes, such as stunting, an ETEC vaccine might have indirect effects on decreasing
1228 antimicrobial resistance, increasing herd (community) immunity, and protecting from all-cause diarrhea
1229 (275). Although several promising oral and parenteral candidate ETEC vaccines have been tested and are
1230 in the pipeline at different stages of preclinical and clinical development (see Table 7 below), currently no
1231 licensed vaccines against ETEC diarrhea exist. The development of vaccines against these infections has
1232 been hampered by technical challenges, insufficient support for coordination of research and development
1233 efforts, and a poorly defined market to incentivise investment in product development. In addition, infants
1234 and children under 5 years in LMICs (the target age group for an ETEC vaccine) have proven difficult to
1235 immunize effectively against enteric pathogens (241, 281, 283). The precise reasons for this are under
1236 investigation, but likely relate to poor sanitation and hygiene conditions prevalent in LMICs (see the
1237 rotavirus and poliovirus section below for further discussion on this topic); as well as compromised gut
1238 health associated with stunting, EED, and malnutrition. To help frame the development of ETEC vaccine
1239 preferred product characteristics (PPCs), WHO recently developed a guidance document to help
1240 developers target their vaccine development efforts (240). The primary goal of the PPCs was to help
1241 guide the development of a safe, effective, and affordable ETEC vaccine that reduces moderate-to-severe
1242 diarrheal disease and morbidity in infants and children under 5 years of age in LMICs.

1243 The main strategies for the development of ETEC vaccines have been to induce immune responses
1244 against CF antigens and against one or both of the ETEC toxins (279). Birth cohort studies in LMICs,
1245 Phase 2b CHIM studies, and limited Phase 2 trials in travelers have indicated that both types of antigens
1246 contribute to protective immunity (241, 242, 248, 281–285). Therefore, vaccines have been designed to
1247 express some of the most prevalent CF antigens based on the epidemiological data described above. The
1248 fact that ETEC strains produce two different toxins also presents a challenge for vaccines. Including the
1249 B-subunit of LT from ETEC in vaccines is feasible because this subunit has no toxin activity. Mutant
1250 forms of the A-subunit of ETEC LT that have lost their toxigenic activity are often used in vaccines, but
1251 they may not completely mimic the antigenicity of native toxin. Mutant forms of LT, such as double-
1252 mutant R192G/L211A (dmLT), can not only serve as safe vaccine antigens but have also been shown to
1253 have adjuvant activity for co-administered ETEC antigens, and may also make these vaccines more
1254 protective (281, 283, 286–288). dmLT has also recently been shown to adjuvant responses to ETEC
1255 vaccine antigens when they are given parenterally (by the intradermal or intramuscular routes) (287). The
1256 ST of ETEC is homologous to two human proteins and is poorly immunogenic in its native form. It has
1257 been proposed that a vaccine that contains five of the CF antigens and LT could provide protection
1258 against 80 percent of global ETEC strains (279). Table 7, compiled from Zhang et al., Walker, Bourgeois
1259 et al., and Fleckenstein et al., describes the candidate ETEC vaccines in development (242, 279, 281,
1260 289).

Table 7. Candidate enterotoxigenic *Escherichia coli* vaccines in development.

Type of vaccine		Candidate	Description	Stage of development	Developer(s)
Live-attenuated		ACE527	Composed of three attenuated strains of ETEC that collectively express six CF antigens and the B-subunit of ETEC LT	Phase 2b	Acambis and PATH (no longer in clinical development)
Whole-cell inactivated		ETVAX	An *E. coli* K-12 strain that over-expresses four CF antigens of ETEC and a hybrid LT/CTB antigen, adjuvanted with dmLT	Phase 2b	Scandinavian Biopharma
Live hybrid-vectored		Shig-ETEC hybrid	ShigETEC LPS-free cell expressing conserved ETEC and *Shigella* antigens	Phase 1	EveliQure
		Shigella hybrid	Several different live-attenuated candidate *Shigella* vaccines have been engineered to express ETEC CF antigens	Preclinical	Center for Vaccine Development, University of Maryland
Subunit	Anti-toxin	Dukoral CTB	There is antigenic similarity between the CTB found in the cholera vaccine Dukoral and LT of ETEC	Licensed for prevention of cholera	Valneva
		dmLT	dmLT of ETEC	Phase 1	Tulane University, PATH, and NIH
		LT-patch	ETEC LT delivered transcutaneously with a skin patch	Phase 3	Intercell (no longer in clinical development)
		ST[a]	Various mutant toxoids of ETEC ST designed to improve immunogenicity while maintaining safety	Preclinical	University of Bergen
	Fimbriae	Fimbrial tip adhesin CfaE	Various constructs designed to block adhesion of ETEC to the intestinal epithelium by inducing antibodies to the tips of fimbriae	Phase 2b	US Naval Medical Research Center
		Adhesin-toxoid fusion	A CF consensus peptide fused to dmLT-ST mutants	Preclinical	Johns Hopkins University and University of Illinois
	Novel proteins	Alternative virulence factors[a]	YghJ, a protein secreted by the same pathway as ETEC LT; EatA, a serine protease that degrades mucin and promotes ETEC access to mucosal surfaces; EtpA, a secondary adhesin	Preclinical	Washington University School of Medicine and GlyProVac

Table 7. Candidate enterotoxigenic *Escherichia coli* vaccines in development.

Type of vaccine	Candidate	Description	Stage of development	Developer(s)

Abbreviations: CF, colonization factor; CTB, cholera toxin B-subunit; dmLT, double-mutant heat-labile toxin; *E. coli*, *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; LPS, lipopolysaccharide; LT, heat-labile toxin; NIH, US National Institutes of Health; ST, heat-stable toxin.

[a] ST toxoid and conserved ETEC proteins can be used in combination with other cellular or subunit vaccine options.

A promising live-attenuated ETEC vaccine candidate, ACE527, under development by PATH, was halted for lack of funding. It is comprised of three attenuated strains of ETEC that have been genetically engineered to express multiple CF antigens. Collectively, the three strains express CFA/I, CS1, CS2, CS3, CS5, and CS6. Each of the three strains expresses the B-subunit of the ETEC LT. In a Phase 1 trial, ACE527 was found to be safe and well tolerated up to a dose of 10^{11} colony forming units. Strong immune responses were elicited to each of the three ETEC strains and to the B-subunit of LT (290). A Phase 2 trial was then conducted in the ETEC human challenge model (291). The ACE527 vaccine candidate provided substantial protection against the duration of diarrhea and was associated with reduced stool shedding of the challenge strain, an indicator of reduced colonization and reduced stool volume. The vaccine candidate showed significant protection against severe diarrhea (protective efficacy = 41 percent; $p = 0.03$). In a subsequent Phase 2b immunization and challenge study, the protective efficacy of this vaccine candidate was improved by adding a third dose of vaccine to the immunization regimen and also by adding the dmLT adjuvant to the vaccine formulation (284). The adjuvanted vaccine candidate provided significant protection against severe diarrhea (65.9 percent; $p = 0.01$), as well as protection against diarrhea of any severity (58.5 percent; $p = 0.02$), when subjects were challenged six to seven months after immunization.

An oral, whole-cell inactivated ETEC vaccine candidate is also under development. An early version of this vaccine candidate combined killed ETEC bacteria expressing five CF antigens with recombinant cholera toxin B (rCTB). It showed short-term efficacy in adult travelers to Mexico and Guatemala (285), but was not efficacious in infants in Egypt or Bangladesh (264). The candidate was reformulated to increase its CF antigen content and to include a hybrid LT/CTB antigen. The reformulated vaccine candidate, called ETVAX, is delivered with dmLT, a mucosal adjuvant. In a Phase 1 trial in 129 Swedish adults, the modified candidate was safe and showed improved immunogenicity, especially to CS6, when it was administered with 10 µg of the dmLT adjuvant (288). In more recent clinical studies, the safety and immunogenicity of ETVAX was assessed in infants and young children in Bangladesh (286). This was also the first evaluation of the dmLT adjuvant in a vaccine candidate in this age group. The data showed that infants and children can develop significant intestinal immune responses to this vaccine candidate. Further, the frequency, magnitude, and breadth of these responses could be improved in infants by giving ETVAX with dmLT. The results also suggested that any reduction in immune response due to giving lower amounts of vaccine in infants could be reversed by including dmLT. In addition, infants given ETVAX with dmLT developed an immune response rapidly after the first dose, while infants given the vaccine candidate alone needed two doses to develop a response. Encouraging ETVAX results were also recently obtained in a Phase 2b study conducted in Finnish travelers spending 12 days in Benin as part of a cultural exchange program. The study results confirm the excellent safety profile and positive

immunogenicity of ETVAX. While not reaching the study's protective efficacy goal of 70 percent, the overall results indicate that ETVAX remains a strong ETEC candidate vaccine that warrants further clinical investigation. ETVAX was 56 percent efficacious against all severe diarrhea, independent of pathogen (p = 0.025) (292). In addition, ETVAX was 52 percent protective against more moderate forms of ETEC diarrhea, which can also impact on daily travel-related activities. Among participants with severe diarrhea, significantly fewer participants that received the candidate vaccine received antibiotic or antisecretory drug treatment compared to placebo recipients (p = 0.03), indicating that ETVAX reduced illness severity in the few breakthrough cases that occurred. Given the protective efficacy results against ETEC in Benin and the encouraging results of the descending age study in Bangladeshi infants and young children, ETVAX is moving forward in clinical development with Phase 1 and 2b studies in progress in Zambia and The Gambia in infants and young children (Pan African Clinical Trials Registry Trial No. PACTR201905764389804) (293).

Another approach to ETEC vaccine development has been the construction of hybrid vectors expressing ETEC antigens. For example, a polyantigen comprised of ETEC CFs, LT, and ST is being expressed in the live-attenuated strain of *Salmonella* Typhi ZH9, with the intent to develop a vaccine against both typhoid fever and ETEC. The vaccine has been shown to induce antibodies against the ETEC CF and both of the ETEC toxins in mice (279, 281, 294). Live-attenuated hybrid vaccines based on *Shigella flexneri* strains expressing ETEC antigens or *Shigella* mutants designed to unmask conserved antigens shared between ETEC and *Shigella* are also under development, by the Center for Vaccine Development at the University of Maryland and by EveliQure in Austria, with both candidates moving into Phase 1 studies during the 2021 time frame.

Subunit vaccine candidates against ETEC toxins are also under development, with CF antigens and a detoxified form of LT being the primary targets. One approach is to use a conserved protein or peptide component of that protein that is located at the tips of fimbriae. The adhesive protein (adhesin) is involved in the first step of ETEC attachment to mucosal epithelial cells. Antibodies against it might block attachment by a wide spectrum of ETEC strains. Two versions of a vaccine based on this concept are under development by the US Naval Medical Research Center (NMRC) and the University of Illinois working in collaboration with the Johns Hopkins University Bloomberg School of Public Health (281, 287, 295). The Navy program is the most advanced, with Phase 1 and 2b trials of two candidate antigens completed. Parenteral delivery of the CfaE adhesin by the intradermal route with mLT induced strong serum and mucosal responses to the adhesin and reduced the severity and incidence of ETEC illness in a CHIM involving challenge with the H10407 strain of ETEC. In addition, intramuscular immunization with CssBA and mLT was also highly immunogenic, inducing both systemic and mucosal immunity as well (296). Funding is currently being sought for a follow-on immunization and challenge study with this antigen-adjuvant combination. A CF consensus peptide fused to dmLT-ST mutants is in preclinical development and has shown encouraging immunogenicity in mouse and rabbit models, as well as showing protective efficacy in the ETEC piglet model (275). Plans are being made to produce pilot lots of this vaccine candidate under current Good Manufacturing Practice so clinical testing can begin.

Novel virulence factors are also being considered for incorporation into candidate ETEC vaccines. Two of these, described in Table 7, are in preclinical development at the Washington University School of Medicine and in Europe by GlyProVac in Denmark (281, 287, 294).

1336 **Human challenge studies with enterotoxigenic *Escherichia coli***

1337 The human challenge model for ETEC has been used for more than 40 years. The initial study with the
1338 model was reported in 1971. The study's collaborators at the University of Maryland and the US military
1339 Walter Reed Army Institute of Research (WRAIR) established that two ETEC strains isolated from US
1340 soldiers in Vietnam with acute, watery diarrhea could cause disease in healthy volunteers. Since that time,
1341 at least 32 human challenge studies have been performed using 14 different strains of ETEC and
1342 including nearly 600 human volunteers (297–303).

1343 Table 8 describes the ETEC strains that have been used in the human challenge model. Collectively,
1344 strains expressing colonization factors CFA/I, CS1, CS2, CS3, CS5, CS6, CS17, CS19, and CS21 have
1345 been used. Strains expressing LT, ST, or both have been included in these studies. The challenge strains
1346 come from eight different countries. The institutions involved in the development of ETEC challenge
1347 strains have included the University of Maryland, Johns Hopkins University, WRAIR, the US Army
1348 Medical Research Institute of Infectious Diseases, NMRC, the University of Texas, and the University of
1349 Bergen, Norway.

Table 8. Strains of enterotoxigenic *Escherichia coli* used in the human challenge model.

Challenge strain	Colonization factors and toxins	Country of origin	Strain developer(s)
214-4	CF unknown, ST	Mexico	CVD
B2C	CS2, CS3, LT/ST	Vietnam	CVD and WRAIR
B7A	CS6, LT/ST	Vietnam	CVD, WRAIR, and NMRC
DS26-1	CS19, LT	Saudi Arabia	JHU and NMRC
E24377A	CS1, CS3, LT/ST	Egypt	CVD and WRAIR
E2528-C1	CF unknown, LT	Caribbean	CVD
H10407	CFA/I, LT/ST	Bangladesh	University of Texas Medical School, WRAIR, and CVD
H1765	CFAII, LT/ST	Bangladesh	University of Texas Medical School
LSN03-016011/A	CS17, LT	Turkey	JHU and NMRC
TD255-C4	CF unknown, LT	Mexico	CVD
TW10598	CS2, CS3, CS21, LT/ST	Guinea-Bissau	University of Bergen and CVD
TW10722	CS5, CS6, ST	Guinea-Bissau	University of Bergen and CVD
TW11681	CFA/I, CS21, ST	Guinea-Bissau	University of Bergen and CVD
WS0115A	CS19, LT/ST	Egypt	JHU and NMRC

Abbreviations: CF, colonization factor; CS, coli surface antigen; CVD, Center for Vaccine Development, University of Maryland; JHU, Johns Hopkins University; LT, heat-labile toxin; NMRC, US Naval Medical Research Center; ST, heat-stable toxin; WRAIR, Walter Reed Army Institute of Research.

1350 Table 9 summarizes 30 studies that have been performed with the ETEC human challenge model (300).
1351 These studies have been the major sources of information about ETEC pathogenesis and the human
1352 immune response to infection with ETEC. Products that have been evaluated in ETEC human challenge
1353 studies include probiotic *Lactobacillus*, bovine colostrum and immunoglobulin G, and bismuth
1354 subsalicylate for the prevention of diarrhea; live-attenuated, whole-inactivated, and subunit candidate
1355 vaccines; and antibiotics (283, 297–303). Recently, the model has been improved through the

1356 development of a severity score that gives another outcome measure when evaluating treatment and
1357 preventive interventions (304) and the development of a new ST-only ETEC challenge strain (TW10722)
1358 that will help in the development of ST toxoids and ST toxoid-containing vaccines (305, 306). In
1359 addition, new applications of the model include microbiota studies and application of advanced systems
1360 biology tools to better understand the factors contributing to innate and adaptive immunity parameters
1361 triggered by ETEC infection (298, 299, 307–309).

Table 9. Human challenge studies with enterotoxigenic *Escherichia coli.*

Challenge strain(s)	Number of volunteers	Purpose of study	Institution(s)	Reference[a]
B2C B7A	24	Establish ETEC as a cause of diarrhea	CVD and WRAIR	DuPont, 1971
214-4	17	Establish ST as a virulence factor	CVD	Levine, 1977
H10407	13	Role of CF antigens in pathogenesis and immunity	University of Texas Medical School	Evans and Satterwhite, 1978
B7A E2528-C1	29	Capacity of prior infection to protect from homologous or heterologous challenge	CVD	Levine, 1979
H10407 214-4 TD255-C4	13	Explore whether person-to-person transmission of ETEC can occur	CVD	Levine, 1980
TD255-C4 214-4 B7A	48	Capacity of *Lactobacillus* to prevent diarrhea in volunteers after challenge with ETEC strains	CVD	Clements, 1981
H10407 B7A	47	Compare two antibiotics for treatment of diarrhea in ETEC-challenged volunteers	CVD	Black, 1982
H10407	26	Evaluate a candidate vaccine antigen, somatic pili, for protection from disease in ETEC-challenged volunteers	CVD	Levine, 1982
H10407	16	Evaluate bismuth subsalicylate for protection from diarrhea in ETEC-challenged volunteers	University of Texas Medical School	Graham, 1983
H10407 H1765	11	Evaluate purified CFs as candidate vaccine antigens for protection from disease in ETEC-challenged volunteers	University of Texas Medical School	Evans, 1984
E24377A	14	Evaluate CS1 and CS3 colonization factors as antigens for protection from disease in ETEC-challenged volunteers	CVD	Levine, 1984
E24377A	6	Evaluate fimbriae antigens as vaccines	CVD	Levine, 1986
H10407	14	Evaluate whole-inactivated ETEC vaccines for protection from challenge	University of Texas Medical School	Evans, 1988
H10407	20	Protection by milk immunoglobulin concentrate against ETEC challenge	University of Maryland School of Medicine	Tacket, 1988

Table 9. Human challenge studies with enterotoxigenic *Escherichia coli*.

Challenge strain(s)	Number of volunteers	Purpose of study	Institution(s)	Reference[a]
E24377A	10	Vaccination with CF encapsulated in microspheres, followed by challenge with ETEC	University of Maryland School of Medicine	Tacket, 1994
H10407	10	Milk immunoglobulin with activity against CF antigens can protect from challenge	University of Maryland School of Medicine	Freedman, 1998
E24377A	10	Milk immunoglobulin does not protect from challenge when administered with a standard meal	University of Maryland School of Medicine	Tacket, 1999
B7A H10407	32	Pathogenicity of ETEC expressing CFA/I or CS6 in human volunteers, and capacity of ciprofloxacin to resolve symptoms of disease and prevent shedding	US Army Medical Research Institute of Infectious Diseases	Coster, 2007
E24377A	20	Protective efficacy of transcutaneous immunization with ETEC LT in a challenge study	JHU and Iomai	McKenzie, 2007
E24377A	16	Efficacy of live-attenuated vaccine strain PTL-003 in protection from challenge	JHU and Acambis	McKenzie, 2008
LSN03-016011/A WS0115A DS26-1	38	Experimental challenge with ETEC expressing CFs CS17 and CS19	JHU and NMRC	McKenzie, 2011 (302)
LSN0-016011/A	36	Protective efficacy of anti-CS17 bovine colostrum passively administered to volunteers	JHU and NMRC	Savarino, 2019 (310)
H10407	45	Dose de-escalation study for ETEC challenge strain H10407	JHU and Gothenburg University	Harro, 2011 (301)
H10407	30	Examine lower challenge doses in an effort to refine the model	JHU and PATH	Chakraborty, 2018 (298)
H10407	36	Protective efficacy of ACE527 plus dmLT	JHU and PATH	Harro, 2019 (284)
TW10598	30	Develop a new ETEC challenge strain for use in vaccine studies	University of Bergen	Skrede, 2014 (303)
TW11681	27	Develop a new ST-producing challenge strain for use in vaccine studies	University of Bergen	Sakkestad, 2019 (297)
TW10722	21	Develop a new ST-producing challenge strain for use in vaccine studies	University of Bergen	Sakkestad, 2019 (305)

1362

Table 9. Human challenge studies with enterotoxigenic *Escherichia coli*.

B7A	30	Protective efficacy of bovine IgG antibodies against CS6 expressing *E. coli* passively administered to volunteers	JHU and NMRC	Talaat, 2020 (311)

Abbreviations: CF, colonization factor; CS, coli surface antigen; CVD, Center for Vaccine Development, University of Maryland; dmLT, double-mutant heat-labile toxin; *E. coli*, *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; IgG, immunoglobulin G; JHU, Johns Hopkins University; LT, heat-labile toxin; NMRC, US Naval Medical Research Center; ST, heat-stable toxin; WRAIR, Walter Reed Army Institute of Research.

[a] See Porter et al. 2011 (300) for the complete references for the challenge studies published prior to 2011.

Summary

The status of ETEC as a pathogen of public health importance in much of the world is now firmly established. No vaccine is available against the pathogen, but significant progress is being made with both promising inactivated whole-cell and subunit vaccine candidates moving further into clinical development. A human challenge model, in use for nearly five decades, has been the principal source of our current knowledge of ETEC pathogenesis and has guided antigen selection for the current candidate vaccines in development. The model has undergone a number of improvements in recent years, which has added value to it as an important research tool in the development new preventive interventions for ETEC. The recent successful development of an ST-only expressing challenge strain, in particular, is a significant advance. This advance in the ETEC challenge model portfolio of strains is very timely since efforts to development a safe and immunogenic ST toxoid for inclusion in future ETEC vaccine formulations has accelerated in recent years. Genetic attenuation of the ST toxin by targeted amino acid substitutions has shown promise in yielding safe and immunogenic toxoids in animal models (306), but these encouraging observations need to be confirmed in Phase 1 and Phase 2b studies. In this regard, we now have a well-characterized human challenge strain that will help support this important vaccine development effort.

The application of the human challenge model to the discovery of correlates of protective immunity has been limited. CHIM study results indicate that both anti-CF and anti-LT toxin immunity can contribute to protection, but more work is needed to better define correlates of protection.

Candidate vaccines against ETEC are in clinical development now that could advance to large field trials based on the results in human challenge studies. These include the inactivated whole-cell ETVAX vaccine, which is poised to begin Phase 3 trials in LMICs and in travelers in the 2021/2022 time frame. With continued funding, NMRC's adhesin-based subunit vaccine approach is also close to moving into late-stage clinical development.

The antigenic diversity of ETEC strains has only limited representation among the most widely used ETEC challenge strains. Nearly 80 percent of the human challenge studies have used just three strains (H10407, E24377A, and B7A). All three express both LT and ST. The CF antigens collectively expressed by the three strains are CFA/I, CS1, CS3, and CS6. The further development of the LSN03-016011/A strain by Johns Hopkins University and NMRC has provided the field with it first LT-only CS17-positive strain.

1393 As indicated above, a major dilemma facing the field of ETEC vaccine development is whether or not
1394 vaccines should induce immunity to ST. Seventy-five percent of ETEC strains express ST, and it is
1395 unlikely that the diarrheal disease induced by ETEC can be completely abrogated by vaccines that do not
1396 induce immunity to ST. However, ST is poorly immunogenic in humans, which may be due in part to the
1397 fact that it closely resembles a self-antigen. A few candidate vaccines in early development appear
1398 promising but they have not moved into human studies. The availability now of a new ST-only ETEC for
1399 future CHIMs studies will play an important role in evaluating any ST toxoids. In addition recent
1400 proteomic microarray analysis of serum and fecal extracts from subjects experimentally infected with
1401 ETEC strains, as well as analysis of samples from adults and children in LMICs, suggests that other
1402 protein antigens, such as EtpA, EatA, and YghJ, if added to future vaccine formulations along with CS6,
1403 could significantly improve coverage against ETEC strains that produce ST toxin only (289, 295, 312,
1404 313) and the ST only strain will also play an important role in evaluating these novel vaccine constructs.

1405

Shigella

Epidemiology, diversity, and public health impact of *Shigella*

Shigella is a major cause of diarrheal disease worldwide. In the Global Enteric Multicenter Study (GEMS) (314–317), *Shigella* was among the four most common enteric pathogens with attributable risk for moderate-to-severe diarrhea. Reanalysis of these GEMS data using molecular diagnostics indicates that *Shigella* remains the most attributable cause of moderate-to-severe diarrhea in children younger than 5 (315–317). *Shigella* was the second leading cause of diarrheal mortality in 2016 among all ages, and the leading bacterial cause of diarrhea, accounting for approximately 212,000 deaths. Although *Shigella* infections occur worldwide, across all age groups, with broad geographical distribution, the greatest burden is among children in low- and middle-income countries (LMICs), where it is estimated to be responsible for between 28,000 (318) and 64,000 deaths (317) among children under 5 years of age. The incidence of *Shigella* disease is highest among infants and children living in the World Health Organization's (WHO) African and Eastern Mediterranean Regions (319–328).

Historically, the field has held that dysentery (bloody diarrhea) was the main presentation for *Shigella* infection, but increasingly sensitive molecular diagnostic tools indicate that *Shigella* contributes to the burden of non-bloody diarrhea almost as much as it does to bloody diarrhea (321, 322). In addition, growing evidence shows that repeated, non-fatal, moderate-to-severe *Shigella* infections contribute to an increasing number of negative health outcomes in children living in LMICs, including stunting, severe malnutrition, and several metabolic disorders developing later in life (316, 320, 323–331), as well as mortality resulting from other infectious diseases (320, 326). In addition, there is growing evidence of increasing antimicrobial-resistant *Shigella*, and it is accordingly included on the WHO and US Centers for Disease Control and Prevention antimicrobial resistance threats lists (262, 332). In light of increasing evidence showing that *Shigella* can present as non-bloody diarrhea, leading to misdiagnosis and inappropriate administration of antibiotics, this becomes an even greater concern. Not surprisingly, in a recent report highlighting the potential role of vaccines in combating antimicrobial resistance, the Boston Consulting Group and Wellcome Trust recommended accelerated exploration of multi-pathogen combined vaccines against high-risk antimicrobial-resistant pathogens like *Shigella* and *Campylobacter* (262).

Finally, *Shigella* is also a major illness among military personnel deployed to LMICs and travelers to endemic areas. Research is ongoing to study long-term health issues related to *Shigella* infections in these populations, and there is already some indication in military populations that *Shigella* may increase incidence of irritable bowel syndrome (IBS) post-infection (333–337).

Four distinct species of *Shigella* are recognized, each with wide global distribution. The most prevalent are *Shigella flexneri* and *Shigella sonnei*, which together comprise about 90 percent of moderate-to-severe diarrhea cases due to *Shigella*. *S. sonnei* predominates mostly in high-income countries, whereas *S. flexneri* tends to predominate in LMICs.

Approximately 50 serotypes of *Shigella* are recognized, which poses a significant challenge with respect to strain selection for vaccines and controlled human infection model (CHIM) studies. Fortunately, recent multi-year, multi-country studies of the distribution and prevalence of *Shigella* serotypes have been

1445 conducted, as well as a large study focused on mainland China (Table 10) (338). These studies, together
1446 with earlier data (339), provide a solid estimate of the most prevalent serotypes worldwide.

1447 In association with the GEMS studies in Africa and South Asia, a total of 1,130 *Shigella* isolates were
1448 serotyped (340). *S. flexneri* accounted for nearly 70 percent of isolates. Five of the 15 known serotypes of
1449 *S. flexneri* comprised 90 percent of the identified *S. flexneri* strains. These serotypes were 2a, 6, 2b, 3a,
1450 and 1b, in order of prevalence. *S. sonnei* comprised 23.7 percent of isolates, while *S. dysenteriae* and *S.*
1451 *boydii* comprised 5 percent and 5.4 percent, respectively. These results were in close accord with the data
1452 published from 1966 to 1997, as reviewed by Kotloff et al. (339).

1453 In addition, results of the GEMS and an earlier von Seidlein et al. study were also are in close accord (see
1454 Table 10). Both studies identified 2a, 3a, and 6 as the most prevalent serotypes of *S. flexneri* and both
1455 studies found that about one in four isolates was *S. sonnei*.

Table 10. Recent studies reporting the prevalence of *Shigella* serotypes.

Group	Species	Serotypes	Prevalence estimates (%)			Most prevalent serotypes
			GEMS study	von Seidlein study	Chang study	
A	*Shigella dysenteriae*	15	5.0	4.0	N/A	N/A
B	*Shigella flexneri*	15	69.5	68.0	76.2	2a, 3a, and 6
C	*Shigella boydii*	19	5.4	6.0	N/A	N/A
D	*Shigella sonnei*	1	23.7	22.0	21.3	N/A

Abbreviations: GEMS, Global Enteric Multicenter Study; N/A, information not available.

1456 The recommendation for the future development of *Shigella* vaccines emerging from this body of work is
1457 that a quadrivalent vaccine comprised of *S. sonnei* and *S. flexneri* 2a, 3a, and 6 could provide substantial
1458 global coverage. Stockpiles of an *S. dysenteriae* serotype 1 vaccine could be considered as a practical
1459 approach to contain future outbreaks (340).

1460 **Pathogenesis of *Shigella***

1461 *Shigella* is highly infectious. It is transmitted from person to person or by ingesting contaminated food or
1462 water. As few as 100 organisms can cause disease. Infection with *Shigella* leads to an acute intestinal
1463 infection of variable severity (341, 342). At the mild end of the spectrum, a watery diarrhea ensues
1464 followed by an inflammatory bacillary dysentery of varying severity, which can include fever, abdominal
1465 cramps, and stools containing blood, sheets of white blood cells, and mucus. The histopathology of tissue
1466 biopsies from infected individuals shows invasion of the epithelium by inflammatory infiltrates, edema,
1467 and most importantly, regions where the colonic epithelium is actually eroded, with the formation of
1468 microscopic abscesses. Disease is usually self-limiting, but severe cases require oral rehydration and
1469 antibiotic treatment. The more severe sequelae of *Shigella* infection can include hemolytic uremic
1470 syndrome, IBS, and reactive arthritis (ReA) (341, 342). As indicated above, the high global burden of
1471 *Shigella* infection also makes this enteropathogen a significant contributor to environmental enteric
1472 dysfunction and stunting among infants and young children living in *Shigella*-endemic areas. It is also
1473 suspected that the significant gut inflammation induced by *Shigella* infection contributes to more long-
1474 term sequelae in LMICs, including several metabolic disorders affecting health and productivity later in
1475 life, such as diabetes, obesity, and hypertension (320, 324, 326, 328–330).

1476 *Shigella* species encode three different toxins that are important in pathogenesis. ShET1 is
1477 chromosomally encoded and found only in *S. flexneri* serotypes 2a and 2b. It is a structural homologue of
1478 cholera toxin and heat-labile toxin of enterotoxigenic *Escherichia coli*. ShET2 is encoded on a virulence
1479 plasmid and is found in all serotypes. ShET1 and ShET2 cause the watery diarrhea that is the prodrome of
1480 disease (343–347).

1481 The most severe form of *Shigella* disease is caused by infection with *S. dysenteriae* serotype 1, which is
1482 the only *Shigella* serotype that encodes Shiga toxin. The action of this toxin results in vascular lesions in
1483 the colon, kidney, and central nervous system. The resulting hemolytic uremic syndrome can be life
1484 threatening (339).

1485 *Shigella* are facultative intracellular pathogens and a constellation of chromosomal-encoded and plasmid-
1486 encoded genes are involved in the processes, whereby *Shigella* enter cells, further invade the colonic
1487 epithelium, and manipulate and evade host immune responses. The salient features of the invasion process
1488 have been reviewed by Schroeder and Hilbi (342).

1489 We have only a limited understanding of how the human immune system contains and eliminates *Shigella*
1490 infection. How the carbohydrate portion of lipopolysaccharide (LPS) is presented to the immune system
1491 in a form that evokes effective cellular and humoral immune responses is unclear (341). The assumption
1492 is that humoral and cellular responses to *Shigella* serotype-specific LPS (and possibly to other antigens
1493 including invasion proteins) work together to make subjects immune to shigellosis (334–337, 343–346,
1494 348–353). Field studies and results from recent CHIM studies indicate that higher levels of serum
1495 immunoglobulin A or immunoglobulin G antibodies to LPS and invasion proteins are associated with a
1496 reduced risk of shigellosis (343–346, 348, 349, 354, 355). In addition, recent immune profiling of
1497 antigen-specific antibody responses to conserved invasion proteins done using newly developed
1498 proteomic arrays, as well as similar analysis by more traditional enzyme-linked immunosorbent assay
1499 methods, indicates that antibodies to IpaB and D may also contribute to protection and that the immune
1500 profiles associated with protection against *S. flexneri* and *S. sonnei* may differ (346, 356) (see more
1501 details below).

1502 **The *Shigella* human challenge model**

1503 The *Shigella* human challenge model was established with the study of Shaughnessy et al. in 1946 using
1504 strains FW I through FW V (357). These strains were later classified as *S. flexneri* 2a*.* Since that time, at
1505 least 18 different *Shigella* challenge studies have been conducted involving more than 700 human
1506 volunteers, using 47 strain/dose combinations (358). The available challenge strains and their utilization
1507 are described in Table 11. The majority of the challenge studies have used *S. flexneri* 2a strains. In the
1508 more recent studies with *S. flexneri* 2a strain 2457T, much lower challenge doses were used than in the
1509 initial study with FW I through FW V strains. Nearly 200 volunteers have been challenged with *S. sonnei*.
1510 The challenge studies with strains of *S. dysenteriae* are few in number and less recent.

Table 11. *Shigella* human challenge models.

Species	Serotype	Strain(s)	Typical dose range evaluated (CFU)	Number of challenges administered
Shigella flexneri	2a	2457T	10^2 to 10^4	456

Table 11. *Shigella* human challenge models.

Species	Serotype	Strain(s)	Typical dose range evaluated (CFU)	Number of challenges administered
		FW I through FW V	10^8 to 10^{10}	
Shigella sonnei	N/A	53G	5 x 10^2	178
Shigella dysenteriae	1	A-1, M-131	10^2 to 10^4	44

Abbreviations: CFU, colony forming units; N/A, not applicable, as *S. sonnei* has a single serotype.

1511 The main difficulty with the *Shigella* human challenge models has been the lack of an obvious
1512 relationship between the challenge dose and attack rates for diarrhea and dysentery (358). The attack rates
1513 for doses of *S. flexneri* strain 2457T have been evaluated in 14 different studies and the attack rates for *S.*
1514 *sonnei* strain 53G have been evaluated in five studies. Even with similar doses, little consistency in attack
1515 rates has been seen. During the course of this work, the delivery of challenge inoculate in buffer has
1516 gradually replaced the earlier use of milk or water, and a grading scale for the severity of diarrhea was
1517 developed by Black et al. (359). A standard dose has not been adopted to enable comparability among
1518 studies.

1519 Variability in attack rates across studies may impact the utility of the human challenge model for
1520 discerning differences between *Shigella* species and serotypes with respect to pathogenesis. It may also
1521 make the evaluation of candidate *Shigella* vaccines more difficult, since many of the candidate vaccines
1522 may reduce the severity of disease rather than prevent infection *per se*. The model has been consistent,
1523 however, with respect to re-challenge studies designed to determine whether infection confers protection
1524 from disease. Two studies with *S. flexneri* strain 2457T (360, 361) showed a reduction in disease of about
1525 65 percent upon re-challenge with the same strain, and prior infection protected 67 percent of volunteers
1526 from disease upon re-challenge with *S. sonnei* strain 53G (362).

1527 *Shigella* CHIM studies are usually performed in naïve adults from high-income countries, although the
1528 major target population is children and adults in LMICs. A dose-escalation human challenge study was
1529 conducted in Thailand with *S. sonnei* strain 53G, the first conducted in an endemic region (363). The
1530 study was performed in three groups of 12 volunteers each to determine the dose that yielded an attack
1531 rate of at least 70 percent in Thai subjects. An attack rate of 75 percent was observed with a challenge
1532 dose of 1,680 colony forming units (CFU). A reasonable correlation was seen between the shedding of the
1533 challenge strain in stool samples and the development of clinical disease in the study volunteers. Further
1534 studies in adults in LMICs are being explored, but it remains to be determined what relevance CHIM
1535 studies in adults may have for pediatric populations in these settings.

1536 The known post-infection sequelae of *Shigella*, including ReA and IBS, may need to be specifically
1537 addressed in human challenge studies. A meta-analysis of three different epidemiological studies showed
1538 a 7-fold increase in risk of IBS following acute gastroenteritis caused by *Shigella* (364). The extent to
1539 which early treatment of shigellosis in the human challenge model may help to reduce the risk of sequelae
1540 like IBS and ReA is not clear. Historically, subjects enrolling in *Shigella* challenge studies have been
1541 screened for human leukocyte antigen (HLA) B27, since this has been shown to be a risk factor for ReA
1542 development. The consensus view is that vaccination with *Shigella* vaccines will not be associated with a
1543 higher risk of ReA, even in HLA B27-positive individuals (364). Other ways in which the performance of

1544 the *Shigella* challenge model might be improved include additional standardization with respect to the
1545 preparation, administration, and dose range of challenge strains. To this end, an *S. sonnei* CHIM using a
1546 lyophilized lot of strain 53G was established (ClinicalTrials.gov Identifier NCT02816346) (356). A dose
1547 in the 1,500 to 2,000 CFU range of 53G was selected as the dose for future challenge studies using this
1548 product. This model will enable direct comparison of study results between institutions and ensure better
1549 consistency over time in the challenge inoculum. Improvements should also include establishing
1550 consistent case definitions for diarrhea and dysentery. The case definitions for diarrhea and dysentery
1551 could then be systematically aggregated with other clinical and microbiological data to generate a
1552 composite score for the severity of disease. Expanding the range of challenge strains to include *S. flexneri*
1553 serotypes 3a and 6 may also be useful. A strong effort to provide standardized guidance for use of the
1554 *Shigella* CHIM was published in a series of papers from a workshop to provide a consensus report on this
1555 subject (343–345).

1556 In addition to permitting identification of promising vaccine candidates, CHIM studies enable exploration
1557 into immunological markers of vaccine-induced immunity. For example, a core *Shigella* proteome
1558 microarray consisting of more than 2,000 antigen targets common to all *Shigella* species was used to
1559 assess serum samples from volunteers immunized with killed, attenuated, and wild-type *S. flexneri* 2a
1560 (346). These studies identified a series of type III secretion system proteins for which immunoreactivity
1561 against was associated with clinical protection.

1562 ***Shigella* vaccine development**

1563 The current vaccine pipeline for *Shigella* includes both oral (live-attenuated and inactivated whole-cell) as
1564 well as subunit candidates for parenteral administration. Live-attenuated vaccines against *Shigella* were
1565 the first to be evaluated clinically. The difficulty with this approach has been to maintain immunogenicity
1566 while minimizing reactogenicity. A series of clinical trials with different live-attenuated vaccines
1567 developed at the Center for Vaccine Development (CVD) at the University of Maryland has led to
1568 selection of strain CVD 1208. This strain, a GuaBA auxotroph in which ShET1 and ShET2 toxins have
1569 been deleted, combines low reactogenicity with robust immune responses. Adaptation of this strain to
1570 growth on soy media to meet regulatory requirements generated the vaccine candidate CVD 1208S (365).
1571 An attenuated vaccine based on *S. sonnei* with the toxin deletions and a VirG mutation to attenuate
1572 through prevention of intercellular spreading was constructed at the US military Walter Reed Army
1573 Institute of Research. This strain was selected for the stability of its invasiveness plasmid, a plasmid that
1574 is required for expression of O-antigen in this serotype. The attenuated *S. sonnei* vaccine has completed a
1575 Phase 2b trial (334, 335, 337).

1576 Glycoconjugate vaccines represent another major approach to *Shigella* vaccine development. Originally
1577 developed at the US National Institutes of Health (NIH), in this type of vaccine O polysaccharides from
1578 relevant *Shigella* serotypes are covalently linked to a carrier protein. The NIH prototype vaccine provided
1579 74 percent protection against *S. sonnei* outbreaks at Israeli army bases. Unfortunately, the vaccine was not
1580 shown to be efficacious in 1- to 4-year-old Israeli children (366). Current work with this approach
1581 involves synthetic conjugates as well as a bioconjugate (334, 337, 348–351). Another novel approach to
1582 "O" antigen-based parenteral *Shigella* vaccines is the Generalized Modules for Membrane Antigens
1583 (GMMA) technology from GlaxoSmithKline (335). A prototype bioconjugate vaccine developed by
1584 LimmaTech in Switzerland that targeted *S. flexneri* 2a was recently shown to be highly immunogenic and

1585 to induce protection against more severe shigellosis in a Phase 2b immunization and challenge study
1586 (354). Similar Phase 2b immunization and challenge studies to assess the protective potential of the
1587 synthetic conjugate and GMMA approaches was also recently completed with results suggesting that the
1588 immunogenicity of the vaccine needed to be improved (348, 349, 367). In addition, a promising new
1589 subunit vaccine candidate, Invaplex$_{\text{AR-DETOX}}$, recently entered into early-stage clinical development. The
1590 vaccine is unique in that it is the only *Shigella* vaccine in clinical development that attempts to induce
1591 both "O" LPS antigen and conserved Ipa protein responses. It also relies on an MsbB mutation in the lipid
1592 portion of its LPS antigens to make it less reactogenic for parenteral delivery. Initial safety and
1593 immunogenicity results have been very encouraging, and more in-depth immunological studies are still
1594 ongoing. (353, 368).

1595 In addition to the vaccines described above, a wide range of *Shigella* vaccine candidates is in the
1596 preclinical stage or in Phase 1 trials. Some new vaccine candidates seek to exploit conserved antigens,
1597 such as proteins involved in the cellular invasion process in addition to or instead of O-antigen. Table 12,
1598 adapted from recent reviews by Mani et al. and Walker et al. (334, 337), describes these candidate
1599 vaccines.

Table 12. *Shigella* vaccine candidates.

Vaccine type	Candidate	Current stage	Developer(s)
Attenuated	CVD 1208S based on *S. flexneri* 2a	Phase 1	CVD
	WRSS1 based on *S. sonnei*	Phase 2b	WRAIR
	ShigETEC LPS-free cell	Phase 1	EveliQure
Vectored	Ty21a typhoid vaccine expressing O-antigens of *S. sonnei* or a combination of *S. sonnei*, *S. flexneri* 2a and 3a, and *S. dysenteriae* O-antigen	Preclinical	Protein Potential
Inactivated	Sf2aWC, a formalin-killed *S. flexneri* 2a whole-cell vaccine	Phase 1	WRAIR and PATH
	Shigella Truncated Mutant (352)	Preclinical	IVI and PATH
Conjugate	Chemical glycoconjugates of *S. sonnei* and *S. flexneri* 2a	Phase 3	NIH Institute for Child Health and Human Development
	Recombinant bioconjugate	Phase 2b	LimmaTech Biologics and GSK
	Synthetic glycoconjugate	Phase 2	Institut Pasteur
Serotype-dependent mixture	Invaplex$_{\text{AR-DETOX}}$, a chemically defined product consisting of invasion plasmid antigens and O-antigens	Phase 1	WRAIR
	GMMA consisting of *Shigella* outer membrane vesicles from an overproducing strain	Phase 2	GSK
	Outer membrane vesicles: naturally secreted outer membrane vesicles of *Shigella*	Preclinical	University of Navarra, Spain
	IpaD/IpaB (DB) fusion	Preclinical	PATH
	PSSP-1 (Pan-*Shigella* surface protein 1)	Preclinical	IVI

Table 12. *Shigella* vaccine candidates.

Vaccine type	Candidate	Current stage	Developer(s)
Serotype-independent proteins	34kDa OMP, an outer membrane protein conserved across multiple *Shigella* species	Preclinical	National Institute of Cholera and Enteric Diseases, India

Abbreviations: CVD, Center for Vaccine Development, University of Maryland; ETEC, enterotoxigenic *Escherichia coli*; GMMA, Generalized Modules for Membrane Antigens; GSK, GlaxoSmithKline; IVI, International Vaccine Institute, South Korea; LPS, lipopolysaccharide; NIH, US National Institutes of Health; WRAIR, Walter Reed Army Institute of Research.

Current *Shigella* vaccine candidates need to provide coverage for up to four serotypes, and the availability of a CHIM for each of these will greatly facilitate their development. Early proof-of-concept work has largely involved *S. flexneri* 2a and *S. sonnei*, so CHIMs for these two strains have been established. However, additional work remains to standardize these models to improve consistency and allow comparisons of vaccine candidates across studies. It is expected that as candidates for these strains advance, it will be possible to find field sites permitting Phase 3 evaluation of protective efficacy. Licensure studies for other strains may not be feasible in the field, so determination of the effectiveness of a vaccine component against these strains could depend on results from a CHIM trial. These less common strains such as *S. flexneri* 3a and 6 and possibly *S. dysenteriae* need to be included among the current challenge strains.

Summary

Diarrheal disease from enteric pathogens is widely acknowledged to be a major worldwide threat to public health. *Shigella* is one of the most important enteric pathogens for which no safe and effective vaccine is currently available. The development of *Shigella* vaccines faces several important challenges, including the high diversity of *Shigella* serotypes and the fact that the protective immunity in communities and in human challenge studies has been serotype specific. Correlates of protective immunity probably include O-antigen-directed antibodies, but the details of the types of responses to be elicited for protective vaccines remain largely undefined beyond that. Recent evidence from Ndungo et al. (346) and Clarkson et al. (356) suggesting that antibody responses to both LPS and conserved protein antigens, like IpaB and IpaC, may contribute to protection needs further investigation.

Human challenge models have played a critical role in defining the pathogenesis and virulence of *Shigella* species as well as in assessing vaccine potential of various candidates. The challenge models will be even more valuable if the diversity of challenge strains can be expanded, and if studies can be consolidated against standardized and readily available challenge strains.

1625 # *Campylobacter*

1626 **Epidemiology and public health impact of *Campylobacter jejuni***

1627 *Campylobacter jejuni* is a common enteric bacterial pathogen thought to be responsible for a significant
1628 fraction of diarrhea cases worldwide. The Global Enteric Multicenter Study found that *Campylobacter*
1629 infections in India, Bangladesh, and Pakistan were common causes of severe diarrhea in infants and
1630 young children (369). Surveillance data are incomplete in many resource-limited countries. One report in
1631 2002 suggested that 40 to 60 percent of children under the age of 5 in these countries will have
1632 experienced at least one episode of campylobacteriosis (370). Post-infection sequelae include Guillain-
1633 Barré syndrome (GBS), reactive arthritis (ReA), Crohn's disease, and irritable bowel syndrome (IBS).
1634 More recent burden studies indicate that even asymptomatic *Campylobacter* infection among infants and
1635 young children in low- and middle-income countries (LMICs) can be associated with negative health
1636 outcomes, including an increase in intestinal permeability and both intestinal and systemic inflammation,
1637 which can contribute to an increased risk of children developing stunting and environmental enteric
1638 dysfunction (371). Alarmingly, *Campylobacter* strains from LMICs have become increasingly antibiotic
1639 resistant. In 2017, the US Centers for Disease Control and Prevention along with the World Health
1640 Organization designated multi-antimicrobial-resistant *Campylobacter* as a serious threat to public health
1641 (262). In addition, a recent Wellcome Trust report urged that better prevention and control measures, like
1642 vaccines, be prioritized for accelerated development (262).

1643 **Pathogenesis and diversity of *Campylobacter jejuni***

1644 In LMIC settings, disease features most commonly reported are watery stool, fever, abdominal pain,
1645 vomiting, dehydration, and presence of fecal leukocytes. Patients are also often underweight and
1646 malnourished, which may be secondary to repeated infections or a marker of a poor nutrition state. In
1647 addition to these acute disease effects, however, a number of chronic sequelae have been identified.

1648 Among these chronic sequelae, *C. jejuni* is the most frequent pathogen associated with GBS (372, 373),
1649 which is considered to be the leading cause of acute flaccid paralysis worldwide (given the success of
1650 polio vaccination campaigns). An estimated 0.1 percent of *Campylobacter*-infected individuals develop
1651 GBS, which is a serious neurological disorder that can cause a wide range of symptoms, from mild
1652 weakness of the extremities to near-complete paralysis and respiratory insufficiency. Molecular mimicry
1653 between lipooligosaccharides (LOS) produced by *Campylobacter* and an auto-immune reaction against
1654 human nerve gangliosides is the cause of GBS following campylobacteriosis. Most patients recover in a
1655 few months, but the case fatality rate can be as high as 3 percent. About 20 percent of GBS patients
1656 experience ongoing functional disability and more than half report severe fatigue for more than one year
1657 (374). One recent study attributed about 30 percent of studied cases of GBS to recent infection with
1658 *C. jejuni* (375). In LMICs, *Campylobacter*-associated GBS may be more common among younger age
1659 groups than adults (376). Aside from GBS, other studies in LMICs have identified stunting, microbiome
1660 changes, functional bowel disorders, and reactive arthropathies as other chronic consequences of
1661 *Campylobacter* infection and disease (372, 373). As indicated above, these negative health outcomes can
1662 also result from chronic asymptomatic infections with *Campylobacter*, which lead to gut inflammation
1663 and a change in gut permeability (371).

1664 ReA associated with *Campylobacter* infection is also thought to have an immunological basis. The
1665 incidence of ReA can be as high as 7 percent in community outbreaks of campylobacteriosis. Guerry et al.
1666 estimate that the post-infective attributable risk of ReA ranges from 1 to 5 percent (377). Like ReA
1667 caused by other pathogens of the gastrointestinal tract, ReA following *Campylobacter* infection is thought
1668 to be associated with HLA B27. Joint symptoms arise about two weeks after infection and usually resolve
1669 completely, although the symptoms may be chronic or relapsing in 5 percent of cases (377).

1670 Although diseases like IBS and Crohn's are sequelae of many cases of campylobacteriosis, data regarding
1671 the degree to which *Campylobacter* is the causative agent are conflicting. Guerry et al. indicate that
1672 between 1 and 10 percent of the risk of IBS is attributable to *Campylobacter* infection. They cite recent
1673 evidence that *C. jejuni* can breach the intestinal barrier in susceptible individuals, which may set up
1674 conditions for chronic inflammatory responses (377).

1675 *C. jejuni* strains are highly diverse and the basis for this diversity is quite complex. Unlike other bacterial
1676 pathogens of the intestinal tract, campylobacteria have a polysaccharide capsule. The capsular
1677 polysaccharides (CPS) expressed on the surface of *C. jejuni* strains are highly variable. More than 40
1678 years ago, Penner and Hennessy developed a serotyping system based on a passive slide agglutination test
1679 for heat-stable antigens (378). This system has underpinned our understanding of the role of *C. jejuni*
1680 polysaccharides in epidemiology, pathogenesis, and immunity (377, 379, 380). Forty-seven serotypes, or
1681 serotype complexes, of *C. jejuni* are recognized. The basis for the observed serotypes of *Campylobacter* is
1682 not the typical lipopolysaccharides called "O-antigens." Campylobacteria synthesize an LOS, which is
1683 linked to the bacterial cell wall in a different way than O-antigens. The Penner typing system is based
1684 primarily on CPS, but the LOS can also contribute to serotype specificity (380).

1685 In addition to polysaccharide antigens, several *Campylobacter* protein targets have been explored as
1686 potential antigens, including flagellin (381), flagellin-secreted proteins (382), and the outer membrane
1687 protein PorA (383). These may serve as important alternative approaches, given the complexity of
1688 *Campylobacter* polysaccharide expression and variation.

1689 Differences in serotype distribution between LMICs versus high-income countries may also play an
1690 important role in differences in long-term sequelae between these settings (377, 380). Such differences
1691 may provide important guidance for the selection of *Campylobacter* strains for inclusion in candidate
1692 vaccines and for selecting challenge strains for use in controlled human infection models (CHIMs), as
1693 described in the following sections.

1694 **Controlled human infection model studies with *Campylobacter***

1695 Challenge strain selection for use in CHIM studies with *Campylobacter* has evolved over the years.
1696 Experimental human infections with *Campylobacter* were first reported between 1988 and 1998 (384).
1697 The challenge strain used was 81-176, a prototype isolate that was also used for preparing candidate
1698 inactivated whole-cell vaccine.

1699 In 2004, Prendergast et al. reported results from the serological characterization of LOS from strain 81-
1700 176 (379). The LOS of this strain exhibited ganglioside mimicry. Prendergast et al. then studied human
1701 volunteers previously challenged with strain 81-176 and volunteers that had received a whole-inactivated
1702 candidate *Campylobacter* vaccine based on strain 81-176. No detectable, sustained increase in anti-

1703 ganglioside antibody responses were seen in the vaccinated or experimentally challenged volunteers,
1704 although weak and transient anti-ganglioside antibody responses were observed in some of the volunteers
1705 in challenge studies. In six volunteers from a previous challenge study, the *Campylobacter* strains present
1706 in stools were examined and found to have undergone phase variation in their LOS structures.

1707 In 2009, Tribble et al. reported on another study using strain 81-176 (385), which evaluated the
1708 relationship of dose to disease severity as well as the level and duration of protection from re-infection
1709 after challenge. The authors observed an illness-dose effect in the study and short-term protection from
1710 re-infection. When subjects were challenged one year after the initial challenge, less severe illness
1711 resulted following re-challenge. Production of IFN-γ by peripheral blood mononuclear cells after
1712 re-stimulation with *Campylobacter* antigens was associated with protection from illness.

1713 The *Campylobacter* human challenge model has been re-developed since 2009. New challenge strains
1714 without the potential for ganglioside mimicry have been selected for use in the *Campylobacter* challenge
1715 model (385, 386). Table 13 describes the criteria used for selecting additional challenge strains. The
1716 selection criteria were the avoidance of the molecular mimicry for human antigens, propensity for
1717 invasion of epithelial cells, passage history and provenance, and serotype.

Table 13. Selection criteria for *Campylobacter* strains to be used in human challenge.

Challenge strain	Source	Serotype	Glycolipid mimicry for GBS antigens	Invasiveness for human epithelial cells *in vitro*
81-176	N/A	HS23/36	Yes	High
RM1221	N/A	N/A	No	N/A
TGH9011	Laboratory passaged	HS-3	No	N/A
BH-01-142	Primary isolate	HS-3	No	Low
CG8421	Primary isolate	HS23/36	No	Low

Abbreviations: GBS, Guillain-Barré syndrome; N/A, information not available.

1718 None of the new challenge strains have the potential for the ganglioside mimicry that underlies GBS.
1719 Along the way, however, one of the potential challenge strains (RM1221) was found to have LOS that
1720 mimic human P blood group antigens. Another potential challenge strain (TGH9011) contains an LOS
1721 core that lacks all glycolipid mimicry. However, no information is available on the disease symptoms that
1722 TGH9011 has caused and it has been passaged in the laboratory for 25 years or more. The strains
1723 ultimately selected for CHIM studies were BH-01-142 and CG8421—both primary isolates with minimal
1724 passage in cell culture and low invasiveness for epithelial cells.

1725 Tribble et al. described the initial study with the newly developed challenge model (385). Twenty-three
1726 healthy adults that were *Campylobacter* naïve were challenged with 1 x 10^6 or 1 x 10^5 CFU of strain
1727 CG8421 and followed as inpatients. The attack rates for the two doses were 100 percent and 93 percent,
1728 and the symptoms of disease were diarrhea, abdominal cramps, nausea, and fever. No major safety
1729 concerns arose during the study. However, it should be noted that because of concerns that infection is
1730 possibly associated with more chronic functional bowel disorders, post-challenge surveillance for acute
1731 and more long-term adverse events has been extended in these more recent challenge studies to 90 and
1732 180 days post-challenge (385–387). Volunteers were treated with strain-sensitive antibiotics no later than
1733 six days after challenge and were released after antibiotics were started, symptoms were resolved, and
1734 two consecutive stool cultures were negative for *C. jejuni.* After study completion, however, two of the

volunteers experienced recrudescent infection, which resolved with subsequent antibiotic treatment. The potential for experimental infection to generate asymptomatic carriers and recrudescence would seem to represent a new obstacle to be overcome (385).

Recent data indicate a lack of homologous protection against *C. jejuni* re-challenge (387). Of the 15 volunteers administered *C. jejuni* strain CG8421, 14 developed illness. Eight of the volunteers that developed illness were re-challenged with the same strain three months later. All developed disease with no reduction in severity of illness. Unexpectedly, the immune responses that were seen upon initial infection, which included serum immunoglobulin A (IgA) and immunoglobulin G (IgG), fecal IgA, IgA antibody-secreting cells, and IFN-γ production, were not boosted upon re-challenge. Later, the same investigators examined $CD4^+$ T-cell responses using multi-parameter flow cytometry using cryopreserved cells from the previous infection and re-challenge study with strain CG8421 (388). Primary infection elicited pro-inflammatory cytokines IFN-γ, TNF-α, IL-2, and the chemokine MIP-1β. None of these responses were augmented upon re-infection.

This observation that the immune responses elicited by *Campylobacter* infection in a CHIM study were not boosted upon re-challenge with the same strain represents a further obstacle in trying to understand how *Campylobacter* challenge study results should be interpreted and how useful they may be for vaccine development. That being said, natural immunity clearly develops against *Campylobacter* in field settings; however, protection against illness may be age related or require more than one exposure to be fully protective (389). Another possibility is that the efficient induction of immune memory by *Campylobacter* may require some level of invasiveness for epithelial cells. Strain CG8421 is poorly invasive for human epithelial cells *in vitro* (see Table 13). This hypothesis is further supported by earlier controlled human infection model studies with the 81-176 strain of *Campylobacter*, which is invasive and induces short-term protection against itself (390). The role of invasion in the development of long-term protective immunity needs further investigation and the development of a new mouse disease model for *Campylobacter* may help to make these studies more practical (391). In related work, the mucosal adjuvant double-mutant heat-labile toxin (dmLT) was recently co-administered with a dose of the live-attenuated enterotoxigenic *Escherichia coli* (ETEC) vaccine ACE527 (392). This vaccine was not protective upon re-challenge when given without the adjuvant, but provided strong protection six to seven months after immunization, which included dmLT when challenged with a virulent ETEC strain. These findings could suggest using dmLT with the immunizing challenge with non-invasive strains like CG8421, followed by homologous challenge.

The *Campylobacter* CHIM has been used to gain insights into the pathogenesis of *Campylobacter* and how this important bacterial pathogen adapts to the gut environment during the infection and disease process (393), as well as into the mechanism associated with the rapid development of macrolide resistance *in vivo* (394). The model has also been used to get an early assessment of vaccine efficacy (see the vaccine development section below) and also to evaluate the ability of a new drug, rifaximin, to provide antibiotic prophylaxis against *Campylobacter* (395). While this study did not demonstrate effective prophylaxis with rifaximin, analysis of gut microbiome from participants indicated potentially significant associations between composition and disease susceptibility (396).

1774 ***Campylobacter* vaccine development**

1775 No licensed vaccine against *Campylobacter* currently exists and development may need to overcome
1776 several challenges (397). The first challenge is identifying those types of vaccines that are most likely to
1777 achieve safety, immunogenicity, and strain coverage. Selecting strains for live-attenuated or whole-cell
1778 inactivated *Campylobacter* vaccines is particularly challenging because they must have no potential to
1779 elicit anti-ganglioside antibodies in humans. Furthermore, vaccines that can do this and that are limited to
1780 a single strain of *Campylobacter* will somehow need to confer immunity to conserved protein antigens,
1781 rather than to the highly variable polysaccharide antigens that are immunodominant.

1782 Another potential pitfall in *Campylobacter* vaccine development is molecular mimicry. Whether or not
1783 the full range of human antigens that can be mimicked by *Campylobacter* is sufficiently understood is
1784 unclear. As a case in point, the examination of four potential challenge strains for CHIM studies revealed
1785 that one of them, RM1221, unexpectedly mimicked a human blood group antigen (see Table 13) (398).
1786 Since *Campylobacter* has a polysaccharide capsule, conjugate vaccines would seem to be a good option;
1787 however, the same proscription against ganglioside and other molecular mimicry would also pertain to the
1788 CPS included in the vaccine.

1789 Given the number of globally prevalent serotypes (377, 380) a vaccine based on CPS would need to be at
1790 least 8-valent (399). Fortunately, conserved, protective antigens that could be delivered as vectored or
1791 subunit vaccines have recently been identified. Two approaches utilizing highly conserved antigens to
1792 immunize against *Campylobacter* are in preclinical development. One is based on the homology between
1793 cholera toxin B-subunit (CTB) and a 53kDa major outer membrane protein, PorA, of *Campylobacter*
1794 (383). CTB has been shown to reduce colonization of adult mice challenged with *C. jejuni* (400). The
1795 other approach utilizes a conserved N-glycan heptasaccharide of *Campylobacter* for immunization. This
1796 antigen was displayed on *Escherichia coli* to immunize chickens and induced up to a 10-log reduction in
1797 *C. jejuni* colonization following challenge (401). This effect was obtained with inactivated whole cells as
1798 well as with the live-attenuated strains of the ACE527 ETEC vaccine expressing the heptasaccharide
1799 antigen (Szymanski C., personal communication). It could be of benefit to compare the anti-
1800 *Campylobacter* potential of the conserved heptasaccharide and CTB antigens in a murine model that has
1801 been recently described to demonstrate intestinal disease following oral challenge (391) and determine the
1802 possibility for synergy between them. Such studies in this small animal model could pave the way for
1803 further clinical studies with these conserved vaccine antigens.

1804 The only *Campylobacter* vaccine candidate that has recently moved from studies in small animals to the
1805 clinic consists of CPS purified from strains 81-176 (HS23/36) and CG8486 ($H54_{complex}$) conjugated to a
1806 recombinant inactivated diphtheria toxin, CRM_{197}. HS23/26 and $H54_{complex}$ are globally prevalent
1807 serotypes of *Campylobacter* (377). The conjugate has been found to be highly immunogenic in mice, and
1808 has demonstrated efficacy against diarrheal disease following a high-dose challenge with strain 81-176 in
1809 *Aotus nancymaae*, a New World owl monkey species (402). A Phase 1 first-in-human trial was recently
1810 completed (ClinicalTrials.gov Identifier NCT02067676) (403). The vaccine was well tolerated but was
1811 not as immunogenic as preclinical studies had suggested. It is anticipated that an adjuvanted version of
1812 the vaccine will go back into Phase 1 trials shortly (402).

Summary

Campylobacter infection, together with its post-infection sequelae, constitutes a major public health burden. The *Campylobacter* CHIM has been modified to eliminate the potential for GBS and is deemed safe for small-scale studies performed under controlled conditions. The current challenge strains do not represent globally prevalent serotypes, and additional strains, should they become available, could improve the relevance of the model. In the *Campylobacter* challenge model, follow-up of volunteers after study completion is needed to address the potential for recurrent disease and the development of a long-term carrier state. The potential for CHIM studies to cause post-infection sequelae other than GBS is not well understood.

Campylobacter pathogenesis and immunity are incompletely understood. We know little about the relationship of particular serotypes to invasiveness or the potential of different strains to foster post-infection sequelae other than GBS. Also, initial studies with strain CG8421 in the CHIM indicate that infection does not always induce homologous immune protection, or even a measurable memory response (385, 386). Therefore, human challenge with the available strains may provide little information on protective immunity against *Campylobacter*. The invasiveness for epithelial cells may play a significant role in protective immunity but is incompletely understood. Further unclear is how to limit the potential for invasive strains to damage the intestinal epithelium while studying their potential to induce protective immunity. The availability of a new promising mouse disease model may help investigators gain better insights into the factors contributing to longer-term protective immunity (391).

Vaccine development for *Campylobacter* faces a number of challenges, some unique to this particular pathogen. Conjugate vaccines that provide coverage for the major *C. jejuni* serotypes offer an option for further development but face the hurdle of requiring eight serotypes to be included in the vaccine. This may make the vaccine unacceptably complex. Simpler vaccine candidates based on more conserved antigens have been identified, and could enter human trials. *Campylobacter* vaccine development has received relatively little attention compared to other bacterial enteric pathogens, like ETEC and *Shigella*, but it, too, is rapidly demonstrating antibiotic resistance (394, 395). Thus, continued refinement of controlled human infection models to accelerate vaccine development for this important pathogen should certainly be pursued.

1842 # *Salmonella*

1843 **Epidemiology, diversity, and public health impact of four *Salmonella enterica* serovars**

1844 *Salmonella* are pathogenic bacteria that have both an extracellular and an intracellular phase in their life
1845 cycle. *Salmonella* infection is principally the result of ingesting contaminated food or water and disease
1846 results from local invasion of the intestinal mucosa or, in the case of typhoid and paratyphoid fevers, local
1847 invasion followed by a more generalized systemic spread and dissemination of the bacteria (404, 405).
1848 Infected individuals can manifest a carrier state of variable duration that is a source of onward
1849 transmission (406).

1850 Human disease following *Salmonella* infection takes the following main forms that have different degrees
1851 of disease severity: enteric fevers, invasive non-typhoidal *Salmonella* disease (iNTS), and non-typhoidal
1852 *Salmonella* (NTS) gastroenteritis. The enteric fevers, which include typhoid and paratyphoid, carry a
1853 1 percent case fatality rate worldwide and higher case fatality rates in resource-limited settings and among
1854 younger age groups (406). iNTS has a case fatality rate up to 25 percent, mostly in children younger than
1855 2 years of age and in immunocompromised adults in Africa. NTS gastroenteritis is a more prevalent,
1856 usually mild, self-limiting diarrheal disease with an estimated 0.1 percent case fatality rate (405, 407,
1857 408). The rapid rise in prevalence of antimicrobial-resistant strains among *Salmonella* enteric pathogens
1858 has served to re-energize vaccine development efforts (262, 409–411). Three vaccines against typhoid
1859 fever are licensed for global use, while vaccine development efforts for paratyphoid and iNTS are being
1860 accelerated (406, 409, 412). This acceleration in vaccine development efforts has been driven by a
1861 growing interest among emerging-market manufacturers and global health vaccine developers to fill gaps
1862 in development and production capacity.

1863 *Salmonella* nomenclature is summarized in Table 14. All of the organisms shown in the table belong to
1864 *Salmonella enterica* subspecies 1 and are referred to as serovars. Each serovar of *Salmonella* contains
1865 multiple serotypes.

1866 The *S.* Typhimurium and *S.* Enteritidis serovars represent an extremely diverse collection, with more than
1867 1,000 serotypes described in humans and a similar number of serotypes in animals (262, 404, 405, 410).
1868 *S.* Typhimurium and *S.* Enteritidis are a common cause of gastroenteritis in humans. The iNTS bacteria
1869 may represent distinct subsets of the *S.* Typhimurium and *S.* Enteritidis organisms that are opportunistic
1870 pathogens in immune-compromised hosts (262, 405, 409).

Table 14. *Salmonella enterica* serovars that cause severe human disease.

Organism	Disease	Host range	Main geographic concentration	Annual global burden of severe disease
Salmonella Typhi	Enteric fever	Humans	Africa, Asia, Latin America	27 million
Salmonella Paratyphi A, B, C	Enteric fever	Humans	South and Southeast AsiaSwitch	5 million
Salmonella Typhimurium	iNTS	Humans and other warm-blooded animals	Africa, Asia	N/A

Table 14. *Salmonella enterica* serovars that cause severe human disease.

Organism	Disease	Host range	Main geographic concentration	Annual global burden of severe disease
Salmonella Enteritidis	iNTS	Humans and other warm-blooded animals	Africa, Asia	N/A

Abbreviations: iNTS, invasive non-typhoidal *Salmonella* disease; N/A, information not available.

1871 The enteric fevers and iNTS have different geographic distributions (407), as summarized in Table 14.
1872 Overall, the burden of enteric fever is focused in South and Southeast Asia, whereas highest burden of
1873 iNTS is in sub-Saharan Africa. However, the global distribution of the *S. enterica* serovars that cause
1874 severe disease in humans is still fragmented and in continuous flux (412, 413).

1875 The epidemiology of these important *S. enterica* serovars is incompletely documented at present,
1876 especially in Africa (414, 415). For the enteric fevers, this gap is partly due to non-specific case
1877 presentation and lack of a widely applicable, highly sensitive, and specific diagnostic test. Blood culture
1878 is still the standard laboratory means of confirming cases of enteric fever, but the very low numbers of
1879 bacteria in blood at the onset of symptoms reduces sensitivity. A novel assay for the diagnosis of *S*. Typhi
1880 infection in which very short-term blood culture is combined with PCR for detecting *S*. Typhi DNA may
1881 hold promise in this regard (415, 416).

1882 Overall, global estimates of disease burden are sufficiently precise to establish that both enteric fevers and
1883 iNTS are of significant public health impact and warrant use of all available corrective measures. Wain et
1884 al. estimate that typhoid and paratyphoid fevers account for about 1 percent of global mortality from all
1885 causes on an annual basis (416). In the context of diarrhea- and enteric fever-related deaths and health
1886 impact, the recently completed Global Burden of Disease Study estimated that enteric fevers and NTS,
1887 which includes iNTS, accounted for 18 percent of total enteric disease deaths worldwide and for
1888 approximately 19 percent of the total disability-adjusted life years burden per year for diarrheal disease
1889 and enteric fevers (409, 411, 412). The combined typhoid, paratyphoid, and iNTS burden has led to
1890 greater interest in developing a combination vaccine that would provide coverage for all four pathotypes
1891 of invasive *Salmonella* (409, 411, 412).

1892 **Pathogenesis and immunity to *Salmonella enterica* serovars**

1893 Natural typhoid infection is usually associated with the detection of serum antibodies and mucosal
1894 secretory immunoglobulin A (IgA) intestinal antibody against various *S*. Typhi antigens; cell-mediated
1895 immune responses are also measurable (407, 409, 411, 416–419). In areas where typhoid is endemic,
1896 there is an age-related increase in the prevalence and geometric mean titer of anti-virulence antigen (Vi)
1897 antibodies (411, 412). Anti-flagella (H-antigen) serum immunoglobulin G (IgG) antibodies following
1898 natural infection are long lived (411). Trials of an early prototype Vi conjugate vaccine in Vietnam in the
1899 early 1990s demonstrated that the threshold level of IgG antibody to the Vi antigen can be a marker for
1900 protection against typhoid fever (420–422).

1901 Cell-mediated immunity also appears to play a part in protection; it has been observed that peripheral
1902 blood mononuclear leukocytes of otherwise healthy adults residing in typhoid-endemic areas that have no
1903 history of typhoid, proliferate on exposure to *S*. Typhi antigens (411, 412). Upon ingestion, these bacteria

1904 cross the intestinal barrier and multiply within the underlying macrophages, where they may persist for
1905 long periods of time and are shielded from the direct effects of antibodies. They can also take up an
1906 extracellular existence, spread from cell to cell, and enter the blood stream and the lymphatic system.
1907 Antibodies are the key defense against the extracellular phase and bactericidal and opsonophagocytic
1908 antibodies may be the mediator of this defense mechanism (411–413, 417), while T cells are required to
1909 clear intracellular bacteria. Although these basic features pertain to both enteric fevers and iNTS, the
1910 pathogenesis of the two diseases is quite distinct (408, 409, 416, 417, 420). For this reason, the two
1911 diseases may require different approaches for protection with vaccines (415, 416). Pathogenesis and
1912 immunity are poorly understood for *S.* Typhimurium, and *S*. Enteritidis. Human challenge models for
1913 iNTS are under consideration, but must be approached cautiously because of the high risk of
1914 complications in immunocompromised individuals.

1915 **Human challenge models with *Salmonella* Typhi and *Salmonella* Paratyphi**

1916 For the enteric fevers, human challenge models have been a key source of information about pathogenesis
1917 and immunity. Much of our knowledge of the pathogenesis of *S*. Typhi comes from an early series of
1918 experimental human challenge studies conducted at the University of Maryland in the 1960s and 1970s.
1919 The use of the early version of the challenge model was suspended in 1974, but a new version was
1920 recently developed (423–426).

1921 The initial human challenge studies with *Salmonella* used the *S.* Typhi Quailes strain, which was isolated
1922 in 1958 from a chronic carrier. This strain expresses a capsular polysaccharide antigen (Vi), the H-antigen
1923 (flagellin), and the O-antigens (lipopolysaccharide [LPS]). The term Vi reflects the fact that the capsular
1924 polysaccharide of *S*. Typhi is a virulence factor. The bacteria were orally administered to 213 volunteers
1925 in a dose-escalation study. The study showed higher attack rates and shorter incubation periods with
1926 increasing doses from 10^3 to 10^9 colony forming units (CFU), and provided the first experimentally
1927 controlled clinical description of typhoid fever. The typhoid fever seen in experimentally infected
1928 volunteers closely resembled that seen in natural infection (413, 423–426).

1929 Subsequent studies at the University of Maryland explored the contributions of Vi and H-antigens to
1930 strain virulence. Wild-type strains that expressed the Vi and the H-antigen were compared to strains that
1931 expressed Vi but lacked the H-antigen, and to strains that expressed neither of these antigens. These
1932 studies established that the attack rate for a given dose was halved in the absence of Vi. At higher doses,
1933 however, people infected with strains lacking Vi still developed typhoid.

1934 The early studies with *S*. Typhi were able to establish a 95 percent attack rate and a relatively linear dose-
1935 response curve. A 2-fold shortening of the incubation period with the higher doses occurred. Importantly,
1936 some participants developed bacteremia without fever—a finding postulated to represent early
1937 intracellular sequestration of the organism from the immune system.

1938 The clinical features of the typhoid fever in the volunteers that were challenged with *S*. Typhi were
1939 variable. The antibiotic chloramphenicol was administered to participants promptly after the onset of
1940 fever, which likely prevented them from developing any of the complications known to occur in natural
1941 infection. Intestinal biopsies of some participants showed that enteritis developed during the symptomatic
1942 period, but resolved without scarring in all participants examined.

1943 The early model development work at the University of Maryland also established a role for human
1944 challenge studies in the development of vaccines against *S*. Typhi. Studies in which human volunteers
1945 were administered the live-attenuated oral vaccine Ty21a prior to challenge showed that the vaccine
1946 provided up to 87 percent protective efficacy. In subsequent field trials, this vaccine conferred 96 percent
1947 efficacy over three years of follow-up. Ty21a went on to become the world's only live oral typhoid
1948 vaccine and is still in use today.

1949 Even with two widely available licensed typhoid vaccines, a Ty21a live-attenuated vaccine and a Vi
1950 capsular polysaccharide vaccine (detailed in the next section), typhoid fever is still a major cause of
1951 morbidity and mortality, especially in low-resource countries. According to Waddington et al., "a further
1952 paradigm shift in our understanding of disease pathogenesis and host response is required … to advance
1953 typhoid control" (413). Efforts to shift the paradigm have resulted in a new human challenge model that
1954 differs in several important respects from the earlier version outlined above. The first study using the new
1955 model was conducted through a collaboration between the Center for Vaccine Development (CVD) at the
1956 University of Maryland and the University of Oxford.

1957 The new model can use challenge strains of either of the agents of enteric fever, *S*. Typhi or *S*. Paratyphi.
1958 The *S*. Typhi strain is the same as in the earlier model. Challenge with the newly developed *S*. Paratyphi
1959 A strain is at a target dose of 1–5 x 10^3 CFU (427). Participants are typhoid-naïve volunteers and the
1960 studies are conducted on an outpatient basis, unlike in the historical studies. Table 15 further describes the
1961 challenge strains and their modes of administration. The purpose of the first published study, conducted at
1962 the Centre for Clinical Vaccinology and Tropical Medicine in Oxford, United Kingdom, was to determine
1963 the dose of the Quailes strain of *S*. Typhi required to achieve an attack rate of 60 to 75 percent in typhoid-
1964 naïve volunteers (423).

Table 15. Human challenge models for *Salmonella* Typhi and *Salmonella* Paratyphi A serovars.

Organism	Strain	Method of production and administration
Salmonella Typhi	Quailes	Isolated from the gallbladder of a chronic carrier in 1958. A challenge dose of 10^3 to 10^4 colony forming units in bicarbonate buffer solution is ingested.
Salmonella Paratyphi A	NVGH308	Ingestion of 1–5 x 10^3 colony forming units of the challenge strain in bicarbonate buffer solution.

Note: Both human challenge models have been conducted at the University of Oxford, United Kingdom.

1965 The Quailes strain of *S.* Typhi expresses the Vi antigen and is fully susceptible to the antibiotics used to
1966 treat typhoid fever. A fresh working cell bank was manufactured under Good Manufacturing Practice for
1967 use in this and subsequent studies. The strain in the cryopreserved cell bank was characterized by full
1968 genome sequencing, which confirmed that it contained the expected virulence determinants. The actual
1969 doses (determined by bacterial culture of the challenge inoculum) were 1.3 x 10^3 and 2.0 x 10^4 CFU.

1970 Forty-one healthy adult volunteers 18 to 60 years of age that had not received typhoid vaccination or been
1971 resident for more than six months in typhoid-endemic areas participated in the study. They were carefully
1972 screened for a number of general conditions that would make them ineligible, such as the presence of
1973 gallstones. *S*. Typhi forms biofilms on the surface of gallstones, where the organisms are extracellular but
1974 impervious to immune clearance. Through the biliary system, the bacteria can be reintroduced into the
1975 intestinal tract and are persistently shed (408). The absence of gallstones is thought to reduce, if not

1976 eliminate, the potential to establish a carrier state in challenged volunteers (which was doubly ensured by
1977 antibiotic treatment before study completion in all challenges volunteers).

1978 Volunteers were followed daily for at least 14 days and then treated with antibiotics, or earlier if
1979 indicated. Follow-up visits were at 21, 28, and 60 days after challenge. No volunteers required hospital
1980 admission or intravenous antibiotics or fluids. After antibiotic administration, no shedding or carriage of
1981 the challenge organisms was detected. Typhoid infection was defined either by fever of 38 °C or greater
1982 sustained for more than 12 hours by day 14 after challenge, or by blood culture for the presence of
1983 *S*. Typhi organisms, or both. Attack rates were 55 percent with the low-dose challenge and 65 percent
1984 with the high-dose challenge. The clinical profiles of infection varied considerably and represented a
1985 range of disease from asymptomatic to clearly symptomatic. Two important observations were that
1986 *S*. Typhi organisms were frequently shed in stools before diagnosis (albeit at low levels) and that infected
1987 individuals developed serum antibody responses to H- and O-antigens, but not to Vi antigen. The Vi
1988 antigen is the basis of a licensed typhoid vaccine. According to the human challenge study of Waddington
1989 et al., Vi may represent an antigen to which an immune response is not always elicited by infection with
1990 *S*. Typhi (423–426).

1991 The challenge model described above was also used to evaluate a new challenge strain of *S*. Paratyphi A
1992 (see Table 15). This study targeted up to 80 participants and was completed in 2016. Challenge with the
1993 NGH308 strain at an actual median dose of 2.4 x10^3 CFU, range 2.2–2.8 was well tolerated and
1994 associated with an acceptable safety profile. The resulting spectrum of illness was such that this new
1995 model appears suitable for the assessment of vaccine efficacy using endpoints that include bacteremia and
1996 other enteric and systemic symptoms (427).

1997 These new challenge models have also been instrumental in gaining new insights into the immunology of
1998 immunity to enteric fever (423–426) and in the identification of additional potential immune markers for
1999 protection (serum IgA and IgG_1) and in supporting policy recommendations by the World Health
2000 Organization (WHO) Strategic Advisory Group of Experts on Immunization for the introduction of Vi
2001 conjugate-based anti-typhoid vaccines (412, 423–430).

2002 **Development of vaccines against *Salmonella***

2003 Table 16 describes the historical and the currently licensed vaccines against *S.* Typhi. The first vaccines to
2004 be developed were whole-inactivated preparations of *S.* Typhi bacteria administered parenterally. These
2005 vaccines consistently showed efficacy against typhoid fever in the 70 percent range, but they were too
2006 reactogenic for general use and their manufacture was discontinued (416). Ty21a is a live-attenuated
2007 vaccine developed at the Swiss Serum and Vaccine Institute based on a chemically mutagenized strain of
2008 *S*. Typhi. It is licensed for adults and children over 5 years of age and requires three to four oral doses in
2009 capsule form. The vaccine had 87 percent efficacy in a human challenge model (as earlier described) and
2010 showed field efficacy of 95 percent in Egypt and 67 percent in Chile. A meta-analysis published by WHO
2011 in 2008 concluded that the vaccine is 51 percent effective against typhoid fever. Ty21a is licensed for
2012 international use (416).

Table 16. Currently licensed *Salmonella* Typhi vaccines.

Licensed vaccine and trade name	Description	Developer(s)
Not applicable	Killed whole-cell vaccine	Not currently manufactured due to high rates of reactogenicity
Ty21a (Vivotif®)	Live-attenuated, oral licensed for adults and children older than 5 years of age	Emergent
Vi CPS (various)	Parenteral Vi (capsular polysaccharide antigen) vaccine licensed for adults and children 2 years of age and older	GlaxoSmithKline, Sanofi Pasteur, Bharat Biotech, BioMed, Finlay Institute, and approximately six other manufacturers located in endemic countries
Vi conjugate (Typbar-TCV™, Peda-Typh™, TYPHIBEV®)	Parenteral Vi conjugated to tetanus toxoid (Typbar-TCV™, Peda-Typh™) or diphtheria toxoid CRM_{197} (TYPHIBEV®) carrier protein, licensed in India for adults and children 6 months of age and older	Bharat Biotech (Typbar-TCV™), Bio-Med (Peda-Typh™), Biological E (TYPHIBEV®)

2013 A parenteral vaccine composed of the detergent-purified Vi antigen of *S*. Typhi is also licensed for
2014 international use. The vaccine is 60 to 70 percent efficacious but only for two to three years. It is licensed
2015 for adults and children older than 2 years of age. Since the vaccine is not conjugated to protein, it does not
2016 establish immune memory and cannot be boosted by repeat vaccination. The vaccine is not patent
2017 protected and is manufactured by a number of commercial entities under different trade names. The
2018 Ty21a and Vi polysaccharide vaccines do afford short-term protection for travelers to typhoid-endemic
2019 regions. Vi conjugate vaccines are also licensed in India for in-country use (406, 409, 412).

2020 The limited efficacy and short-term protection afforded by the Ty21a and unconjugated Vi CPS, and the
2021 lack of any vaccine against any of the other *Salmonella* that cause serious disease in humans, has
2022 prompted a resurgence of vaccine development against all four of the important *Salmonella* serovars.
2023 Most of the candidate vaccines in development are focused on *S*. Typhi, but a few candidate vaccines
2024 against *S*. Paratyphi, *S*. Typhimurium, and *S*. Enteritidis are under development. Table 17 describes the
2025 candidate vaccines in development against all of these organisms. Additional details can be found in
2026 recent reviews by Syed et al. (431) and Baliban et al. (432).

Table 17. Candidate vaccines in development against *Salmonella.*

Vaccine type	Vaccine	Development phase	Developer(s)
Salmonella Typhi			
Vi conjugate	Vi-rEPA (recombinant exoprotein A from *Pseudomonas aeruginosa*)	Phase 3	US National Institutes of Health, Lanzhou Institute of Biological Products

Table 17. Candidate vaccines in development against *Salmonella.*

Vaccine type	Vaccine	Development phase	Developer(s)
	Vi-CRM_{197}	Phase 3	EuBiologics, Republic of Korea; PATH
	Vi-diphtheria toxoid	Phase 1 or preclinical	International Vaccine Institute/Shantha Biotechnics; SK Chemicals; PT Biofarma; Finlay Inst.; Incepta, Bangladesh
	Vi-tetanus toxoid	Preclinical	WalVax, China
	Vi conjugated to fusion protein PsaA-PdT	Preclinical	Harvard Medical School
O9 conjugate	O9:DT	Preclinical	International Vaccine Institute (Korea)
LPS-conjugate	O-specific polysaccharide conjugated to diphtheria toxoid	Preclinical	National Institute for Biotechnology and Genetic Engineering (Pakistan)
Live-attenuated oral	M01ZH09	Phase 2	Prokarium
	CVD 909	Phase 2	University of Maryland
Outer membrane protein	OmpC and OmpF	Phase 1	Instituto Mexicano del Seguro Social
Salmonella Paratyphi			
LPS-conjugate	LPS-tetanus toxoid conjugate	Phase 1	US National Institutes of Health
Live-attenuated oral	CVD 1902	Phase 1	University of Maryland
	phoPQ mutant	Preclinical	Celldex Therapeutics
Bivalent *S.* Typhi – *S.* Paratyphi			
Conjugate	Vi and O:2 antigens of *S.* Typhi and *S.* Paratyphi separately conjugated to CRM_{197}	Preclinical	GSK (licensed to Biological E)
Live-attenuated oral bivalent	MO1ZH9 expressing *S.* Paratyphi LPS (Entervax)	Phase 1	Prokarium
Salmonella Typhimurium			
Live-attenuated oral	CVD 1931	Preclinical	University of Maryland
	Regulated delayed attenuation	Preclinical	University of Arizona
	WT05	Phase 1	Microsciences, Wokingham Berkshire
	ruvB mutant	Preclinical	Seoul National University
	Hfq deletion mutant	Preclinical	Indian Institute of Science, Bangalore
	SA186 lacking ZnuABC transporter	Preclinical	Instituto Superiore de Sanita Roma

Table 17. Candidate vaccines in development against *Salmonella.*

Vaccine type	Vaccine	Development phase	Developer(s)
	MT13 TTSS-2 deficient	Preclinical	Kalinga Institute of Industrial Technology, Odisha University
	Adenine methylase mutants	Preclinical	University of California, Santa Barbara
O-protein-conjugate	O:4,12-TT	Preclinical	US National Institutes of Health
	Os-po	Preclinical	National Biotechnology Laboratory, Stockholm
Protein	OmPD outer membrane proteins	Preclinical	University of Birmingham, United Kingdom
Salmonella Enteritidis			
Live-attenuated oral	CVD 1944	Preclinical	University of Maryland
O:9 conjugate	O:4,5/O:9-flagellin	Preclinical	University of Maryland
S. Typhimurium and *S.* Enteritidis			
GMMA	Generalized Modules for Membrane Antigens	Preclinical	GSK

Abbreviations: GMMA, Generalized Modules for Membrane Antigens; GSK, GlaxoSmithKline; LPS, lipopolysaccharide; WHO, World Health Organization.

2027 The current vaccine development efforts are focused on three main approaches. The first is conjugating
2028 the two important polysaccharide surface antigens (Vi and O) to protein carriers to form the basis for new
2029 parenteral vaccines that can be boosted to enhance long-term immunity. This approach recently received a
2030 strong endorsement from WHO, particularly for use in children under 2 years of age (411). Secondly,
2031 several groups are working on developing new live-attenuated vaccines that include different attenuation
2032 strategies. The University of Maryland, Prokarium, and others are investigating potential successors to the
2033 Ty21a vaccine. Finally, other groups are developing subunit vaccines based on bacterial membrane
2034 antigens. Table 17 describes these candidate vaccines (406, 407, 409, 411, 412, 427, 428, 433–437). For
2035 *S.* Typhi, the most clinically advanced candidates are the polysaccharide conjugate vaccines. Two of these
2036 vaccines are licensed in India and the Vi conjugate vaccine from Bharat has also achieved WHO
2037 prequalification. This vaccine has been shown to be protective in both a controlled human infection model
2038 (CHIM) as well as in the field in Nepal. On the strength of this evidence, TCV was deployed in reactive
2039 vaccination campaigns against an outbreak of extensively drug-resistant *S.* Typhi in Pakistan and an
2040 outbreak of multidrug-resistant *S.* Typhi in Zimbabwe (438. In Zimbabwe, within three months of
2041 initiating the campaign there was a decrease in number of blood culture-confirmed typhoid cases (440).
2042 An evaluation of over 200,000 children in the Pakistan campaign found a vaccine effectiveness of 55
2043 percent and 97 percent against extensively drug-resistant *S.* Typhi (441) and a separate, smaller case-
2044 control study in Pakistan found a vaccine effectiveness of 72 percent (442). Similarly, a cluster-
2045 randomized study of over 60,000 children in Bangladesh found an overall vaccine protection of 57
2046 percent (443). A Phase 3 study of over 28,000 children in Malawi compared TCV against meningococcus
2047 A vaccine and found an overall efficacy of 81 percent (444). Two Phase 2 studies in Burkina Faso
2048 confirmed the safety of TCV and its compatibility with other routine immunizations (445, 446). These
2049 results suggest TCV can play a crucial role in mitigating the impact of *S.* Typhi, including antimicrobial-

resistant infections. As indicated above in Table 17, three additional Vi conjugate vaccine candidates are in clinical development.

The LPS-conjugate vaccines (some of which just contain purified O-antigen) are in the preclinical stage. Among live-attenuated vaccines, the most advanced are CVD 909 at the University of Maryland and M01ZH09 from Prokarium.

For *S*. Paratyphi, University of Maryland researchers have developed the live-attenuated vaccine candidate called CVD 1902, which is currently in Phase 1. Similarly, Prokarium has a bivalent live-attenuated Typhi-Paratyphi vaccine in Phase 1 trials. Celldex Therapeutics has another live-attenuated vaccine candidate in the preclinical stage. A bivalent vaccine in which Vi and O:2 antigens of *S*. Typhi and *S*. Paratyphi A are separately conjugated to carrier proteins is in preclinical development at Biological E. The LPS-conjugate vaccine against *S*. Paratyphi A developed at the US National Institutes of Health failed to show boosting with a second dose given six weeks after the first in a Phase 1 trial.

For *S*. Typhimurium, a variety of live-attenuated vaccines are in preclinical stages. The WT05 candidate vaccine, developed by Microsciences in the United Kingdom, has reached Phase 1. O-antigen conjugate and outer membrane vaccine candidates are also in preclinical stages. Candidate vaccines against *S*. Enteritidis include live-attenuated CVD 1944 and an O-antigen conjugate from University of Maryland, both at the preclinical stage. GSK is applying the Generalized Modules for Membrane Antigens approach for a combined *S.* Typhimurium/*S*. Enteritidis vaccine that is in preclinical development.

Summary

Four serovars of *S. enterica* cause a complex spectrum of disease in humans. Infections with these serovars clearly cause considerable human morbidity and mortality and their geographic spread is unpredictable. The public health threat of these enteropathogens is further amplified by their increasing antibiotic resistance (410, 412). We have only a limited understanding of the pathogenesis and natural immune control of these diseases. Adding to the challenge, two licensed typhoid vaccines are either of moderate efficacy (Ty21a) or of limited durability (Vi polysaccharide antigen vaccine)—factors limiting their widespread use. Neither vaccine works well in the age group at highest risk for illness with these pathogens, infants and children less than 2 years of age. Accordingly, WHO recently endorsed further development of glycoconjugate vaccines for typhoid fever (428), and positive efficacy data for this approach in both the CHIM and an initial Phase 3 efficacy trial in Nepal (433, 436) has driven a major development and testing effort in this area that will likely greatly improve our ability to prevent and control invasive salmonellosis in the future.

Progress can be further accelerated toward better control of *Salmonella* diseases if several pressing needs are properly addressed. These include improved diagnostic tests that can be widely applied in field settings, improved understanding of the degree to which live-attenuated vaccines can cross-protect against *S.* Typhi and *S.* Paratyphi, a clarification of the role of antibodies against the Vi polysaccharide play in protection against *S.* Typhi and *S.* Paratyphi, and an understanding of whether it may be feasible to develop vaccines against *S.* Typhimurium and *S.* Enteritidis by extrapolation of vaccine concepts that have been moderately successful for *S.* Typhi and *S.* Paratyphi bacteria.

2089 For vaccine development against all of the *Salmonella* diseases, the lack of information on natural
2090 immunity and the protective immunity developed after vaccination are critical impediments to progress.
2091 Some cross-reaction of the antibody-mediated immunity generated by the licensed vaccine Ty21a and by
2092 the live-attenuated vaccine candidate CVD 909 to *S.* Paratyphi A and B strains does seem to be occurring
2093 (424–426). Additional studies will be needed to determine if these vaccines can provide partial protection
2094 against *S*. Paratyphi. The availability of a new challenge model that includes a strain of *S*. Paratyphi
2095 should be helpful in this regard.

2096 A somewhat confused picture remains with respect to the protection afforded by Vi capsular
2097 polysaccharide vaccines since experimental human infection with a strain that expressed this antigen did
2098 not elicit detectable anti-Vi antibodies (418). Since so much of the vaccine pipeline depends on Vi
2099 antigen conjugate vaccines, some effort toward clarifying such questions might be of benefit to the field.
2100 It is interesting to note that comparison of markers for protection induced by the Vi antigens given alone
2101 versus those associated with protection induced by Vi conjugates to a protein carrier may differ, further
2102 indicating additional studies in this area are needed (434). Similarly, using the new CHIMs to better
2103 understand any of the factors may contribute to a better understanding of iNTS pathogenesis and
2104 immunity, and would also be a welcome add-on to the field.

2105 The careful work that has supported the development of the new human challenge model for the
2106 *Salmonellae* that cause enteric fevers is a fitting tribute to the historical studies that laid much of the
2107 groundwork for the licensed vaccines against typhoid fever. The new model has already helped to
2108 advance the first Vi conjugate vaccine to WHO prequalification and will be a valuable tool in guiding the
2109 development of similar vaccines for paratyphoid and iNTS. Vaccine development for invasive *Salmonella*
2110 disease has a wealth of promising candidates with high probability of success that will lead ultimately to
2111 better control of these complex and diverse bacterial pathogens.

2112

Norovirus

Epidemiology, pathogenesis, and antigenic diversity of norovirus

Norovirus, a small, non-enveloped, single-stranded RNA virus, is a leading cause of outbreaks of acute gastroenteritis worldwide. The Global Enteric Multicenter Study identified norovirus as the third most important viral cause of diarrheal disease in children younger than 1 year of age, behind rotavirus and adenovirus 40/41 (447). Similarly, in the most recent Child Health Epidemiology Reference Group analysis of diarrhea-associated mortality under the age of 5, norovirus was the most common viral cause of diarrhea-associated deaths (448). An estimated 677 million infections and 213,000 deaths occur each year across all age groups due to norovirus infection (448). Disease can be more severe in infants and the elderly. The most common modes of norovirus transmission are ingesting contaminated food or water, or person-to-person contact. According to some estimates, as few as 30 particles are enough to initiate a new infection.

The mechanisms by which norovirus infection causes acute gastroenteritis are poorly understood (449). Disease onset occurs within 24 to 48 hours of infection and includes one or more of the following symptoms: vomiting, watery diarrhea, abdominal cramps, fever, headache, and malaise. Symptoms spontaneously resolve in the great majority of cases within 48 hours of onset. Virus particles may be shed in stools long after the resolution of symptoms (450).

The epidemiology of norovirus infection is complex (451–456). The virus occurs in pandemics, in large and small outbreaks, and in sporadic cases of disease. Outbreaks are more common when humans are in confined settings like cruise ships, military installations, prisons, nursing homes, and childcare facilities. The US CDC estimates that norovirus may cause 18 percent of acute gastroenteritis cases worldwide (451). Significant gaps remain regarding the norovirus disease burden in Africa and parts of Latin America, where studies have been infrequent.

Noroviruses are genetically diverse and have been classified based on the amino acid sequences of their capsid protein VP1, which is the target of binding and neutralizing antibodies. Genogroup I contains eight genotypes, including the prototype Norwalk virus that caused an outbreak in school children in Norwalk, Ohio, United States, in 1972 (449). About 10 percent of human norovirus infections are Genogroup I (454). Genogroup II, which causes up to 80 percent of human infections, contains at least 23 genotypes (454). Genotype GII.4 was associated with seven global outbreaks between 1987 and 2012 (452, 453).

While the propagation of noroviruses in cell culture is not yet routine, producing artificial virus-like particles (VLPs) that faithfully mimic the norovirus capsid structure is possible (452, 457). VLPs representing different genogroups, genotypes, and variants can be produced in laboratories for exploring the impact of genetic diversity on antigenic variation and determining receptor-binding patterns. The receptors for noroviruses are thought to be histo-blood group antigens (HBGA) expressed at mucosal surfaces, including the lumen of the intestinal tract (458–460). Humans that lack a functional FUT2 gene are called non-secretors because they do not express certain blood group antigens in gastrointestinal secretions VLPs representing different genogroups and genotypes of noroviruses have been used to work out the patterns of binding to different blood group antigens and the capacity of serum antibodies from natural and experimental human infections to block VLP binding to these carbohydrate antigens (453, 458, 461).

2153 Having the capacity to grow the virus in cell culture and to have an animal model in which to conduct
2154 preclinical studies of candidate vaccines would be advantageous. Despite considerable effort, the
2155 propagation of noroviruses by conventional cell culture techniques is not yet routine; however, efforts to
2156 overcome these obstacles are making progress (462–466). Human noroviruses do not infect other normal
2157 animal hosts; however, several studies with human norovirus conducted in gnotobiotic (germ-free) piglets
2158 suggest that vaccine candidates and probiotics can at least partially suppress clinical symptoms, viral
2159 replication, and shedding in these animals (467–470).

2160 **Human challenge models for norovirus**

2161 Human challenge studies have been an integral part of norovirus research since the virus was first
2162 described (449). In 1972, a rectal swab from a patient linked to an outbreak of diarrhea in school children
2163 in Norwalk, Ohio, was used to experimentally inoculate volunteers. The purpose was to reproduce the
2164 clinical symptoms of the disease and to identify a small non-enveloped virus, Norwalk virus. Table 18
2165 describes the human challenge models that are currently available and summarizes the collective
2166 experience. The challenge studies have been representative of the most important genogroups and
2167 genotypes of noroviruses infecting humans. Most of the studies have been conducted with noroviruses of
2168 Genogroup I, which is thought to be responsible for about 10 percent of human norovirus infections
2169 (454). Two challenge studies have been reported using Genogroup II noroviruses, which are thought to be
2170 responsible for about 80 percent of infections in humans. The epidemiologically important genotype II.4
2171 noroviruses are represented by a single challenge study. The challenge pools are purified from the stools
2172 of infected individuals and delivered orally. Since secreted HBGA determine human susceptibility to
2173 challenge, study volunteers are often pre-screened for their blood group and secretor phenotype.
2174 Challenge studies are conducted in an inpatient facility. Volunteers are monitored closely following
2175 challenge and discharged after symptoms of infection have resolved.

Table 18. Human challenge models for norovirus.

Strain and genotype	Method of production and administration	Number of studies	Number of volunteers
Norwalk virus, GI.1	A challenge pool was prepared in 2008 from the liquid feces of a single experimentally infected adult by clarification, centrifugation, and serial filtration. The challenge is administered to healthy adults orally. Infection with this challenge pool requires that individuals be secretor positive and lack the blood group B antigen.	8	333
Snow Mountain virus, GII.2	The virus was purified in 2003 from filtrates of a stool sample and administered to healthy adults orally. Infection with this strain is not dependent on ABO blood type or secretor status.	1	15
Cin-1, GII.4	Isolated in 2003 from a stool sample from an individual with gastroenteritis. Cin-1 infection is not dependent on ABO blood group, but non-secretors are resistant to infection. Challenge is delivered to healthy adults orally in sterile water.	1	40

2176 Subsequent challenge studies with noroviruses were reported between 1990 and 2003 using clinical
2177 isolates of genotype GI.1 viruses similar to Norwalk virus (see Table 18). In 1990, Johnson et al. studied
2178 the relationship of pre-existing serum antibody titers to protection from disease (471). Twelve out of 12
2179 human volunteers with serum antibody titers to the challenge virus greater than 1/200 were protected, but

2180 only 19 of 30 volunteers with titers less than 1/100 were protected. The first published studies using VLPs
2181 for the evaluation of serum antibodies following experimental human challenge were published in 1994
2182 (472, 473). Using this more sensitive assay to study the relationship of serum antibodies to protection
2183 from infection in 17 experimentally challenged volunteers, the investigators concluded that pre-existing
2184 antibodies did not protect from infection. Stored samples from these studies were later used to evaluate
2185 virus shedding after experimental challenge by PCR to detect virus genomes and by antigen enzyme-
2186 linked immunosorbent assay to detect viral proteins (450). The results indicated that the two assays were
2187 well correlated early after infection, and that individuals with clinical gastroenteritis after challenge shed
2188 ten times more virus than those that did not become ill. The duration of shedding was four to eight weeks
2189 or longer in some study subjects.

2190 During this same period, the experimental human challenge study reported by Lindesmith et al. verified
2191 the importance of secreted HBGA in norovirus infection (459). They found that individuals that did not
2192 express the FUT2 gene (non-secretors) were fully protected from challenge; and in the same study, they
2193 gathered evidence that mucosal immunoglobulin A (IgA) responses could protect from infection. Later,
2194 this same group studied the reactivity of serum antibodies within and across different genotypes of
2195 Genogroup I viruses (458). They prepared VLPs from two GI.1 viruses, and from GI.2, GI.3, GI.4, and
2196 GI.5 viruses. Serum antibodies from volunteers infected with one of the GI.1 strains in their previous
2197 challenge study (459) showed cross-reactivity within the GI.1 genotype but not to other GI genotypes.

2198 Between 2010 and 2019, four additional human challenge studies were conducted using genotype I.1
2199 viruses (474–477). One was a challenge study that included 34 human volunteers (476) and showed a
2200 correlation between pre-existing receptor-blocking antibodies and the clinical outcome
2201 (+/– gastroenteritis) after challenge. Binding antibody titers were not correlated with the clinical outcome.
2202 In a follow-up study, these investigators used serum antibodies from the volunteers in the previous
2203 challenge study to develop a simple hemagglutination inhibition (HAI) assay using human type O
2204 erythrocytes (478). They showed that the results from the HAI assay were well correlated with those from
2205 the previously used receptor-blocking assay. An HAI titer of greater than 1/40 protected against disease.

2206 Another human challenge study included 57 volunteers and was reported by Atmar et al. in 2014 (477). In
2207 this study, individuals with different HBGA and individuals that were either secretors or non-secretors
2208 were studied for their susceptibility to a GI.1 norovirus challenge. All of the volunteers that had blood
2209 groups A or O antigens were infected. Individuals with blood group B or AB antigens were not infected,
2210 and individuals that were non-secretors were not infected. For susceptible individuals, the challenge dose
2211 required for infection of 50 percent of volunteers (ID_{50}) was calculated to be 1,320 genomic equivalents,
2212 based on a quantitative reverse transcription polymerase chain reaction (RT-PCR) assay.

2213 Consumption of raw seafood such as oysters carries a risk of infection with enteric pathogens, including
2214 norovirus (479). A norovirus human challenge study that enrolled 44 secretor-positive volunteers tested
2215 methods of decontaminating raw oysters (474). Volunteers ingested either oysters seeded with 1×10^4
2216 genomic equivalents of norovirus GI.1 strain 8FIIb treated by different high hydrostatic pressure
2217 processes, or untreated oysters. Whereas 600 MPa at 6°C for five minutes completely eliminated
2218 infectivity from oysters (zero of ten volunteers infected), oysters treated with only 400 MPa retained
2219 infectivity (six of 19 volunteers infected, 32 percent). Among volunteers that consumed seeded, untreated
2220 oysters, 47 percent (seven of 15) were infected. A subsequent analysis of gut microbiota from these

2221 volunteers identified compositional differences associated with symptomatic and asymptomatic infections
2222 (480). A similar study evaluated the persistence of norovirus in water samples stored for various lengths
2223 of time (481). Subjects were challenged with a relatively high inoculum of 6.5 x 10^7 genomic equivalents
2224 of GI.1 norovirus 8FIIb, and ten of 13 were infected and experienced typical clinical symptoms.

2225 A recent norovirus human challenge study with a GI.1 strain was conducted to support future vaccine
2226 development (475). Lot 001-09NV was derived from a volunteer from a previous challenge study with
2227 strain 8FIIa (459). This new lot was purified and characterized to meet rigorous standards for use in future
2228 tests of vaccine candidates. The virulence and clinical symptoms induced by Lot 001-09NV were similar
2229 to previous studies with related strains. For example, of 16 secretor-positive, blood group A or O
2230 individuals challenged with either 3.6 x 10^5 or 1 x 10^6 genomic equivalents of norovirus, nine (56 percent)
2231 met criteria for acute gastroenteritis (diarrhea and/or vomitus) and 11 (69 percent) were infected, based on
2232 shedding norovirus in stool or vomitus by RT-PCR. Furthermore, robust immune responses to GI.1 were
2233 observed in infected volunteers: a 30-fold increase in blocking titers (BT_{50}) and 161-fold increase over
2234 baseline in immunoglobulin G (IgG) titers. Based on these results, Lot 001-09NV should be useful for
2235 future studies testing vaccine candidates in development (482).

2236 Human challenge studies have also been conducted with three genotypes of the Genogroup II norovirus,
2237 namely genotype GII.1, genotype GII.2, and genotype GII.4 noroviruses. In 2005, Lindesmith et al.
2238 reported the results of a study of 15 human volunteers challenged with the Snow Mountain strain of
2239 genotype II.2 (see Table 18) (483). The investigators found that susceptibility to infection was
2240 independent of both the blood type and the secretor phenotype of the volunteers. Nine of 15 volunteers
2241 were infected and seven developed gastroenteritis. IgG antibodies elicited by infection cross-reacted with
2242 VLPs from another GII strain, but not with those from a GI strain. The first human challenge study with a
2243 GII.4 norovirus was reported in 2012 (484). Strain Cin-1 (see Table 18) was used to challenge 23
2244 volunteers with the secretor phenotype and 17 non-secretors. Seventy percent of secretors and 6 percent
2245 of non-secretors became infected. A small pilot study was conducted with GII.1 Hawaii virus (485). The
2246 majority of infected individuals in these three studies developed symptoms of disease.

2247 **The development of norovirus vaccines**

2248 The first conventional norovirus vaccine candidate to enter clinical trials was comprised of VLPs of the
2249 Norwalk virus administered orally (486, 487). It was well tolerated but poorly immunogenic. The vaccine
2250 candidate was then reformulated to include the adjuvant monophosphoryl lipid A (MPL,
2251 GlaxoSmithKline) and chitosan (a mucoadherent agent) and evaluated in two additional Phase 1 trials
2252 using the intranasal route of administration. Results from this study demonstrated that the
2253 immunogenicity of the vaccine candidate improved and that safety was maintained (488).

2254 The adjuvanted Norwalk VLP vaccine candidate then advanced to a Phase 2 challenge study in human
2255 volunteers (489). It contained 100 µg of VLPs produced in a baculovirus expression system, MPL, and
2256 chitosan (Archimedes Development). Ninety-eight volunteers were randomized 1:1 to receive vaccine or
2257 placebo. The vaccine candidate and placebo were administered intranasally in two doses three weeks
2258 apart. Three weeks after the second dose, 84 of the 90 volunteers that had received two doses of vaccine
2259 volunteered for the challenge study and were admitted to an inpatient facility. The next day, they received
2260 a homologous strain challenge dose approximately ten times the ID_{50}. Volunteers were cared for and

2261 evaluated for at least four days or until the symptoms of disease resolved. The vaccine showed 47 percent
2262 efficacy against the development of disease, and 26 percent efficacy against infection (489).

2263 A bivalent vaccine candidate similar to the adjuvanted Norwalk VLP vaccine was also evaluated in a
2264 human challenge study (490). It contained VLPs of Norwalk virus (genotype GI.1) and VLPs of a
2265 consensus sequence GII.4 strain. The bivalent vaccine was administered intramuscularly in MPL adjuvant
2266 adsorbed to aluminum hydroxide. The vaccine dose contained 50 µg of each VLP. The dosing schedule
2267 was zero and four weeks. At least one month after receiving vaccine or placebo, volunteers were admitted
2268 to an inpatient challenge facility and challenged with the Cin-1 strain (see Table 18). The vaccine efficacy
2269 was low against both infection and disease. Per protocol, 54 percent of vaccinees and 63 percent of
2270 controls were infected. The vaccine did moderately reduce vomiting and/or diarrhea, but did not meet the
2271 predefined rates of infection and illness that were the primary study endpoints. Challenge with Norwalk
2272 virus was not performed.

2273 The negative results of the initial human challenge study with the bivalent VLP vaccine were somewhat
2274 unexpected because the earlier study with the monovalent VLP vaccine seemed to show a moderate level
2275 of protection against the development of disease. Although the bivalent vaccine contained VLPs of
2276 Norwalk virus, the challenge study was apparently not designed to include a homologous challenge with
2277 Norwalk virus, making it difficult to link to the results from the earlier study of the monovalent vaccine.
2278 The GII.4 vaccine strain was an artificially derived consensus strain and it did not exactly match the GII.4
2279 challenge strain. The GII.4 vaccine and challenge strains were mismatched by 19 amino acids in the P2
2280 domain of major capsid protein. Prior to the human challenge study, the GII.4 consensus VLPs were used
2281 to immunize rabbits. Hyperimmune sera from immunized rabbits showed variable, but generally good,
2282 cross-reactivity among different GII.4 strains (491). The cross-reactivity of serum antibodies from human
2283 volunteers vaccinated with the GII.4 consensus VLPs to the challenge strain, however, has not yet been
2284 reported. It may also be important that the bivalent VLP vaccine was administered by the intramuscular
2285 route, while the monovalent vaccine was delivered intranasally.

2286 The bivalent VLP vaccine candidate TAK-214 was advanced further and recently completed a Phase 2b
2287 clinical study in the United States (492). Approximately 4,700 adults 18–49 years of age were
2288 randomized 1:1 to receive either vaccine or placebo and monitored for acute gastroenteritis for 45 days.
2289 Because only six cases of GI.1 or GII.4 norovirus were detected, the study had insufficient statistical
2290 power to assess the primary endpoint. Therefore, the alpha was amended prior to unblinding in order to
2291 provide sufficient power for a secondary endpoint that included all norovirus genogroups detected in the
2292 study subjects, most of which were GII.2. In this secondary analysis, the vaccine demonstrated a
2293 protective efficacy against moderate or severe disease of 62 percent ($p = 0.0097$). In a separate Phase 2
2294 study of TAK-214 in roughly 300 adults over 60 years of age, safety and immunogenicity were similar to
2295 those seen in younger adults and were unaffected by a second dose after 28 days or by addition of MPL
2296 (493). The main sponsor of these studies was Takeda Pharmaceutical Company Limited.

2297 A nonreplicating recombinant adenovirus vector expressing VP1 from a GI.1 strain, designated VXA-
2298 G1.1-NN, was recently tested for safety and immunogenicity in a first-in-human Phase 1 clinical study of
2299 66 healthy adult volunteers (482). This vaccine candidate was found to be safe, well tolerated, and
2300 substantially immunogenic. For example, 78 percent of vaccinees showed a greater than 2-fold rise in
2301 BT_{50}. The candidate is formulated as an oral tablet that is stable at room temperature to simplify delivery.

2302 VXA-G1.1-NN has also been tested along with a GII.4 VP1-expressing adenoviral vector (VXA-G2.4-
2303 NS) in a Phase 1b study. Both the monovalent and combined bivalent vaccines were safe, well tolerated,
2304 and immunogenic, based on IgA antibody-secreting cell response rates: 78 percent for GI.1 and
2305 93 percent for GII.4 (494–496). The main sponsor of these studies was Vaxart, Inc.

2306 Eight additional candidate norovirus vaccines based on whole VLPs or smaller protruding domain (P)
2307 particles produced from just the protruding portion of VP1 are in preclinical development, along with two
2308 adenovirus-vectored norovirus vaccines (452, 457, 468, 497–500). Table 19 summarizes this information
2309 and indicates the institutions involved.

Table 19. Candidate norovirus vaccines in development.

Vaccine type	Phase	Number of candidates	Vaccine developer(s)
Bivalent GI.1/GII.4 virus-like particles (TAK-214)	Clinical Phase 2b	1	Takeda Pharmaceutical Company Limited
Nonreplicating recombinant adenovirus vectors (VXA-G1.1-NN, VXA-G2.4-NS)	Clinical Phase 1b	2	Vaxart, Inc.; University of Maryland
Virus-like particles and protruding domain (P) particles	Preclinical	8	Arizona State University; University of North Carolina; Virginia-Maryland Regional College of Veterinary Medicine; Cincinnati Children's Hospital Medical Center; US National Institute of Allergy and Infectious Diseases; UMN Pharma Inc.; Nanotherapeutics; Kunming University of Science and Technology; Tampere University
Recombinant viral vectors (adenovirus)	Preclinical	2	Chinese Center for Disease Control; Ohio State University

2310 **Summary**

2311 An eminent group of physicians and virologists published the first human challenge study with norovirus
2312 in 1974 (501). Forty years later, human challenge models are a cornerstone of norovirus research and
2313 vaccine development. Three different human challenge models are available, representing viruses of the
2314 Genogroup I and II strains circulating in humans. More than 300 human volunteers have been
2315 experimentally challenged in research studies that provide basic information about norovirus
2316 pathogenesis, the role of serum antibodies in protection from infection and disease, and the role of HBGA
2317 in susceptibility to infection. This information is of fundamental importance for the development of
2318 norovirus vaccines. The foundation for norovirus vaccine development has been laid without growing a
2319 single norovirus in the laboratory and without the support of an animal model of infection. Human
2320 challenge studies have seamlessly woven into the clinical development of the first candidate vaccines
2321 against noroviruses.

2322 A careful analysis of strains from norovirus outbreaks in humans over 30 years has provided a clearer
2323 understanding of norovirus diversity and the challenge that such diversity poses to vaccine development.

Some in the scientific community have proposed that vaccines against the highly variable GII.4 noroviruses may require frequent replacement—a strategy analogous to that used for seasonal influenza vaccines (497). Indeed, the studies discussed here, including the experimental human challenge with GII.4 virus after vaccination, do suggest that protection may be more difficult among the highly variable GII.4 viruses than for the less variable GI noroviruses.

Fortunately, the leading candidate vaccines against norovirus are based on VLPs—a vaccine type that is relatively cost-effective to produce, heat stable in solution, and amenable to long-term storage in a dry powder for intranasal immunization (457). In the future, VLP vaccines against some of the norovirus genogroups and genotypes could be mass produced, stockpiled, and used to contain outbreaks, as well as for mass vaccination campaigns in the most susceptible age groups. The VLP platform may provide the much-needed flexibility for reformulation required for many important diseases caused by highly variable pathogens like noroviruses.

Cryptosporidium

Epidemiology, diversity, pathogenesis, and public health impact of *Cryptosporidium*

The Global Enteric Multicenter Study identified the apicomplexan parasite *Cryptosporidium* as the second most frequent cause of childhood diarrhea worldwide (502). In the Institute for Health Metrics and Evaluation's recent Global Burden of Disease Study, *Cryptosporidium* was estimated to be responsible for approximately 7 percent of all diarrhea-associated deaths worldwide and to account for 8 percent of all diarrhea-associated disability adjusted life years (503, 504). Despite these concerning estimates, these figures still likely under-estimate the true disease burden. The development of improved quantitative molecular diagnostic methods may serve to further increase disease estimates and further heighten concerns about the role of *Cryptosporidium* as a global enteric pathogen (505). Contamination of surface water with *Cryptosporidium* continues to be the source of outbreaks of disease in high- and low-resource countries alike (506). No vaccine against *Cryptosporidium* has been developed and while substantial progress has been made in advancing preclinical drug candidates (507–510), no widely available, highly effective drugs exist to treat the disease. The sole US Food and Drug Administration (FDA)-approved treatment, nitazoxanide, is efficacious in otherwise healthy adults, but poorly efficacious in malnourished children and ineffective in severely immunocompromised patients (511), therefore its uptake in low-resource settings has been limited.

Two species, *Cryptosporidium hominis* and *Cryptosporidium parvum*, cause the great majority of human infections. The former is naturally transmitted only in humans, while the latter is common in domesticated animals and can be transmitted to humans zoonotically, as well as person to person. In the general population, rates of seropositivity to *Cryptosporidium* range from 25 to 60 percent in different populations and locations (512). While surveillance remains incomplete, *C. hominis* accounts for roughly 80 percent of infections worldwide (513).

Cryptosporidium is highly contagious. Infected people and animals typically shed 10^9 oocysts per gram of feces, and ingestion of only a few oocysts from contaminated food or water is sufficient to establish infection. Outbreaks associated with contaminated surface water are common. The largest reported outbreak was in the US city of Milwaukee, Wisconsin, in 1993, with more than 400,000 infections. Outbreaks in high-income countries remain common (506, 514).

Infection with *Cryptosporidium* is associated with a wide spectrum of disease (515). Malnourished children in low-resource countries and immunocompromised individuals or those under immune suppressive treatments are the most vulnerable. In most cases, ingestion of oocysts leads to a watery diarrhea that typically lasts one to two weeks in healthy adults, but can persist for weeks beyond that in malnourished and immunocompromised individuals. Microscopically, *Cryptosporidium* infection is associated with a flattening of the intestinal villi, which may contribute to the malnutrition, environmental enteric dysfunction, and growth retardation seen in infected children. Long-term effects on cognitive and motor development can also be associated with *Cryptosporidium* infection. The parasite can disseminate to other organs and result in significant mortality in immunocompromised hosts (512, 516). Respiratory infection and symptoms have also been well-documented, including in HIV negative adults and children (517–521), however their relationship with intestinal infection and symptoms remains unclear.

2376 **The *Cryptosporidium* human challenge model**

2377 The initial human challenge studies with *Cryptosporidium* conducted in the 1990s and early 2000s were
2378 designed to determine the number of oocysts of *C. parvum* required to establish infection and disease, and
2379 to explore phenotypic diversity of isolates from different animal species. As shown in Table 20, oocysts
2380 are either purified from the stools of infected animals (*C. parvum*) or isolated from humans and
2381 propagated in specialized, gnotobiotic pigs (*C. hominis*). Several methods have been reported for
2382 perpetual propagation of *C. hominis* or *C. parvum* in cell culture (522–524), but these are not widely
2383 available nor used routinely. More recent epidemiology studies highlighting the burden of
2384 *Cryptosporidium* in low- and middle-income countries (LMICs) have rekindled the interest in this model
2385 for the purposes of testing drug and vaccine candidates for treatment or prevention of infection with the
2386 parasite (525). Studies of the natural history of cryptosporidiosis in LMIC populations demonstrate there
2387 are fewer infections among older children (502), suggesting that adaptive immunity induced by a vaccine
2388 is achievable. However, comparatively little is understood about the immune response to the parasite;
2389 therefore, controlled human infection model (CHIM) studies could play an important role in closing these
2390 gaps and supporting development of vaccine candidates.

Table 20. The human challenge model for *Cryptosporidium*.

Organism	Strain(s)	Method of production and administration	Stock producer
Cryptosporidium parvum	Iowa, Moredun, TAMU, UCP	Oocysts are purified from feces of experimentally infected calves. Adults are orally exposed to measured numbers of oocysts.	Challenge stock prepared at the University of Arizona. Challenges performed at the University of Texas Health Science Center.
Cryptosporidium hominis	TU502	An isolate from a child with cryptosporidiosis has been propagated in gnotobiotic piglets and purified from feces. Adults are orally exposed to measured numbers of oocysts.	Challenge stock prepared at Tufts University School of Medicine. Challenges performed at the University of Texas Health Science Center.

2391 The initial human challenge study with *C. parvum* was published by DuPont et al. in 1995 (526). The
2392 volunteers for the study were healthy adults that were seronegative for *C. parvum.* Infection was defined
2393 by diarrheal illness and by the detection of oocysts in stools. Follow-up was intensive for the first two
2394 weeks and was continued at longer intervals for an additional six weeks.

2395 Doses ranging from 3×10^1 to 1×10^6 oocysts of the Iowa strain of *C. parvum* were used to challenge 29
2396 volunteers, and 62 percent of the challenged volunteers became infected. The calculated dose for infection
2397 of 50 percent of volunteers (ID_{50}) was 132 oocysts. Many, but not all, of the subjects that excreted oocysts
2398 also had symptoms of infection, including abdominal pain, cramps, and diarrhea. The number of ingested
2399 oocysts did not markedly affect the incubation period nor the severity or duration of illness.

2400 These same investigators more closely examined the relationships between patterns of oocyst excretion,
2401 challenge dose, and symptoms of disease in infected volunteers. (527). The patterns of oocyst shedding in
2402 volunteers with disease symptoms and diarrhea, with disease symptoms without diarrhea, and without
2403 disease symptoms altogether were compared. Due to the presence of oocyst shedding in volunteers

2404 previously thought not to be shedding, the investigators revised the 50 percent infectious dose (ID_{50}) for
2405 *C. parvum* to 83 oocysts (528).

2406 In this study, the lack of any obvious relationship between challenge dose and the development of disease
2407 symptoms, diarrhea, or the pattern of oocyst shedding was evident. The patterns and duration of shedding
2408 were also quite variable. Eight different challenge stocks isolated at different times were used in the
2409 study, which may account for some of the variability observed.

2410 The final report in this series appeared in 1998 (529). The characteristic antibody responses that
2411 developed during experimental *C. parvum* infection were studied by immunoblot of oocyst proteins.
2412 Serum immunoglobulin G antibodies to proteins with molecular weights of 27 kDa, 17 kDa, and 15 kDa
2413 were those most commonly observed. Some of these antigens were subsequently used in serological
2414 screening assays (530).

2415 During the studies with the Iowa strain of *C. parvum* described above, many of the volunteers underwent
2416 upper endoscopy with jejunal biopsy at both pre- and post-challenge time points. These samples were
2417 used to study the human mucosal immune response to *Cryptosporidium* infection and were correlated
2418 with the severity of disease symptoms when possible. A series of six articles appeared between 2000 and
2419 2003 reporting the results of these studies, and overall results are summarized in a review published in
2420 2008 (531).

2421 A number of subsequent studies were conducted with the human challenge model for C*ryptosporidium*
2422 between 1999 and 2006. The impact of pre-existing immunity on experimental infection with *C. parvum*
2423 was examined in a study by Chappell et al. (528). Human volunteers challenged with four different strains
2424 of *C. parvum* and one strain of *C. hominis* were examined for disease development and oocyst shedding
2425 (532–534). The details and results of these studies are summarized in Table 21.

2426 In considering the re-establishment of the *Cryptosporidium* CHIM (525), one element that merits careful
2427 consideration is long-term safety risks of volunteers. In the studies conducted in the 1990s and early
2428 2000s, there were no reported safety assessments of adverse events related to challenge beyond 60 days.
2429 However, there have been several recent reports of increased incidence of chronic intestinal disorders
2430 among populations that experienced naturally occurring outbreaks of cryptosporidiosis. These have
2431 included diarrhea, abdominal pain, nausea, or even a formal diagnosis of irritable bowel syndrome
2432 according to the Rome III criteria (535–539). These symptoms persisted for several months up to two
2433 years in approximately 10 to 30 percent of subjects after the initial acute infection with *Cryptosporidium*.
2434 In case-control studies that also enrolled subjects not infected with *Cryptosporidium*, the relative risks for
2435 these chronic intestinal symptoms typically ranged from 1.5- to 3-fold increased risk. While the precise
2436 mechanisms remain to be determined, a full accounting of all associated risk factors is warranted. These
2437 observations suggest that future volunteers in a *Cryptosporidium* CHIM would need to undergo a rigorous
2438 informed consent process and be carefully screened for risk factors that might predispose them to long-
2439 term sequelae as a result of the challenge. Treating all volunteers with nitazoxanide before discharge may
2440 also mitigate longer-term risks.

Table 21. Experimental human challenge studies with *Cryptosporidium*.

Species	Strain	Species of origin	Passage	Volunteers		Oocyst dose range	Attack rate (%)	ID_{50}
				Sero-status	N			
Crypto-sporidium parvum	Iowa	Bovine	Bovine	Negative	29	30–1,000,00	62	87
	UCP	Bovine	Bovine	Negative	17	500–10,000	59	1,042
	TAMU	Equine	Human→Bovine	Negative	14	10–500	86	9
	Moredun	Cerine	Ovine→Bovine	Negative	16	100–3,000	69	300
	Iowa	Bovine	Bovine	Positive	17	500–50,000	62	1,880
Crypto-sporidium hominis	TU502	Human	Porcine	Negative	21	10–500	76	10

Abbreviation: ID_{50}, 50 percent infectious dose.

Note: Shaded boxes indicate those challenge strains that either originated in humans, or were passaged through a human, and high attack rates that these strains produced in the human challenge model with low 50 percent infectious doses.

2441 The *C. parvum* strains that have been used for human challenge studies differ with respect to their species
2442 of origin and their passage history (Table 21). The Iowa and UCP strains have a similar provenance. Both
2443 were isolated from calves, in the US states of Iowa and Maryland, respectively, and the two strains have
2444 been passaged exclusively in calves. A 10-fold difference in ID_{50} was seen for these strains in the human
2445 challenge model. Strain TAMU was accidentally transmitted to a human during the necropsy of a foal and
2446 was subsequently propagated in calves. The ID_{50} for this strain in the human challenge model was 10-fold
2447 lower than the ID_{50} for the Iowa strain, and 100-fold lower than the ID_{50} for the UCP strain. The Moredun
2448 strain was isolated from a red deer in Scotland. It was multiply passaged in sheep and then in calves. The
2449 ID_{50} for the Moredun strain was intermediate between that of the Iowa and UCP strains (532, 533).

2450 The Iowa strain of *C. parvum* has been evaluated in human volunteers with, or without, serologic
2451 evidence of prior exposure to *C. parvum* (527, 540). Table 21 shows that the ID_{50} increased 20-fold in
2452 volunteers with pre-existing immunity to *C. parvum.* One challenge study has been conducted with
2453 *C. hominis.* The challenge strain, called TU502, was isolated from a human infection and passaged in
2454 gnotobiotic pigs. In human volunteers without prior exposure to *C. hominis*, the ID_{50} was quite low (Table
2455 21). Heterologous cross-protection between species has not been investigated in humans, but studies in
2456 gnotobiotic pigs suggest partial cross-protection may occur (541, 542).

2457 Collectively, these studies raised the total number of human volunteers that have been safely
2458 experimentally challenged with *Cryptosporidium* to more than 100. The highest attack rates and lowest
2459 ID_{50} were observed for the two strains that had replicated in humans, TAMU and TU502. These results
2460 are highlighted in grey in Table 21. Prior exposure to *C. parvum* was protective against re-challenge,
2461 increasing the ID_{50} for the Iowa strain by about 20-fold. Clinical experience with *C. hominus* is limited,
2462 but the one strain that has been evaluated had a high attack rate and a low ID_{50} in seronegative human
2463 volunteers.

2464 While no vaccine candidates have been tested against experimental human infection with
2465 *Cryptosporidium*, the challenge model has been used to evaluate bovine hyperimmune colostrum (543).

2466 Briefly, pregnant cows were immunized with inactivated *C. parvum* oocysts and the resulting colostrum
2467 was partially purified to yield a concentrate rich in immunoglobulins against *Cryptosporidium*.
2468 Volunteers received 10 grams colostrum or nonfat milk placebo before challenge and then three times
2469 daily for five days after. There was a non-significant trend towards efficacy as measured by a reduction in
2470 the incidence of diarrhea, however the study suffered because of a lower than expected attack rate of only
2471 44 percent infection with the challenge dose of 10,000 oocysts, as compared to a 100 percent attack rate
2472 (4 out of 4 volunteers infected) in a pilot study with the same dose. Future efforts to standardize the model
2473 with a consistent attack rate will be needed to ensure it is suitable for evaluating both therapeutic and
2474 vaccine candidates.

2475 **The development of vaccines against *Cryptosporidium***

2476 The field of vaccine development against *Cryptosporidium* is at the stage of antigen discovery and
2477 exploratory studies in animal models (512, 515, 516, 544). Table 22 summarizes the status of the
2478 *Cryptosporidium* vaccine pipeline and more details can be found in a recent review by Lemieux et al.
2479 (545). Two main approaches have been pursued for selecting antigens to be included in vaccines. In one
2480 approach, proteins that are exposed on the surface of the sporozoite stage of parasite development are
2481 under consideration. These include the apical complex proteins that mediate attachment and invasion
2482 (512, 516). The lead target antigens seem to be gp40/15 and cp23. The gp40/15 glycoprotein is cleaved
2483 into two subunits that remain covalently attached on the surface of the parasite, and the surface-expressed
2484 form of gp40/15 mediates binding to epithelial cells and is associated with the parasitophorous vacuole
2485 (546). Cp23, another surface glycoprotein, is also the target of some candidate vaccines. A very large and
2486 heavily glycosylated protein, called gp900, is under consideration because it binds to intestinal epithelial
2487 cells and inhibits *C. parvum* attachment *in vitro*. An antigenic fragment of gp900 (designated SA40), as
2488 well as a fragment of another antigenic sporozoite protein, Cpa135 (designated SA35), were determined
2489 to have a protective effect as maternal antigens in a BALB/c neonatal mouse model (547).

Table 22. Candidate *Cryptosporidium* vaccines in preclinical development.

Vaccine type	Phase	Number of candidates	Vaccine developers
DNA vaccines	Preclinical	6	Emory University; Chinese Academy of Sciences; Jilin University; Tsinghua University; Shandong Institute of Parasitic Diseases
Recombinant bacterial expression vectors	Preclinical	8	University of Virginia; Virginia Commonwealth University; Instituto Nacional de Tecnología Agropecuaria; Istituto Superiore di Sanita

2490 The second approach to *Cryptosporidium* vaccines is the use of reverse genetics to identify potential
2491 target antigens. Using the complete genome sequences of *C. parvum* and *C. hominis*, a reverse genetics
2492 approach has identified three additional antigens: Cp15, profilin, and apyrase (548). An acidic ribosomal
2493 protein that is somehow surface expressed is being investigated in the form of a DNA vaccine (549) or in
2494 combination with Cp23 (550). Some of these proteins are being expressed in vectors like *Salmonella*,
2495 recombinant vaccinia, and *Lactobacillus* (512, 551). Others are being expressed in related protozoan
2496 parasites such as *Toxoplasma gondii* (552) or *Tetrahymena thermophila* (553). In another study, a reverse
2497 genetic approach identified three glycosylphophatidylinositol-anchored proteins: GP60, CpH1, and
2498 CpSUB1. These were validated by demonstrating that they bound to sera from *Cryptosporidium*-infected

2499 neonatal calves (554). Furthermore, naturally occurring maternal antibodies against these proteins were
2500 found to be present in bovine colostrum and efficiently transferred to neonatal calves.

2501 In the animal health field, cryptosporidiosis is also a serious problem among livestock animals, especially
2502 cows. There are no FDA- or US Department of Agriculture-approved vaccines for prevention of
2503 cryptosporidiosis in animals; however, there is one preparation marketed as a feed additive, BoviCare®-cp
2504 (Huvepharma), which may stimulate a protective immune response. BoviCare-cp is composed of digested
2505 *C. parvum* proteins and is recommended to be administered to neonatal calves twice daily for the first
2506 seven days of life. Unpublished research suggests that BoviCare-cp reduces diarrhea and oocyst shedding
2507 in neonatal calves experimentally challenged with *C. parvum* (Welter M., personal communication).
2508 Additional studies are needed to verify these results, and it remains to be determined if this product is
2509 efficacious in humans.

2510 **Summary**

2511 The intestinal parasite *Cryptosporidium* came to light as an important intestinal pathogen in the era of
2512 HIV/AIDS. For decades, it was generally regarded as an opportunistic pathogen of immune deficiency
2513 and the causative agent of occasional outbreaks due to contaminated water and food. The Global Enteric
2514 Multicenter Study (502) brought *Cryptosporidium* into focus as a major cause of morbidity and mortality
2515 globally, especially in children younger than 2 years of age.

2516 At this time, the only licensed vaccine against a parasitic disease is the RTS,S vaccine against
2517 *Plasmodium falciparum* malaria. The initial studies with a human challenge model for *Cryptosporidium*
2518 have provided an avenue to better understand pathogenesis and immunity directly in the human host, and
2519 to support the development of vaccines against a parasitic disease of newly recognized importance at the
2520 earliest possible stage.

2521 Several obstacles must be overcome, however, to develop a human challenge model of broader utility.
2522 The isolation and purification of oocysts from the stools of infected animals is not particularly scalable,
2523 reproducible, or convenient. Furthermore, no capacity is in place to produce standardized, larger-scale
2524 challenge stocks until *Cryptosporidium* can be adapted to cell culture, as has been done for *P. falciparum.*
2525 Both *C. hominus* and *C. parvum* are infectious in neonatal gnotobiotic pigs, both by oral and airborne
2526 routes of transmission. Unfortunately, the challenge doses for oral infection that have been used are five
2527 orders of magnitude higher than the doses required for infecting 50 percent of human volunteers with the
2528 best available challenge strains (see Table 21). The relevance of airborne transmission to the principal
2529 modes of transmission in humans is unclear. The gnotobiotic piglet model is also limited by the fact that
2530 specialized facilities are required and neonatal piglets can only be kept in isolation for a short period of
2531 time.

2532 Additional studies with *C. parvum* challenge strains were planned by investigators at the University of
2533 Vermont in order to develop a repeated low-dose challenge model, to complement the high-dose
2534 challenge model developed previously (Kirkpatrick B., personal communication). Additional studies
2535 using a *C. hominis* challenge, incorporating re-challenge studies to investigate correlates of protective
2536 immunity using highly sophisticated techniques like mass cytometry (to enable high-content
2537 immunophenotyping) and sequencing of antibody genes, were planned in collaboration between the
2538 University of Maryland and Tufts University (Levine M. and Chen W., personal communications).

2539 However, because of barriers in obtaining FDA approval related to consistency of challenge inocula,
2540 these studies are currently on hold. A key limitation is the lack of a source of Good Manufacturing
2541 Practice-certified *Cryptosporidium* oocysts that would be acceptable to the FDA for administration to
2542 CHIM volunteers. Investigators at Radboud University in the Netherlands are also considering
2543 establishing a *C. parvum* challenge model (Sauerwein R., personal communication) and may benefit from
2544 a more permissive regulatory environment in the European Union.

2545 In light of the seminal GEMS results, young children and infants living in low-resource countries where
2546 infection with *Cryptosporidium* is endemic would most benefit from an effective vaccine. An improved
2547 human challenge model for *Cryptosporidium* would also have great utility in the drug development field,
2548 since it would facilitate screening of candidates for clinical effect and early elimination of drug candidates
2549 that do not show a treatment benefit in this model. Several drug candidates are advancing in preclinical
2550 development and may be ready for testing in a *Cryptosporidium* CHIM in the near future (507, 508).

2551

Pseudo-challenge with live-attenuated pathogens

For many pathogens, challenge with fully virulent wild-type strains is unethical because of safety concerns for participants. In a handful of notable examples, live-attenuated strains, such as licensed vaccines with well-established safety records, have been used as so-called “pseudo-challenge” agents to explore key questions that would otherwise be difficult or impractical to study by either challenge with or natural exposure to virulent wild-type strains. Some examples of this can be found with rotavirus (555), polio (556), influenza (557), and dengue (558). Studies using this approach for rotavirus and polio are summarized in the following sections; studies using live-attenuated dengue vaccine candidates and live-attenuated influenza vaccines as challenge strains are discussed in the corresponding sections.

Rotavirus

Epidemiology and public health impact of rotavirus

Despite recent successes in the development and deployment of vaccines, rotavirus remains the most important pathogen causing diarrheal disease among young children worldwide. As with other enteric diseases, the bulk of the burden falls on impoverished children in low- and middle-income countries (LMICs) with poor access to clean water, sanitation, hygiene, and appropriate nutrition. Rotavirus is responsible for approximately 125,000 deaths among children under 5 years old, although this has decreased from around 500,000 in 2000 (559). Much of this success can be attributed to the approval and introduction of four World Health Organization (WHO)-prequalified live oral rotavirus vaccines (LORVs): RotaTeq® (Merck, USA), Rotarix™ (GlaxoSmithKline, Belgium), Rotasiil® (Serum Institute, India), and Rotavac® (Bharat Biotech, India). However, these LORVs have demonstrated only modest efficacy in LMICs (560). The reasons for this observation are still under investigation, and may be multi-factorial, but this points to the need for optimization of existing vaccines and/or development of new ones that perform more effectively in LMIC settings. Pseudo-challenge studies with LORVs offer one method to accelerating the development of new and improved rotavirus vaccines.

Pathogenesis and diversity of rotavirus

Rotavirus is a non-enveloped, double-stranded RNA virus whose genome contains 11 segments encoding six structural proteins, including VP4 and VP7, which form an outer capsid. The two outer capsid proteins are the target of neutralizing antibodies and determine the rotavirus serotypes. Multiple VP7 serotypes (also called “G” serotypes, since VP7 is glycosylated) have been identified. Similarly, a large number of VP4 (or “P” serotypes for protease-sensitive protein) have been described. Given that most of the rotavirus strains have not been characterized by immunological means, the term “genotype” is frequently used, especially for the initial identification of new viral strains. Some of the predominant genotypes associated with acute disease in children include G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (561).

A number of host proteins related to histo-blood group antigens have been implicated as rotavirus receptors. Differences in various polysaccharides can modulate virus binding and entry into the host cell, depending on the G and P serotypes (562).

Rotavirus infects via the fecal-oral route and upon infection and replication in the intestinal epithelium triggers acute secretory diarrhea. In addition to the lytic damage to the cells in the tip of the intestinal

2590 villi, which limits the absorptive capacity, the virally encoded toxin non-structural protein 4 stimulates
2591 chloride secretion (563). Other mechanisms, including activation of calcium signaling may also contribute
2592 to the induction of watery diarrhea (564).

2593 **Rotavirus vaccine development**

2594 While LORVs have been found to be highly efficacious (90 to 95 percent) in high-income country
2595 settings, their efficacy in low-income countries has been substantially lower (40 to 60 percent) (560). The
2596 precise explanation for this remains to be determined, but various factors have been postulated. One of the
2597 most commonly cited is poor immunogenicity because of reduced replication of the LORV in intestinal
2598 epithelial cells of young children with chronic intestinal inflammation, a condition known as
2599 environmental enteric dysfunction (565). Other possible explanations are (i) presence of maternal
2600 antibodies, given that in low-resource settings women have higher levels of these antibodies in
2601 comparison to women living in high-income countries, as antibodies transmitted transplacentally or by
2602 breastfeeding interfere with vaccine virus replication (566); and (ii) genetic differences in receptors and
2603 co-receptors between residents of high-income countries versus LMICs (567, 568).

2604 One proposed approach to overcome this limitation of LORVs is to develop a parenterally administered
2605 rotavirus vaccine that would bypass any deficiencies in mucosal immunity in infants in LMICs (569,
2606 570). There are several use cases for such a vaccine, the simplest being introduction as a stand-alone
2607 product. However, recent criticisms about the already large number of vaccines being added to the WHO
2608 Expanded Programme on Immunization suggest that such an approach could be difficult to implement. To
2609 overcome this criticism, one option would be to combine a parenteral rotavirus vaccine with another
2610 existing parenteral vaccine, such as the pentavalent combination (diphtheria-tetanus-pertussis,
2611 *Haemophilus influenzae* type b, and hepatitis B) or other multivalent combination (hexavalent with
2612 inactivated polio vaccine, etc.). Another, non-mutually exclusive approach would be to use the parenteral
2613 rotavirus vaccine in combination with an LORV in a prime-boost regimen. Experience with other
2614 vaccines, including influenza A H5N1 (571, 572) and typhoid (573), suggests that a prime-boost approach
2615 could lead to a synergistic response that is superior to either vaccine given individually.

2616 There are several parenteral rotavirus vaccine candidates progressing through development. The most
2617 advanced of these is a nonreplicating trivalent subunit rotavirus vaccine consisting of P[4], P[6], and P[8]
2618 VP8s conjugated to P2 tetanus toxoid. This candidate recently advanced to a multi-country pivotal Phase
2619 3 clinical trial (ClinicalTrials.gov Identifier NCT04010448) (574). Another parenteral candidate, earlier in
2620 development, is the heat-inactivated rotavirus vaccine CDC-9 strain (575). Several other parenteral
2621 rotavirus vaccine candidates are at earlier stages of development (576).

2622 **Pseudo-challenge studies with a live oral rotavirus vaccine**

2623 In a recent Phase 1/2 clinical trial of monovalent P2-VP8-P[8] (555), a pseudo-challenge model was
2624 included as part of the study design as a novel approach to demonstrating vaccine efficacy. Either placebo
2625 or the vaccine candidate in three doses of either 30 µg or 60 µg were given to 162 South African infants
2626 at 6, 10, and 14 weeks of age. The primary objectives were to determine vaccine safety/reactogenicity and
2627 immunogenicity. As a secondary objective, the pseudo-challenge was incorporated as an assessment of
2628 vaccine impact on mucosal immunity and viral shedding. The LORV Rotarix was given to vaccinees four
2629 weeks after the third P2-VP8-P[8] dose, and then shedding of live Rotarix was measured by enzyme-

2630 linked immunosorbent assay at five, seven, and nine days post-dose. As exploratory objectives, mucosal
2631 immune responses before and after Rotarix administration were quantitated. Specifically, serum levels of
2632 anti-rotavirus immunoglobulin A (IgA), anti-P2-VP8-P[8] IgA, and immunoglobulin G geometric mean
2633 titers (GMTs) and homotypic neutralizing antibody GMTs were determined (Table 23).

Table 23. Rotarix fecal shedding in infants vaccinated with monovalent P2-VP8-P[8].

	Placebo	P2-VP8-P[8] 30 µg	P2-VP8-P[8] 60 µg	30 µg and 60 µg combined
Number shedding on days 5, 7, or 9	17/44 (39%)	6/45 (13%)	9/46 (20%)	15/91 (17%)
Reduction compared to placebo (95% confidence interval)	Not applicable	66% (21–85)	49% (0–75)	57% (23–76)
p value		0.0087	0.0493	0.0052

Data reproduced from Groome et al. 2017 (555).

2634 In order to determine sample size, it was assumed that 30 percent of placebo recipients would shed
2635 rotavirus. In the final analysis, the most significant result was the number of subjects that shed rotavirus
2636 on any day (5, 7, or 9): 39 percent of placebo recipients as compared to 17 percent of P2-VP8-P[8]
2637 recipients (combined 30 µg and 60 µg groups) shed rotavirus, constituting a 57 percent reduction
2638 ($p = 0.0052$). Of the 32 shedders, 29 shed Rotarix and three shed a G9P[8] strain, presumably the result of
2639 natural infection.

2640 This rotavirus pseudo-challenge approach was also used in another recent Phase 1/2 trial, of trivalent P2-
2641 VP8 (577). This trial had a similar design as the one described above: 558 South African infants were
2642 enrolled at 6 weeks of age; received three doses of trivalent P2-VP8 or placebo at 6, 10, and 14 weeks of
2643 age; and then received Rotarix as a pseudo-challenge four weeks after the last dose of trivalent P2-VP8.
2644 This study tested three dose levels of trivalent P2-VP8: 15 µg, 30 µg, and 90 µg. The reduction in
2645 shedding on any of days 5, 7, or 9 after pseudo-challenge was not significantly different from placebo for
2646 the 15 µg and 30 µg groups, but was significant for the 90 µg group: 45 percent polymerase chain
2647 reaction-confirmed shedding with placebo, versus 27 percent shedding with 90 µg, a 41 percent reduction
2648 (95 percent confidence interval 0.1 to 65, $p = 0.035$) (see Table 24).

Table 24. Rotarix fecal shedding in infants vaccinated with trivalent P2-VP8-P[4],[6],[8].

	Placebo	Trivalent 15 µg	Trivalent 30 µg	Trivalent 90 µg
Number shedding on days 5, 7, or 9	24/53 (45%)	22/52 (42%)	19/56 (34%)	15/56 (27%)
Reduction compared to placebo (95% confidence interval)	Not applicable	7%	25%	41% (0.1–65)
p value		Not significant	Not significant	0.035

Data reproduced from Groome et al. 2020 (577).

2649 These results served as a proof of concept that reduction in shedding of an orally administered live oral
2650 vaccine strain could serve as a valid and informative endpoint in the development of parenterally
2651 administered rotavirus vaccines. Importantly, these modest Phase 1/2 studies provided strong justification
2652 for advancing the vaccine to a much larger Phase 3 study now ongoing (ClinicalTrials.gov Identifier
2653 NCT04010448) (574). The success of this approach also stimulated a pilot study in Zambia that
2654 demonstrated reduction viral shedding after a second dose of Rotarix as a live-attenuated challenge (578).

Polio

Epidemiology and public health impact of polio

Infection with poliovirus is responsible for poliomyelitis (polio), consisting of acute flaccid paralysis that can be fatal as a result of motor neuron damage leading to progressive paralysis. The Global Polio Eradication Initiative (GPEI) has reached the endgame of its campaign, but poliovirus remains endemic in two countries: Afghanistan and Pakistan (579). There are three serotypes of poliovirus (types 1, 2, and 3) and immunization with a single strain does not confer protection against the others. Therefore, a strategy to immunize against all three types is essential for successful eradication. Wild-types 2 and 3 have been eradicated, with the last confirmed cases occurring in 1999 and 2012, respectively. Only type 1 remains circulating in endemic countries, with fewer than 180 cases detected annually over the past five years (580). This represents substantial progress since the start of the GPEI in 1988, when there were 350,000 confirmed cases worldwide (581).

Current vaccination strategy

Much of the progress in the current eradication campaign was achieved with trivalent oral polio vaccine (tOPV), a live-attenuated vaccine composed of all three strains, also known as the Sabin vaccine. However, one of the risks with use of tOPV is vaccine-associated paralytic polio, which occurs at very low rates of two to four per 1 million vaccinations. Another risk is reversion of one of the live-attenuated vaccine-derived strains that begins to circulate in communities with insufficient immune coverage, resulting in circulating vaccine-derived poliovirus (cVDPV). Risk of this reversion is particularly notable with type 2 polio because of the genetic instability of the attenuated strains. In order to eliminate these risks, many countries have switched to use of inactivated polio vaccine (IPV), also known as the Salk vaccine, which contains killed virus and therefore lacks the risk of vaccine-associated paralytic polio and generation of cVDPV. IPV generates excellent humoral immunity; however, its key disadvantage is that it is administered systemically and thus does not confer high levels of mucosal immunity in the intestinal tract, the primary site for polio infection. Therefore, subjects receiving IPV only are still at risk of enteric polio infection and may shed virus if exposed to wild polio, thereby contributing to the chain of transmission. Most countries with high levels of vaccine coverage now use IPV to avoid the risks associated with OPV; however, in LMICs with poor sanitation and hygiene and lower vaccination coverage, use of OPV and its associated risks are still prevalent. In 2016, WHO initiated a phased elimination of OPV, beginning with withdrawal of OPV2 by replacing tOPV with bivalent oral polio vaccine (bOPV, containing only types 1 and 3), and the use of IPV to provide coverage against OPV2. However, during this interim period, subjects that receive IPV and bOPV will still be at risk of shedding type 2 virus in feces if exposed to a type 2 cVDPV. This risk mandates the stockpiling of monovalent oral polio vaccine type 2 (mOPV2) (582) or other short-term measures such as prophylactic or therapeutic antivirals (583) to control an outbreak of cVDPV2. A recent development to counter cVDPV2 outbreaks is the novel OPV2 which is more genetically stable than OPV2 (584, 585).

Pseudo-challenge studies with live-attenuated polio vaccines

While challenge studies intentionally infecting subjects with wild-type polioviruses are obviously unethical, the use of challenge with licensed live-attenuated OPV strains with well-established safety profiles has been applied in two approaches as part of the polio eradication endgame and withdrawal of

2695 OPVs. The first is challenge studies in the target population of naïve infants in order to evaluate the
2696 efficacy of IPV and mixed IPV/OPV vaccination regimens. The second is challenge models performed in
2697 healthy volunteer adult populations with a history of vaccination with IPV only. These have been used in
2698 the development of novel oral polio vaccines, and also therapeutics intended for use in outbreak
2699 scenarios. In both cases, the primary objective of these challenge studies is typically to evaluate intestinal
2700 shedding of the challenge virus as a measure of the extent of mucosal immunity induced by the
2701 vaccination regimen.

2702 **Challenge studies in infants**

2703 A series of Phase 2 and Phase 3 studies have been conducted with infants in LMICs, in which the subjects
2704 received various regimens containing IPV and/or bOPV and then were challenged with either tOPV or
2705 mOPV2. Two recently published comprehensive reviews (556, 586) provide detailed overviews of the
2706 findings of these studies. In general, they found that one or two doses of IPV in combination with bOPV
2707 conferred strong immunity to types 1 and 3, and a moderate level of immunity to type 2. Taken together,
2708 these studies provided robust support for the GPEI strategy of switching from tOPV to a mixed
2709 IPV/bOPV regimen.

2710 **Challenge models in adults**

2711 As part of the strategy of replacing tOPV with bOPV, two novel OPV2 vaccine candidates are under
2712 development. These live-attenuated strains contain several genetic modifications of the standard OPV2
2713 strain that make them more genetically stable and thus better suited for stockpiling as a means to address
2714 cVDPV2 outbreaks and avoid further introduction of cVDPV2. In order to test the safety and efficacy of
2715 these candidates, a Phase 1 study was conducted in healthy adult volunteers that had a well-documented
2716 history of receiving IPV as children (587). This study tested two strains, designated candidate 1 (genotype
2717 S2/cre5/S15domV/rec1/hifi3) and candidate 2 (genotype S2/S15domV/CpG40). Both candidates were
2718 found to be safe and immunogenic. Fecal shedding was measured by a composite index that accounted for
2719 quantity and duration of shedding. This study demonstrated that shed viruses were genetically stable and
2720 remained attenuated. The study was conducted in a purpose-built containment facility at the University of
2721 Antwerp, Belgium, and dubbed "Poliopolis" in order to ensure strict isolation of subjects shedding virus
2722 and prevent accidental release into the environment of any virulent revertants (588). Novel OPV2
2723 candidate 1 was subsequently approved for Emergency Use Listing by WHO (589, 590).

2724 Another approach for mitigating the risk of lack of intestinal immunity with IPV is the co-administration
2725 of an adjuvant that enhances the immune response. Previous work with double-mutant heat-labile toxin
2726 (dmLT) from enterotoxigenic *Escherichia coli* (ETEC) (R192G/L211A) has demonstrated that it is a
2727 potent adjuvant when administered orally (591, 592) and that it also has the potential to stimulate mucosal
2728 immunity of parenterally co-administered antigens, including IPV (593). This effect has been established
2729 in mouse (594) and nonhuman primate preclinical models (Norton B., personal communication) and in a
2730 recent ETEC subunit vaccine Phase 1 clinical study (595).

2731 An ongoing Phase 1 study at the University of Antwerp will examine the response of IPV administered
2732 with or without dmLT to challenge with bOPV (ClinicalTrials.gov Identifier NCT04232943) (596). This
2733 study will determine the safety of dmLT when co-administered with IPV, and the efficacy of the

2734 adjuvanted vaccine as measured by fecal shedding of bOPV, type-specific fecal IgA, polio neutralizing
2735 antibody responses, and other immunological markers.

2736 A similarly designed polio pseudo-challenge model study was conducted to support the development of
2737 pocapavir, a novel small-molecule antiviral that targets polio and is intended for use in cVDPV outbreaks
2738 (597). In this study at the Sahlgrenska University Hospital in Göteborg, Sweden, 144 adults were
2739 enrolled, challenged with mOPV1, and then treated with either pocapavir (1,600 mg per day) or placebo.
2740 Time to cessation of fecal shedding of mOPV1 was the primary efficacy endpoint. Pocapavir was found
2741 to decrease time of shedding by three days compared to placebo (ten versus 13). Unexpectedly, 44 percent
2742 of subjects experienced infection with pocapavir-resistant strains, including several in both the placebo
2743 group as well as in the active treatment group at baseline (i.e., before treatment initiation). The high rate
2744 of transmission of resistant strains was attributed to the close quarters of the subjects in the containment
2745 facility: for example, sleeping six people to a room and sharing dining and lavatory facilities. When
2746 subjects with resistant strains were excluded from the analysis, pocapavir gave a much stronger response
2747 and reduced average duration of shedding to only 5.5 days. A follow-up analysis of fecal samples from a
2748 subset of volunteers from the placebo group in this study demonstrated that these individuals had neither
2749 pre-existing intestinal neutralizing antibodies, nor did they mount strongly neutralizing responses after
2750 mOPV1 challenge (598), underscoring the vulnerability associated with IPV administration. A recent case
2751 study documented the successful use of pocapavir in clearing a vaccine-derived type 3 polio infection in
2752 an immunodeficient infant (599), providing additional validation for the role of antivirals in addressing
2753 VDPV infections.

2754 **Conclusions and future prospects**

2755 As the GPEI moves toward global eradication, challenge studies in the target population of infants in
2756 LMICs as well as challenge models conducted with adult volunteers will both continue to play crucial
2757 roles in developing innovative tools such as novel OPV2 and other interventions that will enable
2758 successful completion of this historical global effort. Meanwhile, pseudo-challenge models with LORVs
2759 have the potential to accelerate development of next-generation parenteral rotavirus vaccines that
2760 overcome limitations of the current approaches.

2761

Respiratory diseases

Influenza

Epidemiology, viral diversity, and public health impact of influenza

Seasonal epidemics of influenza spread rapidly around the world, typically affecting 5 to 15 percent of the population and causing an estimated 5 million cases of severe infection and 290,000 to 650,000 deaths annually (600). Moreover, four influenza pandemics have occurred in the twentieth and early twenty-first centuries in which a strain emerged that had not previously spread appreciably in humans. These pandemics were associated with high rates of infection, morbidity, and mortality. The 1918 to 1923 "Spanish flu" killed an estimated 50 million people, mostly young adults. Mortality from the subsequent pandemics in 1957 ("Asian flu"), 1968 ("Hong Kong flu"), and 2009 (A[H1N1]) caused 2 million, 1 million, and 0.15 to 0.6 million deaths, respectively (601). While existing vaccines are not fully protective, the impact of their widespread application is notable. For example, during the 2012 to 2013 influenza season in the United States, vaccination was calculated to have prevented 6.6 million influenza virus-associated illnesses, 3.2 million medically attended illnesses, and 79,000 hospitalizations, in spite of the fact that less than 45 percent of individuals were vaccinated (602).

The influenza virus is diagrammed in Figure 8. Its genome consists of eight negative, single-stranded RNA segments that are associated with the nucleocapsid protein (NP). The virus has two matrix proteins: M1, which lines the inner surface of the virus envelope and lends rigidity to the structure; and M2, an ion channel-forming peptide that protrudes through the membrane and is partially exposed on the virion surface. The following two proteins coat the outer surface of the virus: neuraminidase (NA), which mediates virus release from infected cells; and hemagglutinin (HA), which binds to the receptor on target cells and mediates virus entry.

Figure 8. Diagram of influenza virus structural elements.

The HA protein is a major target of influenza vaccines. The HA structure (Figure 9) is trimeric, with a globular "head" and an elongated "stem" or "stalk". Each monomer consists of two polypeptide chains (HA1 and HA2) joined by a disulfide bond. HA1 forms the head and is the receptor-binding domain. Broadly neutralizing antibodies against this part of the protein can block receptor engagement. HA2 contains the membrane fusion machinery; a portion of it, called the HA2 endodomain is internal to the virion.

Figure 9. Structure of influenza hemagglutinin with a globular head domain (red) and elongated stalk domain (green).

Genetic variability is the hallmark of RNA viruses. In influenza, two types of variation occur. "Drift" is when mutations rapidly accumulate in the HA and NA proteins and, to a lesser extent, in structural proteins like the matrix and nucleocapsid. "Shift" is when reassortment of the viral RNA segments occurs. The latter takes place when two viruses infect the same cell and RNA segments of their genomes are randomly packaged into progeny virions. Notably, the highly variable, surface-expressed HA and NA

2798 that are key targets of antiviral immunity are encoded on separate RNA segments. Co-infection can
2799 generate new HA/NA combinations in a single round of replication.

2800 Influenza viruses can be classified as A or B, based on the amino acid sequences of their HA protein (see
2801 Figure 10). Influenza B is unique to humans, while influenza A is found in humans and a number of
2802 animal species, most notably birds and pigs. The influenza A viruses are further classified into groups 1
2803 and 2. Each group is comprised of multiple types that correspond to antigenically distinct proteins (603)
2804 and there is further serotype diversity within each subgroup.

2805 **Figure 10. Phylogenetic classification of human influenza A and B viruses according to the**
2806 **hemagglutinin gene.**

2807 Influenza A infections in humans include serotypes H1, H2, H3, H5, H7, and H9, which come from both
2808 groups of the influenza A viruses. The N1, N2, and N9 serotypes of NA) have been found in viruses
2809 spreading in humans. Both strains of influenza B viruses circulate in humans.

2810 Animal reservoirs are a potential source of further diversity of influenza viruses in humans (604). Since
2811 the 1918 pandemic, all of the pandemic influenza strains have had two or more genes of animal origin
2812 (see Table 25). The 1957 Asian flu strain had HA, NA, and PB1 (an RNA polymerase) from birds. The
2813 Hong Kong flu strain had HA, NA, and PB1 genes from birds, but the HA and PB1 were from a different
2814 avian source than those in the Asian flu strain. The 2009 influenza A(H1N1) strain replaced all of the
2815 genes found in the 1918 Spanish flu strain with those of animal origin. This virus combines genes from
2816 two avian and two porcine sources—some shared with previous pandemic strains and others newly
2817 introduced from novel avian and porcine sources.

Table 25. Strains of influenza responsible for pandemics spanning a century.

Years	Pandemic	Strain	Species of origin of HA and NA genes
1918–1923	Spanish flu	H1N1	Human
1957–1958	Asian flu	H2N2	Avian
1968–1969	Hong Kong flu	H3N2	Avian
2009–2010	2009 A(H1N1)	A(H1N1)	Porcine and avian

Abbreviations: HA, hemagglutinin; NA, neuraminidase.

2818 Sporadic human infections with avian, and less frequently, porcine influenza virus continuously occur and
2819 are under close surveillance globally. Reassortment with previously human-adapted influenza strains,
2820 and/or further *do novo* adaptation of new animal strains to humans, are believed necessary to spark global
2821 pandemics.

2822 To address the significant genetic variation in influenza viruses, different vaccine approaches have been
2823 designed for seasonal and pandemic strains. The unpredictability of pandemic influenza outbreaks has led
2824 to influenza vaccine stockpiling against newly identified, novel strains associated with limited outbreaks.
2825 For example, the avian influenza strain A(H5N1) none of whose genes match those of previous pandemic
2826 strains of avian origin has been newly transmitted to humans. By 2020 the virus (termed "bird flu") has
2827 spread from birds to 861 humans in 17 countries causing 455 deaths (605), a fatality rate exceeding
2828 50 percent. Presently, influenza A(H5N1) does not easily spread from human to human The United
2829 Kingdom stockpiled 17 million doses of a vaccine against this strain, should it begin to spread human to

human and spark a new pandemic (606). Another potentially emergent avian strain, influenza A(H7N9), was sporadically observed in humans in mainland China in 2013 and 2014. By 2017, at least 1,564 cases and 612 deaths (a fatality rate of 39.2 percent) had resulted from this strain (607).

Influenza vaccines

For decades, influenza vaccines have been made by growing the virus in eggs and inactivating it with formalin or other alkylating agents (Figure 11). Though highly efficacious, the reactogenicity of unpurified whole-inactivated vaccine led to making “split” preparations, which are better tolerated. More recently, inactivated vaccines are based on the purified HA and NA proteins, which are even less reactogenic (608).

Figure 11. Inactivated influenza A virus vaccine manufacture.

The great success of live-attenuated vaccines for multiple other viral infections led to sustained efforts for more than two decades to develop live-attenuated influenza vaccines (LAIVs) (609). Aviron (now MedImmune/AstraZeneca, Gaithersburg, Maryland, United States) introduced the first licensed LAIV in 2003, Fluenz™ Tetra/FluMist® Quadrivalent, based on a cold-adapted A/Ann Arbor/6/1960 strain (610). A similar cold-adapted vaccine based on a Leningrad influenza A strain, termed Ultravac® (Microgen, Moscow, Russia), was developed by the Saint Petersburg Institute of Experimental Medicine (IEM) and has been used successfully in Russia since 1987 (611). IEM signed an agreement with the World Health Organization (WHO) under which they committed to supply pandemic and seasonal candidate vaccine viruses to various developing-country vaccine manufacturers, including China (Changchun BCHT Biotechnology Co., Ltd.), India (Serum Institute of India Pvt. Ltd.), and Thailand (Government Pharmaceutical Organization, GPO) (612). Serum Institute developed a trivalent LAIV, Nasovac-S, which was proven to be efficacious in studies in children in Bangladesh (613) and was licensed in India and prequalified by WHO (614). GPO (615) used the Russian strain in the development of a pandemic H5N1 vaccine that was shown to be immunogenic and capable of providing significant priming to a later boost with an H5N1 inactivated vaccine (616). IEM’s findings with an H5N2 LAIV were similar (617). Detailed analysis of the immune responses to LAIVs reveals that while their ability to induce hemagglutination inhibition (HI) and neutralizing antibodies is lesser than that of inactivated vaccines, LAIVs induce local immunoglobulin A (IgA) responses detected in nasal or throat swabs, as well as circulating cytotoxic T-cell responses, which are not commonly observed after inactivated vaccines (618). Despite these advantages, LAIVs are not approved for children under 2 years old, due to increased risk of wheezing. Furthermore, FluMist is not approved for adults over 49 years old because of concerns about decreased efficacy in older individuals.

Genetic drift of the HA and/or the NA genes (driven by the human immune response to natural infection and by vaccination) commonly leads to a need to manufacture new vaccines based on the actual circulating strains. WHO maintains a worldwide network of laboratories that, together with five central laboratories, characterize up to 30,000 influenza strains yearly. In February of each year, the decision is made on which strains are to be included in the vaccines for the upcoming influenza season in fall and winter. Moreover, in a given year, the seasonal influenza vaccines that are needed may be different for the Northern and Southern Hemispheres (619). In the United States, the US Food and Drug Administration (FDA) requires that seasonal influenza vaccines be re-licensed each year (620). Seasonal influenza vaccines are tailored to closely match the circulating strains. Since 2013, they have contained both

2871 influenza A and B strains, given their common co-circulation during seasonal outbreaks. Up until a few
2872 years ago, seasonal vaccines were trivalent, including an H1, an H3, and a B strain; in the last few years,
2873 more than one B strain has circulated, leading to the requirement for quadrivalent vaccines including two
2874 A and two B strains. Seasonal vaccines that elicit antibodies to the HA proteins of a hemagglutination
2875 titer greater than 1/10 for children and 1/30 for adults are regarded as highly protective against infection
2876 and disease.

2877 Currently, more than 25 seasonal influenza vaccines are licensed every year in the United States and 12
2878 are prequalified by WHO for use in all member countries (621, 622). Most of them are egg derived, but
2879 the field is progressively moving toward tissue culture-derived vaccines, which have shown similar
2880 profiles of safety and immunogenicity and are less dependent on the growth ability of the virus in eggs
2881 and on maintaining egg production and are of more consistent manufacturing and are not contraindicated
2882 in individuals allergic to egg proteins. It has been claimed that by circumventing the need for egg culture
2883 adaptation, cell-cultured vaccines may result in increased efficacy (623). A cell culture-derived trivalent
2884 inactivated influenza virus vaccine produced by Seqirus (headquartered in the United Kingdom) using the
2885 Madin Darby canine kidney cell line was shown to be efficacious in a large efficacy trial with disease
2886 endpoints (influenza-like illness) (624). This study was followed by the testing of a quadrivalent version
2887 of the vaccine, approved in the European Union (Flucelvax® Tetra) and the United States (Flucelvax®
2888 Quadravalent). The pivotal Phase 3 trials of this vaccine in adult and pediatric populations demonstrated
2889 the immunogenicity of the quadrivalent cell culture-derived vaccine to be non-inferior to that of the
2890 trivalent vaccine against shared strains and superior against the influenza B strain absent from the
2891 trivalent vaccine (625).

2892 Other approaches to influenza vaccination that have led to licensure include the development of
2893 recombinant HA subunit vaccines. Thus, Protein Sciences Corporation (Meriden, Connecticut, United
2894 States) developed a trivalent recombinant subunit HA vaccine, Flublok®, expressed by a chimeric
2895 baculovirus grown in insect cells. The vaccine was well tolerated and immunogenic in adults older than
2896 18 years and even in subjects older than 75 years of age; it demonstrated non-inferiority to split vaccines
2897 and was shown to be efficacious in a field trial against drifted influenza viruses (626). Flublok was
2898 approved by the FDA on a Fast Track Designation. The coverage of this vaccine was recently expanded
2899 to include a second influenza B strain to generate Flublok® Quadrivalent (627). Of note is the increased
2900 immunogenicity of HA subunit vaccines when formulated as nanoparticles, which have potential
2901 advantages over inactivated vaccines. Nanoparticles stimulate the innate and adaptive immune systems,
2902 exhibit antigen depot effect, extend the period for antigen processing and presentation, and exert an
2903 intrinsic adjuvant effect on inducing robust immune responses (628). NanoFlu™, by Novavax, Inc.
2904 (Gaithersburg, Maryland, United States), is a nanoparticle influenza vaccine produced in insect cell lines
2905 infected by a chimeric baculovirus carrying the HA genes of the four selected seasonal strains (629). A
2906 study of NanoFlu was carried out in 2,652 healthy adults aged 65 and older that received either
2907 quadrivalent NanoFlu or Fluzone® (Sanofi Pasteur Inc., Swiftwater, Pennsylvania, United States).
2908 According to a press release, NanoFlu outperformed the Sanofi vaccine on measures of immunogenicity,
2909 including significantly higher geometric mean titers and seroconversion rates across all four strains in the
2910 vaccines; however, the study results have yet to be published (630).

2911 Meta-analyses across multiple trials of the various seasonal vaccine candidates, including those noted
2912 above, reveals little difference with regard to safety or immunogenicity across all the vaccine candidates

2913 available. It has been well established that HI titers ≥1:10 are associated with clinical protection, and thus,
2914 demonstrating that a new vaccine induces such titers is frequently sufficient for licensure (631), as long as
2915 it is produced in a similar way to vaccines previously licensed on the basis of clinical efficacy. The real
2916 challenge for current influenza vaccines is the frequent mismatching between the strains selected for the
2917 season and the multiple viral strains that end up circulating, leading to a wide variability in their efficacy.
2918 For example, the efficacy of seasonal vaccines ranged from 10 percent in the 2004 to 2005 season to
2919 60 percent in the 2010 to 2011 season, with the biggest factor explaining the difference noted as mismatch
2920 between the vaccine strains and the circulating strains (632). Between 2000 and 2011, influenza B
2921 vaccine strains did not match circulating strains in six influenza seasons. Increased rates of influenza
2922 activity were observed in the United States in the fall of 2014, and this was attributed to poor vaccine
2923 effectiveness as a result of a mismatch of the H3 component of the current influenza vaccine to
2924 circulating strains, leading to an overall effectiveness of 23 percent (632).

2925 To date, WHO has prequalified 15 trivalent, 8 quadrivalent inactivated vaccines and one live-attenuated
2926 influenza vaccines. (633), The immunogenicity of seasonal influenza vaccines in children and the elderly,
2927 as well as that of vaccines based on pandemic strains, is known to be diminished with respect to that for
2928 seasonal vaccines in adults. Several adjuvants have been used to address this limitation, including
2929 aluminum hydroxide (alum), MF59, AS03, AF03, virosomes, heat-labile enterotoxin (LT), and more
2930 recently the Matrix-M™ nanoparticle approach by Novavax, Inc. (622). While generally safe and well
2931 tolerated, some rare adverse events have discouraged the widespread use of some of these adjuvants; for
2932 example, the linkage of LT with Bell's Palsy.

2933 **Universal influenza vaccines**

2934 A large effort is underway to develop vaccines that would not need seasonal reformulation. Immunity to
2935 more conserved viral proteins or domains is the target for developing more broadly protective, or even
2936 universal, influenza vaccines. The most obvious targets are proteins that are both expressed on the surface
2937 of virions (hence accessible to antibodies) and more conserved than the highly variable "head" portion of
2938 HA. The HA "stalk," NA, and the ion channel M2 protein all fall into this category. A vaccine could also
2939 target the internal structural proteins of the virus, like NP and M1. In this case, the vaccine-mediated
2940 protection would more likely result from the action of cytotoxic T cells. Cytotoxic lymphocytes can
2941 specifically recognize and kill virally infected cells when fragments of viral proteins are presented on
2942 human leukocyte antigen (HLA) molecules on the cell surface.

2943 The isolation and characterization of broadly neutralizing antibodies targeting epitopes in the HA stem
2944 region is a significant advancement toward identifying targets for universal influenza vaccines. Isolated
2945 from the plasmablasts of infected patients or those experimentally vaccinated against the pandemic 2009
2946 influenza A(H1N1) strain (634), these antibodies are extensively affinity matured, suggesting that they
2947 originate from memory B cells. Chimeric HA molecules with the head of one strain and the stem of
2948 another have been used to separately evaluate the capacity of candidate vaccines to boost antibody
2949 responses to the more conserved stem, rather than to the head region (635). A study by Ellebedy et al.
2950 found that the best way to boost anti-stem antibody responses was to immunize with a strain to which the
2951 volunteers were completely naïve. Immunization in a background of pre-existing immunity mainly
2952 boosted strain-specific responses to the head region (636).

2953 The concept that has emerged from Ellebedy's research is that vaccines based on influenza strains that
2954 have not caused pandemics in humans, but which have sporadically appeared (like influenza A[H5N1] or
2955 A[H7N9] bird flu strains) could establish stronger humoral immune memory to the conserved HA stem.
2956 This stronger humoral immune memory to the more conserved portions of HA could, in turn, provide
2957 broader protection against pandemic and seasonal influenza strains.

2958 Other conserved antigens that are exposed on the virion surface are being actively investigated as targets
2959 of humoral immunity, including the NA (637) and the M2 matrix ion channel protein. A number of
2960 subunit vaccines based on the influenza matrix protein are in development (606, 638). Most of them
2961 contain only the M2 external domain. This approach is amenable only to influenza A viruses, given that
2962 the M2 of influenza B viruses has a different structure and the external portion is too short to be
2963 immunogenic.

2964 Other approaches under development include the construction of synthetic peptides that represent mixes
2965 or string of B- and/or T-cell epitopes from influenza proteins; epitope-based Multimeric-001 (M-001)
2966 vaccine candidate is currently being evaluated in clinical trials. Results from this clinical trial are not yet
2967 available (639). A somewhat opposite strategy is the "computationally optimized broadly reactive
2968 antigen" approach (640), in which combinations of H1 HA antigens, some naturally derived sequences,
2969 and other computer-generated optimal sequences are mixed, with the expectation of inducing responses
2970 against diverse H1 influenza strains. Experiments in mice have been promising. A nanoparticle "mosaic"
2971 influenza vaccine based on the HA hypervariable receptor-binding domain from sequences of H1N1
2972 influenza strains isolated over a span of more than 90 years is being developed at the National Institutes
2973 of Health Vaccine Research center (641). Another example of epitope-based vaccines is the development
2974 of a liposome nanoparticle subunit vaccine by Dhakal et al. (642).

2975 Among the first of the potentially broadly protective candidate vaccines to be evaluated in human
2976 challenge studies are those using a vectored approach. The MVA-NP+M1 vaccine expresses NP and M1
2977 from an influenza A(H3N2) strain in the modified vaccinia virus Ankara (MVA) vector. This vector has
2978 been extensively attenuated through serial passage in tissue culture. NP and M1 are expressed as a fusion
2979 protein. Fifteen volunteers were administered the vaccine, evaluated for cellular immune responses, and
2980 challenged 30 days later with an influenza A strain. Three of 11 vaccine recipients and five of 11 controls
2981 developed influenza. In vaccine recipients, the symptoms of the disease were blunted and viruses were
2982 shed for a shorter period of time (643). A recent study of the MVA-NP+M1 vaccine, newly manufactured
2983 on an immortalized cell line, has been reported (644).

2984 The same NP and M1 antigens were expressed in a replication-deficient chimpanzee adenovirus (Ad)
2985 vector (645). This vaccine, called ChAdOx1 NP+M1, was evaluated in a Phase 1 study, either alone or
2986 with an MVA-NP+M1 boost. Although the chimpanzee Ad vaccine produced a similar magnitude of T-
2987 cell responses to that seen with MVA-NP+M1, boosting with MVA-NP+M1 did not significantly
2988 augment the responses. However, when given in combination with seasonal influenza vaccine, an increase
2989 in the antibody response and boosting of T-cell responses were observed (646). A summary of the many
2990 novel approaches for influenza vaccine development are diagrammed in Figure 12 (647). The general
2991 expectation is that effective delivery of the wider range of influenza viral antigens that comprise a
2992 universal influenza vaccine will likely require novel vaccine types as well (648).

2993 **Figure 12. Range of current and new vaccine approaches against influenza A virus.**

2994 Several RNA vaccines for influenza virus are under development. They include synthetic mRNA
2995 molecules encoding only the HA, as well as virally derived self-amplifying RNA (sa-RNA) candidates
2996 that encode both the antigen of interest and proteins involved in RNA replication. When tested head to
2997 head, both were protective, but the protection achieved by sa-RNA required only 1.25 µg sa-RNA
2998 compared to 80 µg for the mRNA vaccine. An sa-RNA trivalent vaccine made from H1N1, H3N2 (X31),
2999 and B (Massachusetts) protected mice against sequentially administered H1N1 and H3N2 challenges
3000 (649).

3001 **The influenza human challenge model**

3002 The experience with formal experimental influenza infection to human volunteers is extensive and goes
3003 back to 1937, when Smorodintseff et al. (650) infected 72 volunteers with a human influenza virus
3004 passaged in ferrets and mice for maintenance. The challenge was safe and well tolerated; only
3005 approximately 20 percent of the subjects had symptoms and they were of mild intensity. Between 1948
3006 and 1988 thousands of quarantined volunteers were infected with respiratory viruses at the UK Medical
3007 Research Council Common Cold Unit in Salisbury. Carrat et al. compiled the results of 56 human
3008 challenge studies that included 1,280 participants, conducted in the 1990s to early 2000s (651). In most of
3009 the studies, volunteers were excluded if they had pre-existing antibodies against the challenge strain
3010 (usually <1:6). The challenge was mostly given intranasally at doses that ranged from 3×10^{10} to $7.2 \times$
3011 10^{10} $TCID_{50}$ (50 percent tissue culture infectious dose). Participants were quarantined for seven to nine
3012 days and closely followed for clinical signs and symptoms. Virus shedding was evaluated daily using
3013 nasal washes. The review by Carrat et al. provides an overview of important virological and clinical
3014 features of influenza infection in individuals with low HI titers to the challenge strain, and over a narrow
3015 dose range for the challenge inoculate (651). Overall, 88.2 percent of challenged volunteers developed
3016 influenza infection. Virus shedding was evaluated using nasal washes. Clinical illness was defined by
3017 upper and lower respiratory symptoms, ear symptoms, and fever. The clinical and virological features of
3018 infection with four different influenza challenge strains are summarized in Table 26.

3019 In the early 2000s, influenza human challenge studies came to a halt as a consequence of an adverse
3020 outcome in a 21-year-old healthy subject challenged with influenza B in an investigation of peramivir for
3021 influenza prophylaxis. The subject presented asymptomatic echocardiogram changes on day 4 after
3022 challenge that returned to normal by day 15, but after traveling to Indonesia, where he developed an upper
3023 respiratory tract infection, an echocardiogram (taken 51 days after challenge) revealed a reduced ejection
3024 fraction, though the subject was symptomatic. Extensive work-up for infectious etiologies revealed
3025 nothing, and repeated echocardiograms showed gradual improvement and return to normal. In spite of the
3026 lack of direct causality linking myocarditis to the influenza challenge, no further influenza challenge
3027 studies were conducted in the United States for nearly a decade (652).

Table 26. Clinical and virologic features of influenza infection in experimentally challenged human volunteers.

Challenge strain	Number of volunteers challenged	Infected participants with virus shedding (%)	Infected participants with clinical illness (%)	Infected participants with fever (%)
Influenza A(H1N1)	532	93.1	69.3	30.9

Table 26. Clinical and virologic features of influenza infection in experimentally challenged human volunteers.

Challenge strain	Number of volunteers challenged	Infected participants with virus shedding (%)	Infected participants with clinical illness (%)	Infected participants with fever (%)
Influenza A(H3N2)	473	92.5	62.3	39.5
Influenza A(H2N2)	86	83.9	77.4	100
Influenza B	189	81.5	62.5	7.0

Virus shedding was observed in more than 80 percent of infected volunteers. Different challenge strains produced somewhat different clinical features. The most variable symptom was fever, which varied from 7 to 100 percent. Adapted from Carrat et al. (651).

3028 **Learning about influenza pathogenesis and immune responses from challenge studies**

3029 Important insight into the pathogenesis of influenza viruses has been gained from challenge studies as
3030 follows:

3031 ***On re-infection:*** Memoli et al. (653) reported the outcome of an intranasal challenge (a re-challenge
3032 experiment with influenza strain, A[H1N1]pdm09). Seven subjects challenged with the virus were re-
3033 challenged one year later. At least three of the seven participants had evidence of repeat infection, and
3034 five of the seven showed clinical evidence, demonstrating that sequential re-infection with the same virus
3035 may not be a rare phenomenon, which raises questions about the strength of the immune memory induced
3036 by infection.

3037 ***On genetic evolution:*** Considerable viral evolution occurs during experimental infection with H3N2. In a
3038 study by Sobel et al. (654) in which the viral stock contained genetic variants that originated during the
3039 passaging process the direct intranasal inoculation resulted in a bottleneck that reduced nonsynonymous
3040 genetic diversity in the HA and NP proteins. Intra-host viral evolution continued over the course of
3041 infection. A separate study Xiao et al. examined the genetic evolution of the virus in 15 subjects receiving
3042 influenza A/California/04/2009(H1N1) and compared it to that of five naturally infected subjects,
3043 demonstrating that intra-host evolution is different for challenged and naturally infected subjects (655).

3044 ***Clinical outcomes:*** Critical insight into what drives influenza-related symptomatology were discerned by
3045 inoculation of influenza A/Wisconsin/67/2005(H3N2) in 17 volunteers. Clinical symptoms were observed
3046 in nine of them. Serially drawn blood samples were tested for 25 cytokines, revealed increased levels of
3047 interleukin (IL)-6, IL-8, IL-15, monocyte chemotactic protein (MCP)-1, and IFN-γ induced protein (IP)-
3048 10 as early as 12 to 29 hours post-inoculation (before symptoms appeared). No such changes were
3049 observed in the subjects that remained asymptomatic. Some inflammatory mediators (MCP-1, IP-10, IL-
3050 15) were expressed in circulating cells, while others (IL-6, IL-8, IFN-α and IFN-γ) were produced at the
3051 site of infection (656). A separate transcriptome analysis of peripheral blood was conducted in 41
3052 volunteers that received an intranasal challenge with influenza A (A/Brisbane/59/2007[H1N1] or
3053 A/Wisconsin/67/2005[H3N2]) (657). Eighteen (44 percent) developed symptoms. A gene signature for
3054 symptomatic influenza capable of detecting 94 percent of infected cases was identified from the analysis.
3055 The gene signature can be detected as early as 29 hours post-exposure, about 38 hours before peak of
3056 clinical symptoms. When used as a tool to investigate patients admitted with clinical influenza, the gene

signature discriminated between 2009 influenza A(H1N1)-infected and non-infected individuals with 92 percent accuracy.

Transmission: The human challenge model for influenza has also been used to study person-to-person transmission of (H1N1)pdm09 providing objective evidence of the effectiveness of such measures as hand-washing and protective masks to prevent influenza transmission (658, 659).

Immune response and correlates of protection: Huang et al. (660) examined the kinetics and magnitude of the B-cell and antibody responses in relation to clinical symptoms and viral shedding in 12 subjects experimentally infected with an influenza A/Brisbane/59/2007(H1N1) strain. The volunteers had little or no detectable HI antibody prior to challenge but developed increasing levels of immunoglobulin G antibody-secreting B cells within seven days of exposure, which was taken as evidence for an anamnestic immune response. At the time this response appeared, HI antibodies were still undetectable. An inverse correlation was seen between the magnitude of the response and the viral load and duration of shedding. The quick induction of a recall response suggests that establishing a memory response by vaccination may be feasible (660). Also of interest is the observation that HI antibodies were not well represented in the memory B-cell compartment. Pre-existing influenza-specific $CD4^+$ T cells to the nucleoprotein and matrix were correlated with protection from disease in a study conducted by Wilkinson et al. (661). An influenza A(H3N2) strain was used for challenging volunteers previously screened to select those with no detectable antibody to the challenge virus. By day 7 post-challenge, large increases were seen in influenza-specific T cells, indicative of a recall response. Virus shedding and severity of disease were correlated with pre-existing $CD4^+$ T cells, but not with $CD8^+$ T cells. The $CD4^+$ T cells showed evidence of cytotoxic activity and responded to peptides from homologous, but also from the heterologous, pandemic strain influenza A(H1N1).

Correlates of protection: In an attempt to discern correlates of protection, Memoli et al. administered wild-type 2009 A(H1N1)pdm influenza A challenge to individuals with HI titers of either >1:40 or <1:40 (662). HI titers of >1:40 were protective against mild and moderate influenza disease. While the baseline HI titer correlated with some reduction in disease severity measures (>1:40 titers), the baseline titers for NA inhibition were better correlated with disease severity. However, in a separate challenge study conducted by Gould et al. using the influenza A/California/2009(H1N1) strain among volunteers pre-selected for low serum HI titers, no correlation was observed between the titers and outcome of the challenge. On the other hand, a weak, but statistically significant correlation was found between nasal and serum IgA and the duration of nasal shedding, pointing to mucosal IgA as a potential correlate of protection (663).

Park et al. explored the potential correlation between anti-influenza hemagglutinin stalk antibodies and protection from A(H1N1) influenza challenge in 65 volunteers (664). The level of anti-stalk antibodies increased in 64 percent of the subjects after challenge; however, baseline anti-stalk antibody titers did not correlate with the symptoms induced by the challenge.

Antibody-dependent cellular cytotoxicity (ADCC) has also been proposed as a correlate of protection for influenza and tested in a human challenge study conducted by Jegaskanda et al. (665). Increases in titers of ADCC-mediating antibodies to recombinant HA or to virus-infected cells were observed in adults and children that received LAIV as well as in experimentally infected adults. Pre-existing ADCC titers ≥320 were associated with lower virus replication and a significant reduction in symptoms.

3098 Taking advantage of the recommended use of LAIV in children, Wright et al. used the vaccine as a
3099 pseudo-challenge agent to examine potential correlates of protection in children (666). After the children
3100 were intranasally immunized with LAIV, a second dose of the same vaccine or of an inactivated influenza
3101 vaccine was provided. The patterns of immunity induced by the two vaccines were very different;
3102 however, the outcome of the second LAIV "challenge" did not correlate with serum or mucosal responses
3103 to the vaccine as tested with a battery of assays that included cytokine and chemokine levels. The authors
3104 concluded that the mechanism of protective immunity to LAIV could not be defined with traditional
3105 methods.

3106 ***Therapeutics:*** The influenza challenge model was also employed to assess the therapeutic efficacy of a
3107 monoclonal antibody against the influenza hemagglutinin stalk region (MHAA4549A). In a study of 100
3108 subjects challenged with an H3N2 virus (influenza A/Wisconsin/67/2005), the antibody, which was
3109 provided to the subjects 24 to 36 hours after the challenge, was well tolerated and significantly reduced
3110 the viral load and tissue culture infectivity of nasopharyngeal secretions. In addition, it had an impact on
3111 influenza symptoms, duration of shedding, and levels of inflammatory markers (667).

3112 **Challenge strains currently under development**

3113 Investigators at the US National Institutes of Health and Pennsylvania State University used cloned wild-
3114 type A(H1N1) strains manufactured under Good Manufacturing Practice (GMP) to conduct a dose-
3115 escalation study in healthy adult volunteers in a quarantine unit (668). Similarly, Watson et al. developed
3116 an H1N1 challenge in the United Kingdom (669) using influenza strain A/California/2009(H1N1). The
3117 agent was manufactured under current GMP conditions and characterized in accordance with regulatory
3118 guidelines. A dose-ascending, open-label clinical study was conducted in 29 healthy young adults sero-
3119 negative to the challenge strain with three increasing doses of virus. Subjects were challenged intranasally
3120 at various doses of the virus and followed for clinical symptoms, immunological responses, and viral
3121 shedding. A dose-dependent increase in clinical signs and symptoms occurred in 75 percent of subjects
3122 receiving the highest dose (3.5×10^6 $TCID_{50}$), classified as mild (all subjects), moderate (50 percent of
3123 subjects), and severe (16 percent of subjects); symptoms peaked four days after infection. Physician-
3124 observed clinical signs were correlated with nasal mucus weight ($p < 0.001$) and subject-reported
3125 symptoms ($p < 0.001$). Viral shedding peaked at $\log_{10}$ 5.16 $TCID_{50}$ three days after inoculation and was
3126 maintained for approximately five days. Symptoms and signs were limited to the upper airways and were
3127 of insufficient severity to be of clinical concern.

3128 Using reverse genetics, Memoli et al. developed a GMP-produced wild-type 2009 A(H1N1)pdm virus to
3129 be used as an intranasal challenge agent (668). Escalating doses were tested to identify a dose leading to
3130 signs of disease in at least 60 percent of volunteers. A dose of 10^7 $TCID_{50}$ caused mild to moderate
3131 disease in 69 percent of subjects. Viral shedding lasted four to five days and significant antibody titer
3132 rises were induced by the challenge. Of interest, viral shedding preceded symptoms by 12 to 24 hours and
3133 terminated two to three days prior to symptom resolution, indicating that individuals may be infectious
3134 before development of symptoms. Most common symptoms were nasal congestion and rhinorrhea, with
3135 fever observed in only 10 percent of the subjects. The Memoli group also developed an influenza
3136 A/Bethesda/MM1/H3N2 under GMP (670). Like the H1N1 predecessor, it was used in a dose-escalation
3137 challenge trial at doses from 10^4 to 10^7 $TCID_{50}$. Clinical symptoms, viral shedding, and immune responses
3138 were evaluated. Only the two higher doses tested (10^6 and 10^7) resulted in disease and viral shedding. Of

3139 37 participants challenged, 16 (43 percent) had viral shedding and 27 (73 percent) developed symptoms,
3140 with 12 participants (32 percent) experiencing mild to moderate influenza disease, defined as shedding
3141 and symptoms. Similar to the H1N1 challenge, viral shedding was seen one to two days after challenge,
3142 preceding the development of clinical symptoms, which peaked on day 3. Only ten participants
3143 (29 percent) had a ≥4-fold rise in HI antibody titer after challenge. The fact that pre-existing HI antibodies
3144 were not present in these subjects (by study design) may indicate that pre-existing immunity factors other
3145 than anti-HA antibody may limit shedding of the two viruses. Nonetheless, this A/Bethesda/MM1/H3N2
3146 challenge virus can be utilized in future studies to further explore pathogenesis and immunity and to
3147 evaluate vaccine candidates.

3148 Fullen et al. (671) reported the GMP manufacture of a separate wild-type H3N2, the
3149 A/Perth/16/2009(H3N2) strain. Four escalating doses were studied in six volunteers each; infection was
3150 observed in approximately 80 percent of subjects receiving 2.5×10^4 to 4.7×10^6 $TCID_{50}$ of virus.
3151 Symptoms, including runny nose and sneezing, developed more rapidly with the higher doses. Muscle
3152 aches and sore throat were only seen with the higher titer, but viral shedding was observed frequently.

3153 As inferred from the studies above, the choice of challenge dose is one of the most critical steps in
3154 controlled human infection model studies. Obviously, wild-type virus strains leading to serious infections
3155 cannot be used; at the other end, excessively attenuated strains may not be useful to recapitulate even mild
3156 cases of infection (672). As an example, a challenge study by Ramos et al. (673), in which a monoclonal
3157 antibody was tested, was hard to interpret because the A(H3N2) strain used was overly attenuated or the
3158 challenge dose was too low. Only two of 24 control (placebo) participants (8 percent) reported fever, and
3159 their nasal secretions averaged only 6 g. In comparison, in a separate challenge study evaluating
3160 oseltamivir for efficacy, 12 of 33 patients (36 percent) receiving placebo had fever after challenge, with
3161 influenza A/Texas/36/91(H1N1) virus, and the mean weight of nasal secretions collected was 12 g (674).

3162 As the field moves forward in developing additional influenza challenge models, it is critical to ensure
3163 consistency, as much as possible, across the criteria for selection of volunteers; the way in which the
3164 inoculum is administered; the collection, treatment, and storage of the samples; clinical scoring of disease
3165 severity; laboratory evaluations; etc., in order to properly analyze the data and compare results across
3166 studies (675).

3167 **Evaluation of novel vaccines in human challenge studies**

3168 Evaluation of vaccine efficacy is the primary goal of developing challenge models, and now that several
3169 challenge agents are available, the time is ripe to conduct vaccination challenge studies. A few examples
3170 are discussed below.

3171 Lambkin-Williams et al. evaluated the efficacy of aerosolized split inactivated vaccine in a challenge
3172 model that uses the A/Panama/2007/1999(H3N2) challenge strain. Subjects immunologically naïve to the
3173 virus were dosed via nasal spray with proteosome-adjuvanted trivalent influenza vaccine or placebo
3174 (676). Vaccine doses of 15 and 30 μg were given either once or twice 14 days apart and the subjects
3175 challenged with A/Panama/2007/1999(H3N2) five weeks later. Immune responses were measured by HI
3176 and nasal wash secretory IgA. In all, 57 to 77 percent of subjects seroconverted to the vaccine, which
3177 exhibited 58 to 82 percent efficacy against any influenza symptoms with seroconversion, 67 to 85 percent
3178 for systemic or lower respiratory illness and seroconversion, and 65 to 100 percent for febrile illness and

seroconversion. The two-dose regimen was found to be superior to the single-dose regimen. The protection observed significantly correlated with pre-challenge HI and mucosal secretory IgA titers

Van Doorn et al. (677) reported the results of a trial of a vaccine candidate termed FLU-v, a peptide vaccine developed by PepTcell (now SEEK, London, United Kingdom) and designed to provide a broadly protective cellular immune response against influenza A and B. Study subjects received one dose of either the vaccine with adjuvant or the adjuvant alone (16 subjects per group) and were challenged 21 days later with A/Wisconsin/67/2005(H3N2) and followed for seven days post-challenge. FLU-v was safe and well tolerated. Subjects in the vaccine group developed FLU-v-specific IFN-δ responses (8.2- to 3.9-fold versus 1.3- to 0.1-fold higher than the control group). The cellular responses observed correlated with reductions in both viral titers and symptoms.

Powell et al. used a challenge model to evaluate the quality of influenza-specific T-cell responses in subjects receiving an MVA vaccine expressing the influenza NP and M1 genes (MVA-NP+M1) (678). The vaccine induced increased expression of Granzyme A, Perforin, and CD57 on HLA A*02 cells recognizing the M1 58–66 peptide before and after challenge. Similar phenotypic changes have been associated with protection against influenza in other studies. The corresponding efficacy data were published separately (606). Two out of 11 vaccinees and five out of 11 control subjects developed laboratory-confirmed influenza, including symptoms and virus shedding; the symptoms were less pronounced in the vaccinees.

Summary

The human challenge models for influenza are safe and provide high rates of infection and disease. They also provide a consistent clinical course and a range of challenge strains with which to evaluate candidate vaccines. Since field trials will only be able to evaluate vaccine-mediated protection against the currently circulating strains, challenge models could be a practical way of comparing the breadth of protection against different influenza serotypes. Efforts to identify target antigens for a universal vaccine are well underway, but the most advanced candidates are still in early stages of development. Universal vaccines will likely include vectored or subunit vaccines, which are unlike the whole-inactivated or live-attenuated seasonal vaccines in current use. The main reason for an approach that differs from seasonal vaccines is because immune responses to the more conserved viral proteins are thought to be subdominant. Eliciting them may require that highly variable proteins (such as the HA1 protein, to which the dominant responses are directed) be removed from some of the vaccines.

3210 # Respiratory syncytial virus

3211 **Epidemiology, diversity, pathogenesis, and public health impact of respiratory syncytial virus**

3212 Respiratory syncytial virus (RSV) is one of the leading causes of mortality in infants and the elderly. A
3213 meta-analysis of available data has indicated that, in the absence of effective interventions, 34 million
3214 new infections with RSV can be expected each year, leading to 3.4 million hospitalizations and 200,000
3215 deaths (679). Ninety-nine percent of those deaths will occur in low-resource countries around the world.
3216 No vaccine or effective drug exists against RSV.

3217 RSV is an enveloped virus with a single-stranded RNA genome. The virus structure is shown in Figure 13
3218 (680). RSV has three transmembrane glycoproteins that are key targets of antiviral antibodies: G, the
3219 attachment protein; F, the fusion protein; and SH, a small hydrophobic protein of unknown function.
3220 Inside the virion are the matrix protein M and two regulatory proteins called M2-1 and M2-2. The RNA
3221 genome is associated with three proteins: nucleocapsid protein (N), a phosphoprotein (P), and a
3222 polymerase (L). The genome also encodes two non-structural proteins called NS1 and NS2.

3223 **Figure 13. Structural diagram of respiratory syncytial virus.**

3224 Two distinct genogroups of RSV are found in humans, termed A and B (681). The Genogroup A and B
3225 viruses have been analyzed with respect to which of the viral proteins are the most variable between the
3226 two genogroups. The G proteins of RSV A and B are the most divergent, with only 53 percent homology
3227 between the two genogroups. The SH and NS1 proteins of RSV A and B strains have 76 and 87 percent
3228 homology, respectively. The F protein, has 89 percent homology between the RSV Genogroup A and B
3229 viruses (3).

3230 The global epidemiology of RSV infection in infants and young children is incompletely described with
3231 respect to the circulation of RSV genogroups and genotypes (682). Overall, about two out of three
3232 infections are Genogroup A and one out of three are Genogroup B. Data from the United States and
3233 Europe indicate that RSV epidemics are seasonal and that strain variation often occurs from season to
3234 season (683).

3235 The majority of individuals become infected with RSV in early life. By 2 years of age, more than
3236 80 percent of children around the world will have experienced RSV infection, two-thirds of them under 1
3237 year of age (684). Following infection with RSV, protective immunity is incomplete and short lived. The
3238 results from experimental human challenge with RSV suggest that previously infected individuals are
3239 susceptible to re-infection (685), however there is limited data from natural infections to confirm this.

3240 RSV infections are associated with a broad spectrum of disease (6). In infants, about two-thirds of
3241 infections are limited to the upper respiratory tract, while one-third of infections also involve the lower
3242 respiratory tract. In the two-thirds of infants with upper respiratory infection, symptoms of disease are
3243 mild, including runny nose and ear infections. The disease is usually self-limiting. Lower respiratory tract
3244 infections (LRTIs) with RSV often lead to bronchiolitis, a condition in which inflammatory immune cells
3245 infiltrate the air spaces, resulting in mucus production, shedding of damaged epithelial cells, and edema of
3246 the wall of the airway. The resulting obstruction of the airway is the major cause of infant mortality from

RSV (6). RSV infections are also important causes of morbidity and mortality in the elderly, where repeated episodes of RSV infection may occur in a given individual within a single year (686).

The status of the human immune system is thought to have a major impact on the severity of disease. Our current understanding suggests that differences in the innate immune response to infection in infants under 1 year of age, as compared to immune response in older children, underlie the greater mortality of RSV infection in young infants (6). Co-infections with bacterial respiratory pathogens including pneumococcus and *Haemophilus* are risk factors for severe disease in young children (687–690). The mortality of RSV in older adults is ascribed to weakened immunity, which permits re-infection even in the short period after an episode of RSV, when there is usually a level of protective immunity. Since protective immunity is incomplete and re-infections are common, human infections with RSV and the immune response generated provide few clues to guide the development of RSV vaccines and offer little information about the impact of RSV's genetic diversity on protective immunity.

Respiratory syncytial virus vaccine development

Respiratory syncytial virus vaccine development has proceeded cautiously since an initial serious misstep in the 1960s, when the administration of a formalin-inactivated RSV (FI-RSV) vaccine candidate to infants and children was associated with more severe disease upon subsequent RSV infection. In that trial, 26 percent of vaccine recipients were hospitalized with severe lower respiratory infections and two deaths were attributed to vaccination (691, 692).

The basic scientific explanation underlying the enhanced disease seen in infants in the FI-RSV vaccine trials continues to be rigorously pursued. Much has been learned in the last 50 years about protective, as well as dysregulated, immunity in RSV infection (693). Protecting against severe RSV disease with antibodies is clearly possible under some circumstances. The RSV-neutralizing monoclonal antibody directed against the fusion (F) glycoprotein, palivizumab, reduces hospitalization for bronchiolitis by 50 to 60 percent in RSV-infected infants when passively administered in the first few months of life. Neutralizing antibody titers in recently infected adults may reduce the symptoms of subsequent infection, but the protective effect of neutralizing antibodies is short lived (693).

Considerable evidence supports the hypothesis that T-cell immunity is dysregulated in RSV infection (6, 693). The type 1 interferons are key innate defenses against viral infections, and the NS1 and NS2 proteins of RSV suppress production of type 1 interferons by immune cells. RSV infection of lung epithelial cells *in vitro* sends inhibitory signals to $CD8^+$ T cells, which would otherwise eliminate virus-infected cells. The immune response to the harmful formalin-inactivated vaccine was biased away from a normally protective Th1 type, cytotoxic T-cell response, and toward Th2 and Th17 responses. The catalogue of potential interactions of RSV with the human immune system is long (6) and understanding is still limited.

RSV infection itself does not lead to the enhanced disease upon re-infection that was seen with the FI-RSV in the 1960s. Accordingly, subsequent RSV vaccine development was re-focused toward live-attenuated RSV vaccines, which should provoke immune responses more similar to those following natural infection. The collective safety experience with this approach has been acceptable, with no evidence of enhanced disease upon first RSV infection in several hundred study participants that participated in trials of live-attenuated RSV vaccines, as determined through extended follow-up (694).

3287 The first series of live-attenuated vaccines to be developed were cold-passaged, temperature-sensitive (ts)
3288 mutants of an RSV Genogroup A strain (695). Use of attenuated viruses with a ts phenotype is intended to
3289 restrict viral replication to the upper respiratory tract. One vaccine, called cpts-248/404, was considered
3290 sufficiently attenuated for more extensive evaluation in infants and young children. The vaccine was
3291 infectious in the absence of pre-existing immunity to RSV but did not infect RSV-seropositive children.
3292 The vaccine did not cause lower respiratory tract illness but did induce a level of nasal congestion in the
3293 youngest age group that interfered with feeding.

3294 The second series of live-attenuated RSV vaccines used reverse genetics, whereby attenuating mutations
3295 and deletions could be specifically introduced into viral genome (696). Mutations were inserted
3296 individually or in combinations stepwise in a series of viruses that were progressively more attenuated.
3297 Several of these constructs have entered clinical trials. While the initial constructs yielded a highly
3298 attenuated virus that was well tolerated, safe, and moderately immunogenic, evaluation of nasal wash
3299 isolates from recipients identified a number of specimens exhibiting loss of the ts phenotype. Constructs
3300 to stabilize them were subsequently evaluated separately, for instance in a study of the strain RSVcps2;
3301 25 of 34 vaccinees (85 percent) were infected by the vaccine; 77 percent shed vaccine virus, but only
3302 59 percent developed a ≥4-fold rise in RSV serum-neutralizing antibody titers (697). Reversal of the
3303 attenuation was not detected.

3304 Additional mutations to stabilize the ts phenotype include deletion of either the M2-2 gene (698) or the
3305 NS2 gene (699). These candidates have shown acceptable stability *in vitro* and have been tested clinically
3306 in infants and young children. In the clinical trial, M2-2-deleted virus was shed by 95 percent of
3307 vaccinees; approximately 90 percent presented a ≥4-fold increase in neutralizing antibodies. NS2 is an
3308 interferon antagonist whose deletion diminishes RSV replication, while the increased interferon response
3309 to infection may enhance the adaptive immune response. Mutations in the NS2 gene (an interferon
3310 antagonist) accompanied by an additional deletion of codon 1313 was restricted in replication, well
3311 tolerated, immunogenic, and primed for potent antibody responses after natural exposure to wild-type
3312 RSV. At a dose of 10^5 PFU, the vaccine was over-attenuated, but at a 10^6 PFU dose it was well tolerated,
3313 able to replicate in 90 percent of vaccinees, and immunogenic (80 percent of the initially seronegative
3314 children. 1:64).

3315 A double M2-2 / NS2 construct (700) tested one intranasal dose (10^5 PFU) of the double-mutant (n = 21)
3316 or placebo (n = 11) in children 6 to 24 months old. All 21 vaccinees were infected with vaccine (based on
3317 either shedding or rise in serum RSV antibodies), and 20 shed the virus (based on positivity of nasal wash
3318 by both immuno-plaque assay and quantitative polymerase chain reaction). Serum RSV-neutralizing
3319 antibodies and anti-RSV F immunoglobulin G increased ≥4-fold in 95 percent and 100 percent of
3320 vaccinees, respectively. Mild upper respiratory symptoms and/or fever occurred in 76 percent of
3321 vaccinees and 18 percent of placebo recipients.

3322 In spite of the several advances made to developed attenuated strains, safety concerns and the limited
3323 market existing in industrial countries have deterred the development of any of the candidates discussed
3324 above in advanced clinical trials.

3325 Due to successful prophylactic treatment of RSV infection with the F protein-directed antibody
3326 palivizumab, several groups have taken a structural-based approach to create nanoparticles, bacteria-like
3327 particles, and subunit vaccines using engineered forms of RSV F protein, hoping to elicit a similar

3328 protective response against severe disease (701–704). The F protein exists in a metastable pre-fusion and
3329 stable post-fusion state, with both epitopes targeted by neutralizing antibodies in natural infection;
3330 however, the pre-fusion form is the major target. In preclinical studies, immunization of mice and Rhesus
3331 macaques with an F protein containing a stabilized antigenic site Ø (a conformation exposing multiple
3332 strong antigenic targets), elicited significant RSV-specific neutralizing activity (704). GlaxoSmithKline
3333 (formerly Novartis) is developing both post-fusion F protein (705, 706) and an uncleaved monomeric
3334 form of F protein that retains the pre-fusion antigenic targets as subunit vaccine candidates.

3335 Since the disease burden and mortality from RSV is highest in early infancy, RSV vaccines eliciting
3336 strong RSV-neutralizing responses are attractive candidates for maternal immunization so that high levels
3337 of protective antibodies can be passively transferred to infants before birth. Novavax completed the first
3338 maternal immunization efficacy trial of their nanoparticle RSV vaccine in approximately 4,600 pregnant
3339 women. The results from this multi-country, randomized, controlled trial showed the vaccine was safe,
3340 but it failed to meet its primary efficacy endpoint (707). The observed global vaccine efficacy in reducing
3341 medium to severe RSV LRTI was 39.4 percent (95 percent confidence interval [CI] 5.3 to 61.2), with
3342 44.4 percent (95 percent CI 19.6 to 61.5) reduction in hospitalizations. While considerably more efficacy
3343 was observed in South Africa sub-cohorts, the study was not sufficiently powered to establish if the
3344 higher efficacy reflected variations within the confidence interval. Of note, two additional pre-specified
3345 endpoints showed vaccine efficacy: 25.3 percent against all-cause LRTI and 39.1 percent against all-
3346 cause LRTI with significant hypoxemia (less than 92 percent oxygen saturation). Other maternal
3347 immunogens include vaccine candidates from GlaxoSmithKline (708), Pfizer (709), and the NIAID
3348 Vaccine Research Center (710).

3349 A number of vectored and subunit vaccines against RSV are in the preclinical and clinical stages of
3350 development. These are reviewed in Loomis and Johnson (711), Morrison and Walsh (712), and Aranda
3351 and Polack (713). Some examples of candidate RSV vaccines are shown in Table 27. The current
3352 vectored approaches reaching the clinics include adenovirus, bacillus Calmette-Guérin, and Sendai virus
3353 vectors expressing RSV proteins, particle vaccines including nanoparticles, and live-attenuated vaccines.
3354 These candidate vaccines may enable the evaluation of individual RSV genes for their capacity to elicit
3355 and propensity to interfere with cellular and humoral anti-RSV immune responses. They can also
3356 contribute to rational antigen selection for a new generation of candidate RSV vaccines.

Table 27. Examples of candidate respiratory syncytial virus vaccines in development.

Vaccine type	Vaccine(s)	Developer(s)	Phase
Live-attenuated	Ts, ΔNS, ΔM2, other	LID/NIAID/NIH; MedImmune, LLC; Sanofi Pasteur	1
Live chimeric	BCG-RSV	Pontificia Universidad Católica de Chile	1
	Sendai-RSV	St. Jude Children’s Research Hospital, United States	1
Particle-based	Nanoparticle	Novavax, Inc.[a]	3
Subunit protein	F	GSK[a]	2
		Pfizer Inc.[a]; Janssen Vaccines; VRC/NIAID/NIH[a]	1
	G	Beijing Advaccine Biotechnology	1

Table 27. Examples of candidate respiratory syncytial virus vaccines in development.

Vaccine type	Vaccine(s)	Developer(s)	Phase
Recombinant vectors	Adeno	Vaxart, Inc.	1
		GSK; Janssen Vaccines	2
	Modified vaccinia virus Ankara	Bavarian Nordic A/S	2

Abbreviations: BCG, bacillus Calmette-Guérin; GSK, GlaxoSmithKline; LID, Laboratory of Infectious Diseases; NIAID, US National Institute of Allergy and Infectious Diseases; NIH, US National Institutes of Health; RSV, respiratory syncytial virus; VRC, Vaccine Research Center.

[a] Development of target protective effects in infants by immunizing pregnant women in the third trimester.

From the PATH Vaccine Resource Library (714).

3357 **The respiratory syncytial virus human challenge model**

3358 The development of a human challenge model for RSV began in the 1970s (see Table 28). The initial
3359 work with the model was facilitated by the availability of a challenge pool of an RSV Genogroup A,
3360 genotype 2 virus that had been passaged multiple times in tissue culture (715). From 1981 to 2004, human
3361 challenge studies were conducted to investigate the infectivity of RSV by different routes of inoculation
3362 (715) and evaluate the relationship of the challenge dose to symptoms of disease, virus shedding, and the
3363 development of neutralizing antibodies (716).

Table 28. Respiratory syncytial virus challenge strains.

Challenge strain	Method of production and administration	Stock producers
RSV A2	An RSV Genogroup A strain was isolated from a pediatric patient in Melbourne, Australia. The virus was expanded by three passages in Vero cells in culture. Healthy adults were challenged via intranasal instillation.	Walter Reed Army Institute of Research and US National Institute of Allergy and Infectious Diseases
RSV A Memphis-37	An RSV Genogroup A strain was isolated from a pediatric patient in Tennessee, United States, and produced in Vero cells. Healthy adults were challenged via intranasal instillation.	Meridian Life Sciences, Inc. and Retroscreen Virology, Ltd.

Abbreviation: RSV, respiratory syncytial virus.

3364 Studies with the RSV challenge model waned for nearly a decade, but by 2010 the model had undergone
3365 further development with the availability of a new challenge strain called Memphis-37 (717). As
3366 described in Table 28, Memphis-37 is a contemporary clinical isolate that was minimally passaged in cell
3367 culture.

3368 DeVincenzo et al. (717) used a Memphis-37 challenge strain manufactured under Good Manufacturing
3369 Practice to evaluate the relationship of challenge dose to infection rate, viral load, and severity of disease;
3370 36 healthy adults with exceptionally low neutralizing antibody titers against RSV were quarantined for 13
3371 days following intranasal inoculation of the virus. Over a 500-fold dose range, 77 percent of volunteers
3372 became infected, no significant association was detected between challenge dose and infection rate or
3373 peak viral load. Viral load correlated with the severity of disease, which was limited to the upper

3374 respiratory tract and the weight of nasal mucus secreted. The production of cytokines after challenge was
3375 also evaluated in the study in order to gather evidence pertaining to the prevailing view of RSV immune
3376 pathogenesis as an aberrantly expanded Th2 response. Two of the Th2-biased cytokines (IL-4 and IL-10)
3377 did not correlate with disease severity, while the levels of several other pro-inflammatory cytokines and
3378 chemokines did track with viral load and disease severity. They suggested that, rather than an immune
3379 mechanism, viral load may be the main correlate of the severity of disease. Perhaps important, however,
3380 is that the study volunteers may not be representative of the general population with respect to pre-
3381 existing immunity to RSV.

3382 The newly developed Memphis-37 RSV challenge model has achieved a high attack rate and high degree
3383 of consistency in the disease course observed, has established objective and subjective measures of
3384 disease severity; and has benefitted from the use of a well-characterized, safe, and appropriate challenge
3385 strain manufactured under Good Manufacturing Practice. However, it is noteworthy, however, that the
3386 peak viral load in adults after challenge was about 50-fold lower than that seen in naturally infected
3387 infants, which may be explained by the presence of pre-existing cellular and humoral immunity that was
3388 present even in volunteers with low pre-existing serum neutralizing antibodies.

3389 A full genome sequence of Memphis-37 was recently published (718) showing that Memphis-37 is a
3390 representative strain within the RSV A genogroup. Of interest, the G protein of RSV A viruses contains a
3391 highly conserved amino acid motif (CWAIC) that mimics a human chemokine. This mimicry may
3392 interfere with leukocyte migration to the lung (719).

3393 The Memphis model has been used for the assessment of anti-RSV drugs, including GS-5806, a viral
3394 entry inhibitor that blocks the fusion of virus with the cell membrane (720), as well as JNJ-53718768,
3395 another fusion inhibitor. GS-5806 was associated with reduced viral load, mucus weight, and respiratory
3396 tract symptoms. A limitation of using the challenge model to assess antivirals is the narrow window for
3397 testing the drugs; they have to be given very early to see any effect, while in the case of a natural infection
3398 they will likely be given at a point when the disease is already established.

3399 The Memphis-37 model will be of great value to assess the ability of vaccine to block infection as
3400 opposed to prevent severe disease. The new wave of vectored and subunit RSV vaccines in preclinical
3401 development could benefit from the use of human challenges. Such studies could evaluate the potential of
3402 individual RSV proteins, presented to the immune system in different ways, to maintain a high safety
3403 profile while overcoming the interference with effective immunity that seems to be a hallmark of RSV
3404 infection.

3405 Until recently RSV challenge models had not been applied to vaccine development, in part because of the
3406 lack of induction of lower respiratory tract disease, however a recent study demonstrated the value of the
3407 model in vaccine development. Sadoff et al. (721) challenged 53 subjects intranasally with the Memphis
3408 37b RSV strain; 27 of them had received a single intramuscular dose of an Ad26 recombinant virus
3409 carrying a prefusion construct of the RSV F protein and had 26 received placebo four weeks earlier. RSV
3410 neutralizing antibody titers increased 5.8-fold by 28 days after vaccination. Post-challenge results include
3411 a minor drop in RSV infection rates (65.4 percent among placebos versus 40.7 percent among vaccinees);
3412 however, viral load (VL-AUC: area under the curve of qRT-PCR and quantitative culture) and disease
3413 severity were lower among the vaccinated subjects in comparison to the placebo recipients as follows:
3414 median VL-AUC qRT-PCR: 0.0 versus 236.0; median VL-AUC quantitative culture: 0.0 versus 109;

median RSV AUC clinical severity score 35 versus 167). The study represents a breakthrough that would hopefully lead to other developers using the model.

In addition to its practical use in drug and vaccine development, our understanding of the early immune response to RSV has advanced considerably thanks to the use of the challenge model. An interesting finding is the absence of immunoglobulin A (IgA) memory cells after experimental infection, which matches with the known poor and short-lived protection derived from infection (722). Nonetheless, a tight connection exists between IgA and protection from infection, since individuals that do have preexisting mucosal IgA appear to be protected from infection. However, among those that become infected, preexisting IgA levels did not influence the outcome of disease. These findings have been confirmed, with the addition that circulating neutralizing antibodies are also (along with IgA) associated with lower infectivity and higher viral replication. Again, when subjects are infected, no significant protection from disease evolution is observed. When subjects with very low preexisting titers of neutralizing antibody were selected and challenged with RSV, 35 of 42 (77 percent) were infected. Here again, pre-challenge nasal IgA antibodies, as well as neutralizing antibody, were lower among those infected. A trend between the diminished mucosal IgA and disease outcome was suspected but not found. In this study as well, no correlation was found between pre-challenge IgA levels and disease outcome.

The relationship between T-cell immunity and RSV infection has been difficult to understand. While it is suspected that depletion of T cells (e.g., immune-compromised subjects) increases the susceptibility to infection, T cells have also been associated with disease enhancement. Jozwick, Chiu, and their group at Imperial College (723) investigated systemic and local RSV-specific $CD8^+$ T cells in 49 RSV-challenged subjects: 26 (53 percent) developed infection (polymerase chain reaction positive), with viral load peaking at approximately seven days; 17 suffered symptoms of the common cold (lower respiratory tract); and nine had no symptoms. From 24 of the subjects, they were able to obtain serial samples of bronchial brushings, biopsies, and lavages, which were examined for RSV antigen-specific $CD8^+$ T cells using peptide-specific tetramers. Inflammation was observed in the materials collected and antigen-specific $CD8^+$ T-cell proliferation and activation was present. Circulating antigen-specific activated $CD8^+$ T cells also expanded within ten days of the challenge and viral load measures were inversely correlated with $CD8^+$ proliferation, insinuating a role for $CD8^+$ T cells in detaining viral infection. The subset of resident memory $CD8^+$ T cells was selectively expanded and associated with reduced disease. Rapid recovery was observed in spite of the extensive lower airway inflammation with persistent viral antigen and cellular infiltrates.

The Imperial College team investigated the presence, frequency, and specificity of $CD4^+$ T cells in bronchoalveolar lavage from subjects exposed to the same Memphis-37 challenge strain (724). The frequency of RSV-specific $CD4^+$ T cells strongly correlated with local C-X-C motif chemokine. The team was able to identify 39 epitopes in the virus and quantitate five of them using MHC II tetramers. The tetramers revealed enrichment of resident memory $CD4^+$ T cells in the lower airway. These cells displayed progressive differentiation, down-regulation of costimulatory molecules, and elevated CXCR3 expression.

Summary

The development of RSV vaccines has been particularly challenging. The obstacles have been many, including the apparent immune dysregulation in RSV infection, the lack of protective immunity in natural

3456 infection, and the difficulty involved in extrapolating experimental results from RSV-exposed adults to
3457 RSV-naïve infants. For live-attenuated vaccines, safety has been maintained, but maintaining a stable
3458 attenuated phenotype with some of the vaccine constructs has been difficult. The difficulties in evaluating
3459 RSV vaccines in young infants in field trials may very well be offset by the development of robust
3460 challenge models; however, the field is in need of additional challenge strains, including some of higher
3461 virulence than those currently in use. The potential that vectored and subunit vaccines overcome some of
3462 the many obstacles to vaccine development could be realized with the broader application of human
3463 challenge studies at the appropriate point in the clinical development process. Meanwhile, the field is
3464 effectively using the model to identify potential correlates of protection.

3465

Pneumococcus

Epidemiology, diversity, and pathogenesis of pneumococcus

In the year 2000, before the introduction of the first of the pneumococcal conjugate vaccines (PCVs), the bacterium *Streptococcus pneumoniae* (also called pneumococcus) caused an estimated 14.5 million episodes of serious disease and 826,000 deaths in children less than 5 years of age (725). This amounted to 11 percent of all deaths in children under 5, most of them in low- and middle-income countries.

While worldwide introduction of PCVs is far from complete, it was estimated that in 2015, 294,000 pneumococcal deaths occurred among children not infected with HIV (726). Less conservative assumptions result in pneumococcal death estimates as high as 515,000, half of which occurred in India, Nigeria, the Democratic Republic of the Congo, Pakistan, China, and South Sudan. Altogether, they estimated 3.7 million episodes of severe pneumococcus globally in children in 2015. Pneumococcus also causes serious disease in the elderly.

Pneumococcal bacteria exist as chains of varying length surrounded by capsular polysaccharides (CPS) which allow the bacteria to attach to the nasopharyngeal mucosa and establish colonization. Colonizing bacteria can also exist in biofilms in the mucosa (727), in which they exist as aggregates encased in an extracellular matrix. In biofilms, the bacteria are incompletely encapsulated, metabolically less active, and resistant to antimicrobials. Colonization of the nasopharynx with pneumococcus is frequent in infants and young children, and generally declines with age. Rates of colonization may reach 40 percent in children and 15 percent in adults (728). Colonization leads to a carrier state in which the bacteria persist without causing serious disease. A fraction of colonized individuals goes on to develop invasive pneumococcal diseases (IPDs), which include bacteremia, meningitis, and bone and joint infections. Pneumococcal pneumonia, a leading cause of severe pneumonia in children, is accompanied by bacteremia and considered invasive in up to 25 percent of cases (729). Pneumococcus can also develop non-invasive diseases such as otitis media. The factors that govern the transition from an essentially benign carrier state to serious disease are incompletely understood (730). Properties of the bacteria themselves, the effect of co-infection with other bacteria, and the host inflammatory response are thought to be contributing factors. Whether or not bacteria in biofilms are an important source of transmission or progression to invasive disease is an open question.

Close to 100 serotypes of pneumococcus have been identified worldwide on the basis of the binding of serum antibodies to their CPS. The CPS are essential for colonization and are key virulence factors for these bacteria. Immunity mediated by antibodies against the CPS is largely serotype specific.

Development of pneumococcal vaccines

The first pneumococcal vaccine was licensed in 1983. It included purified CPS of 23 of the most common serotypes, and prevented invasive disease in 57 percent of vaccinated individuals (729). CPS of many of the most common serotypes, including 6A, 6B, 14, 19A, and 23F, were poorly immunogenic in children less than 2 years of age, however, and the vaccine did not reduce pneumococcal carriage. The duration of protection was also limited.

3503 Conjugating pneumococcal polysaccharides to highly immunogenic carrier proteins largely overcame
3504 these problems and launched the era of PCVs. Conjugating polysaccharide antigens to proteins recruits
3505 non-cognate $CD4^+$ T-cell help for establishing memory responses and promotes class-switching and
3506 affinity maturation of antibodies. This conversion of the polysaccharide response from T cell-independent
3507 to T cell-dependent is particularly important for children under 2 years old and older adults with declining
3508 immune function, since unconjugated polysaccharides are poorly immunogenic in these vulnerable
3509 populations (731). Between 2000 and 2010, three different PCVs were licensed (see Table 29).

Table 29. Licensed pneumococcal conjugate vaccines for children less than 5 years of age.

Vaccine	Manufacturer	Serotypes[a]	Carrier protein	Indication	Licensure
PCV7 (Prevnar™)	Pfizer Inc.	4, 6B, 9V, 14, 18C, 19F, 23F	CRM_{197}, a non-toxic variant of diphtheria toxin	Prevention of IPD and otitis media due to *S. pneumoniae*	Licensed in 91 countries from 2000 to 2008
PCV13 (Prevnar 13®)	Pfizer Inc.	4, 6B, 9V, 14, 18C, 19F, 23F, **1**, **3**, **5**, **6A**, **7F**, **19A**	CRM_{197}	Prevention of IPD and otitis media due to *S. pneumoniae*	Licensed in 2009/2010 in the United States and Europe
PHiD-CV (Synflorix®)	GSK	4, 6B, 9V, 14, 18C, 19F, 23F, **1**, **5**, **7F**	Non-typeable *H. influenzae* protein D (8 serotypes) and CRM_{197} (2 serotypes)	Prevention of IPD and otitis media due to *S. pneumoniae* and *H. influenzae*	Licensed in more than 60 countries worldwide (not including the United States) in 2009

Abbreviations: GSK, GlaxoSmithKline; *H. influenzae*, *Haemophilus influenzae*; IPD, invasive pneumococcal disease; PCV, pneumococcal conjugate vaccine; *S. pneumoniae*, *Streptococcus pneumoniae*.

[a] Serotypes in PCV13 and PHiD-CV that are not included in PCV7 are in boldface type.

3510 The global and regional distribution of pneumococcus serotypes is a key consideration in the utilization of
3511 licensed vaccines and for the further development of vaccines against the CPS antigens. Although the first
3512 PCV (PCV7) was licensed in 2000, its incorporation into national childhood immunization programs in
3513 the most affected countries in the world has lagged, partly due to incomplete information about the match
3514 of the seven serotypes in the vaccine to the circulating strains in different countries and regions.

3515 The Pneumococcal Global Serotype Project studied how the fraction of IPD in children less than 5 years
3516 of age due to the serotypes included in these vaccines according to geographic region (732). PCV7
3517 showed the most marked regional differences, with better coverage in North America and Europe as
3518 compared to the rest of the world. These disparities were largely, but not completely, abrogated with the
3519 improved strain coverage of the 10-valent and 13-valent vaccines. The latter two vaccines would be
3520 expected to cover at least 70 percent of global IPD episodes in young children (733).

3521 A substantial body of evidence exists to indicate that the licensed PCVs can be highly effective against
3522 the serotypes they contain. Over the first seven years after PCV7 introduction in the United States, a
3523 45 percent decrease occurred in overall IPD incidence, and a 94 percent decrease occurred in the
3524 incidence of IPD with the serotypes included in the vaccine (734).

3525 Primary colonization with non-vaccine serotypes increased in the post-PCV7 era. In addition,
3526 pneumococcus can undergo capsular-switching by an exchange of genes among co-colonizing strains,
3527 further increasing the incidence of non-vaccine serotypes. Together, these mechanisms can lead to
3528 wholesale serotype replacement in which the non-vaccine strains greatly increase in prevalence relative to
3529 the vaccine strains. A systematic review of the evidence in 2011 indicated that extensive serotype
3530 replacement did occur after PCV7 introduction (735). Disease caused by non-vaccine serotypes in the
3531 United States had risen to 88 percent by 2004, as compared to 17 percent before PCV introduction.

3532 In a recent meta-analysis of 68 studies of IPD in children in countries in which PCV7 had been
3533 introduced, serotype 19A was the most predominant cause of childhood IPD, accounting for 21.8 percent
3534 of cases. In countries that introduced higher-valent PCV13, the overall serotype-specific contribution of
3535 19A was lower (14.2 percent). Overall, non-PCV13 serotypes contributed to 42.2 percent of cases. PCVs
3536 with broader valency, such as PCV15 (732) and PCV20 (736) are now in clinical trials. Initial results
3537 from Phase 1/2 studies demonstrated two different preparations of PCV15 have safety profiles
3538 comparable to that of PCV13 (732). Both PCV15 formulations include serotypes 22F and 33F in addition
3539 to those in PCV13, and both induced serotype-specific antibody responses to all 15 serotypes.

3540 A 20-serotype PCV developed by Pfizer that contains seven new serotypes in addition to the 13 included
3541 in PCV13 (737) showed efficacy in a Phase 3 trial and has been approved by the US FDA for use in
3542 subjects 18 years of age and older (738).

3543 A non-conjugated 23-valent polysaccharide vaccine (PNEUMOVAX® 23, Merck) is recommended for
3544 adults 65 years or older. It consists of a mixture of purified unconjugated CPS from *S. pneumoniae* types.
3545 The efficacy of CPS vaccines was studied in South Africa in male 16- to 58-year-old gold miners, where
3546 it showed efficacies of 76 percent (for a 6-valent vaccine) and 92 percent (for a 12-valent vaccine) (739).
3547 Similar results have been observed in additional studies of these vaccines in France and elsewhere.

3548 Pneumococcal vaccines have led to a significant and impressive reduction in not only pneumonia, but also
3549 sepsis and bacteremia in immunized populations. However, there remain concerns that the current vaccine
3550 strategy does not adequately address meningitis, making expansion of the vaccine arsenal an important
3551 goal.

3552 **Development of new pneumococcal vaccines**

3553 While the current PCVs have been very effective in reducing rates of invasive pneumococcal disease,
3554 their effectiveness has been somewhat diminished by the phenomenon of serotype replacement already
3555 noted above (740). Given the high number of serotypes currently known, it is cumbersome and inefficient
3556 to add additional serotypes to the current vaccines. The alternative is to develop vaccines using antigens
3557 different from CPS shared by all *S. pneumoniae* strains, in order to induce a broader response than that of
3558 PCVs, stimulating both humoral and cellular immunity. Such vaccines (e.g., whole-cell vaccine, subunit
3559 proteins, vectored vaccines, etc.) may be simpler and less expensive to manufacture (741). Work on the
3560 development of whole-cell or live-vectored (742) pneumococcal vaccines is also underway. Table 30
3561 below summarizes some of the pneumococcal vaccines in development.

3562 Several studies have failed to conclusively identify specific proteins with protective potential. For
3563 instance, in studies in which the pneumolysin toxoid and PhtD, two purified *S. pneumoniae* proteins, were

3564 combined with polysaccharide conjugates high pre-vaccination Ply and PhtD antibody concentrations
3565 were observed but they were not greatly increased by vaccination in the 2- to 4-year-olds and only
3566 responses to protein D were observed in the infants (743, 744).

3567 The pathway to licensure for protein-based or whole-cell vaccines is uncertain given that correlates of
3568 protection will be difficult to identify and large efficacy superiority trials with disease endpoints (as
3569 opposed to carriage endpoints) will be required. Human challenge studies may play a critical role in
3570 addressing some of these regulatory issues; at a minimum, they may provide proof of concept that such
3571 vaccines are efficacious against one or more of the challenge agents currently in use.

Table 30. Some pneumococcal vaccines in development.

Vaccine type	Institution	Description
Conjugate	Merck	15-valent conjugate vaccine with or without adjuvant (745)
Whole-cell	PATH	Whole-cell vaccine formulated in aluminum hydroxide adjuvant (746)
Vectored	Genocea	PspA-*Lactobacillus lactis* vaccine delivery vehicle
		Multicomponent adenovirus vector
Particle	Genocea	Lactococcal GEM-based vaccine
Subunit	GlaxoSmithKline	Pht proteins
	Sanofi Pasteur	Recombinant PspA
	Genocea	Protein subunit vaccine

Abbreviations: GEM, Gram-positive Enhancer Matrix; Pht, pneumococcal histidine triad; PspA, pneumococcal surface protein A.

3572 A killed whole-cell vaccine based on a non-encapsulated *S. pneumoniae* strain developed by PATH was
3573 safe and well tolerated when given to adults. The vaccine elicited significant increases (defined arbitrarily
3574 as at least a 2-fold rise) in immunoglobulin G (IgG) responses to multiple pneumococcal antigens,
3575 including PspA and Ply. Functional antibody responses were observed with the highest dose of whole-cell
3576 pneumococcal vaccine (0.6 mg). Increases in T-cell cytokine responses, including interleukin 17A, were
3577 also seen among whole-cell pneumococcal vaccines (746). Additional studies have been conducted with
3578 this vaccine in toddlers in Kenya, however further vaccine development is on hold.

3579 **The experimental human carriage model for pneumococcus**

3580 Substantial evidence indicates that the highly effective PCVs in current use substantially reduce
3581 nasopharyngeal carriage of the vaccine strains, as well as invasive and non-invasive pneumococcal
3582 disease in vaccinated individuals (747). Carriage of the vaccine strains is also reduced in unvaccinated
3583 individuals (748), presumably the result of herd immunity. Reduction in the rates of nasopharyngeal
3584 carriage is a key goal for future pneumococcal vaccines and may be a primary clinical endpoint in future
3585 vaccine trials.

3586 Accordingly, a human challenge model has been developed to more broadly evaluate the capacity of
3587 candidate pneumococcal vaccines to reduce rates of carriage and to explore immune responses that may
3588 be involved in the prevention of the carrier state. The features of the currently used model are detailed in
3589 Table 31.

Table 31. The human carriage model for pneumococcus.

Method of production and administration	Challenge strain	Strain provider
Laboratory cultivation is used to freshly prepare and titrate the challenge inoculum. The inoculum is administered to healthy adult volunteers by intranasal instillation in doses ranging from 10,000 to 140,000 colony forming units.	P833, a type 23F clinical isolate	Dr. Jeffrey Weiser, University of Pennsylvania
	BHN418, a type 6B clinical isolate	Dr. Peter Hermans, University of Nijmegen

The initial work on this human challenge model was published in 2002 and 2005 by a group at Baylor University (749, 750). Using a serotype 23F clinical isolate, the investigators found that carriage could be established in six of 14 healthy, uncolonized volunteers. The duration of colonization was variable. Pre-existing serum IgG antibody to the 23F CPS did not correlate with susceptibility to carriage in those subjects inoculated with a 23F strain. A correlation in these volunteers, however, did appear with pre-existing serum IgG and secretory IgA responses to PspA. Seven of the eight subjects that did not become colonized had pre-existing antibody to PspA, which is one of the antigens included in candidate vaccines in development (see Table 30). Also, the investigators found that six out of eight subjects challenged with a serotype 6B clinical isolate became colonized. In a follow-up study, the same group explored responses to a number of other pneumococcal surface proteins, many of which are also under consideration for the development of vaccines. Among the eight proteins evaluated, only the two related choline-binding proteins (PspA and CbpA) generated serum IgG responses in colonized subjects. Unlike PspA, no pre-existing serum IgG antibody responses were detected toward CbpA.

After a decade of inactivity, the model underwent further development in Liverpool, United Kingdom, using the same two clinical isolates (23F and 6B) with non-colonized healthy adult volunteers (751). Paradoxically, the first experimental colonization study failed to establish colonization with either strain in all but one of the 19 volunteers. By 2013, virtually every aspect of the challenge model, including the important preparation and titration of challenge inoculum, had been refined (752). The more developed study procedure was evaluated in 159 volunteers, and carriage could be established in 22 percent of volunteers using dose ranges of 11,100 to 313,000 colony forming units (CFU) per naris for strain 6B and 9,000 to 84,500 CFU per naris for strain 23F. The most common symptoms after challenge were non-specific nasal symptoms. None of the volunteers developed symptoms of pneumococcal disease.

The investigators cautioned that reproducibility can be a problem with the inoculum, which seems to stem partly from the propensity of these encapsulated bacteria to aggregate and partly from the loss of viability of the challenge organisms when suspended in the saline used for intranasal administration. The investigators also provided specific guidance on the nasal wash procedure.

Additional studies have been conducted with this model, including an investigation of the role of carriage in protecting against re-challenge with the homologous strain (753). A dose-response curve was first established for the serotype 6B challenge strain, which showed that up to 60 percent carriage could be established with doses of 40,000 CFU or more. Pre-challenge serum IgG titers to the 6B CPS were not correlated with the establishment of carriage. When carriage was established, carriage density was unrelated to challenge dose. After carriage had returned to undetectable levels, ten volunteers were re-challenged with about 40,000 CFU of the same 6B strain, some up to one year later. All ten volunteers remained carrier negative. The investigators further studied the capacity of the model to examine antibody

responses to several bacterial proteins following the establishment of carriage. They found many bacterial proteins that were immunogenic in humans, which could be potential targets for candidate vaccines.

Several additional publications have reported on using this challenge model (754–756). One stated that pneumococcal carriage does not markedly impact the proportions of other bacteria that colonize the human nasopharynx (757). Other reports suggested that the density and duration of carriage may be influenced by an immunomodulatory response, including the production of transforming growth factor beta 1 and an infiltration of T regulatory cells in the nasopharynx. The investigators suggested that the bacteria themselves may modulate the immune response to facilitate the establishment of the carrier state. Other studies have characterized the colonization "take" and kinetics as well as the symptoms associated with it (minimal to none).

Likewise live influenza vaccination (as compared to parenteral trivalent influenza vaccine [TIV]) induced less symptoms when given before pneumococcus inoculation; with colonization status only affecting the TIV group where more symptoms were reported by colonized participants compared to non-colonized participants following inoculation (n = 12/23 [52.17 percent] versus n = 13/38 [34.21 percent], respectively; $p < 0.05$). When influenza vaccination followed bacterial inoculation: no difference was seen in the symptoms reported between the live-attenuated and trivalent inactivated influenza vaccine groups following inoculation (756).

Taken together, the initial results from the human challenge model show promise for the dissection of immune responses associated with preventing nasopharyngeal carriage of two globally prevalent pneumococcal serotypes, 6B and 23F.

The model has now been used successfully to test the protective effect of PCV13 on colonization by the 6B strain, which was used as the challenge (758). Healthy adults received PCV13 or hepatitis A (control) vaccine. Only minor symptoms were observed after the challenge; colonization rates at any time (the primary endpoint of the study) were 10.4 percent in the PCV13 group and 47.9 percent in the control group, a 78 percent reduction. In addition, the colonization density was reduced by 3-fold. In a subsequent study, the authors examined the role of mucosal IgG to CPS in mediating protection from carriage in the same cohort (759). In the PCV13-vaccinated subjects, IgG levels to the CPS were increased in serum and nasal washes. Nasopharyngeal samples obtained post-vaccination heavily agglutinated pneumococcus compared to pre-vaccination samples among subjects protected, pointing to pneumococcal agglutinating antibodies as the mechanism of protection against carriage acquisition (759). Albeit colonization is not a disease endpoint, it is a surrogate for vaccine-mediated protection and transmission; the impact of PCV on nasopharyngeal colonization in children is well known (760); and thus, the model has significant relevance for the future testing of novel vaccines.

Summary

Pneumococcal conjugate vaccines are one of the great success stories in human vaccine development in recent years. The experience since the introduction of these vaccines, however, has been a case study on the selective pressures imposed by vaccines with incomplete strain coverage, and on the interaction of vaccines and drugs. The common denominator for vaccines of all types to prevent childhood morbidity and mortality from pneumococcus may be the prevention of the carrier state. Including the full spectrum of prevalent strains in a single vaccine construct is not possible at the present time. As a result, the strains

3664 not in the vaccines grow in prevalence and become the dominant circulating strains and eventually, the
3665 vaccines become less effective. Focusing the immune response on more conserved protein antigens may
3666 be needed to overcome the limitations of conjugate vaccines. A human carriage model with the potential
3667 to evaluate such vaccines was developed and successfully tested against a licensed vaccine. The model
3668 generated data suggesting that in non-colonized adult volunteers, establishing carriage may provide short-
3669 term protection against the homologous strain. Further, the model clearly demonstrated its usefulness
3670 when testing the protective effect of a well-known vaccine, PCV13, which could be used as a positive
3671 control in future studies. Vaccination substantially reduced colonization rates and density.

3672

Tuberculosis

Epidemiology, pathogenesis, and public health impact of tuberculosis

Tuberculosis (TB) is an ancient scourge, established in prehistoric human populations before their migration out of Africa (761). In 1882, Robert Koch discovered the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*) and established that it is the causative agent of TB. Today, *Mtb* infection causes more human deaths per year than any other pathogen, including HIV and *Plasmodium falciparum*, the principal agent of human malaria (762). Every year, 10 million people newly develop TB and 1.5 million die of the disease. The incidence of TB infection in 2018 was 1,310 cases per million of the global population, far exceeding the goal of one case per million set jointly by the World Health Organization (WHO) and the Stop TB Partnership. Multidrug-resistant, extensively drug-resistant, and even totally drug-resistant strains are also increasing in prevalence. A more effective TB vaccine is urgently needed.

The life cycle of *Mtb* in the infected host (Figure 14) is important to consider for effective vaccine development because it is partly under host immune control. Gengenbacher et al., describes the life cycle of *Mtb* in humans (763). TB is transmitted by the inhalation of aerosols from the coughs of individuals with active TB. The mycobacteria take up residence in the lung alveoli, where phagocytic cells of the immune system engulf them. Normally, this would lead to a prompt adaptive immune response, but the response is delayed for two to three weeks in the case of *Mtb* infection. During this time, the early innate response to infection recruits macrophages and other immune cells to sites of infection in the lungs, where they become organized as primary granulomas, from which cells come and go. With the development of the adaptive immune response, an infiltration of T cells into the granulomas occurs, which become larger and solid. What happens next is a matter of the balance of immune responses. The granulomas can be static in a standoff with the immune system, but if the response is too inflammatory, the central regions of the granulomas become necrotic and caseous (cheese-like) and exhibit an outgrowth of actively replicating bacteria. In the TB-infected individual, granulomas at all stages can co-exist in the lungs. When the liquefying granulomas sufficiently damage the linings of airways, the bacteria escape through coughing to infect new hosts.

Figure 14. Transmission and pathology of tuberculosis.

In a small fraction of individuals, the infection directly transforms into active TB, and these individuals are an immediate source of new TB cases (Figure 14). The establishment of latent TB, however, is the normal outcome of infection. Some estimates place the reservoir of individuals latently infected with TB at more than 2.33 billion, or about one-third of the world's population (761, 763). Some of these individuals experience life-long latency, but the latent infection reactivates in others, at which point the individual once again becomes infectious to others. Reactivation occurs in 2 to 10 percent of individuals with latent TB each year.

Re-infection of latently infected individuals plays a role in reactivation because a new infection stimulates immune responses that attack and help break down granulomas. In some latently infected individuals, an eventual loss of immune control of the infection occurs. The devastating effects of losing immune control of latent TB in HIV infection are well documented, with continuing active replication and dissemination occurring outside of the lungs. The widely used TB vaccine, bacillus Calmette-Guérin (BCG), prevents the dissemination of *Mtb* outside of the lungs, but does not prevent the establishment of latent infection.

3713 Understanding how many humans are latently infected and what fraction of latently infected individuals
3714 will experience reactivation is very important for the control of TB. Vaccines that either prevent the
3715 establishment of latency or that prevent reactivation from latency will be of critical importance for TB
3716 control. Certain antigens expressed in the *Mtb* bacteria are latent, but are not expressed in actively
3717 replicating *Mtb* bacteria. These genes are encoded in part by a 48-gene region of the *Mtb* genome called
3718 the DosR regulon. A regulon is a group of genes that are turned on or off together, although they may be
3719 widely scattered in the genome of an organism (762, 764).

3720 Importantly, DosR regulon-encoded antigens are present in the BCG genome, but they are not expressed.
3721 In individuals with prior BCG vaccination, these antigens are recognized poorly, if at all.
3722 Correspondingly, vaccination with BCG has little or no capacity to elicit immunity to the latent phase of
3723 *Mtb* infection.

3724 Immunity to latency antigens is readily and strongly detected in individuals with long-term *Mtb* infection
3725 that have not experienced a reactivation. Another group of interesting antigens are those involved in the
3726 resuscitation of *Mtb* after latency; that is, genes that enable the bacteria to return to their metabolically
3727 active state. The antigens these genes encode are termed resuscitation promoting, or *Rpf*, antigens.
3728 Immunity to *Rpf* antigens could curtail the emergence of actively replicating bacteria from a latent
3729 infection (762).

3730 Some of the latency and *Rpf* antigens have been incorporated into candidate TB vaccines. These vaccines
3731 are among the first to target multiple phases of the *Mtb* life cycle, and they have shown their potential to
3732 elicit immune control of latent infection in animal models. Incorporating some of these newly discovered
3733 antigens into the BCG genome, and engineering BCG to resume expression of them, are other potential
3734 strategies to take advantage of these new discoveries.

3735 **Tuberculosis vaccines in development**

3736 The only currently licensed vaccine against TB is BCG, an attenuated strain of *Mycobacterium bovis*.
3737 This vaccine was developed nearly 100 years ago to prevent serious TB disease in infants, which became
3738 common in Europe in the early twentieth century (765). It is the most widely used vaccine worldwide.
3739 When administered to infants shortly after birth, it can prevent the forms of disease that result from TB
3740 dissemination outside the lungs in infants and young children. The protection afforded by BCG
3741 vaccination is highly variable (0 to 80 percent efficacy) and is short lived. Re-vaccination with BCG later
3742 in life does not confer immunity to TB, with or without prior vaccination with BCG.

3743 Table 32 describes some of the candidate vaccines that are in clinical development and was compiled
3744 from a number of recent reviews (762, 764–772). The table does not include the more than 20 candidate
3745 vaccines in preclinical development.

Table 32. Candidate tuberculosis vaccines in clinical development.

Type of vaccine	Candidate	Description	Stage	Developer(s)
BCG replacement	VPM1002	Recombinant BCG with deletion of urease C and expression of listeriolysin O to promote Class 1 presentation	Phase 3	Serum Institute of India Pvt. Ltd. and Vakzine Projekt

Table 32. Candidate tuberculosis vaccines in clinical development.

Type of vaccine	Candidate	Description	Stage	Developer(s)
vaccine for infants				Management GmbH
	MTBVAC	Live, rationally-attenuated *Mtb* with stable, engineered deletions in *phoP* and *fadD26*	Phase 2a	University of Zaragoza, Biofabri, and South African Tuberculosis Initiative
Booster vaccine for infants, children, and adolescents with BCG	MVA85A Aeras485	Ag85A expressed in an MVA vector	Phase 2b	University of Oxford and Aeras
	H4:IC-31 (Aeras-404)	Ag85B/TB10.4 fusion protein with cationic peptide/TLR-9 adjuvant IC31	Phase 2	Aeras, Sanofi Pasteur, and Intercell

3746

Table 32. Candidate tuberculosis vaccines in clinical development.

Type of vaccine	Candidate	Description	Stage	Developer(s)
Booster vaccine for adults with latent TB	MVA85A Aeras 485	Ag85A expressed in an MVA vector	Phase 2b	University of Oxford and Aeras
	M72-AS01$_E$	Rv1196/Rv0125 fusion protein with AS01$_E$ adjuvant	Phase 2a	GlaxoSmithKline
	Ad5 Ag85A	Ag85A expressed in Ad5 vector	Phase 1	McMaster University, Canada
	H56:IC31	Ag85B/ESAT06/Rv2660 fusion protein with cationic peptide/TLR-9 adjuvant IC31	Phase 2a	Statens Serum Institute and Intercell
	ID93:GLA-SE	Rv2608/Rv3619/Rv3620/Rv1813 fusion protein with stable emulsion/TLR-4 adjuvant	Phase 1	Infectious Disease Research Institute
	SRL172	Whole-cell inactivated variant of *Mycobacterium obuense* intended for HIV-infected subjects	Phase 3	Aeras
	DAR-901	Whole-cell inactivated vaccine derived from SRL172	Phase 2	Aeras
Therapeutic vaccine for adults with active TB	*Mycobacterium indicus pranii*	Killed vaccine against leprosy with observed protection against TB	Phase 3	Cadila Pharmaceuticals Ltd., India
	Mycobacterium vaccae	Atypical *Mycobacterium*	Phase 2b	Anhui Zhifei Longcom Biopharmaceutical Co., Ltd., China
	RUTI	Detoxified liposomal fragments of *Mtb*	Phase 2b	Archivel Farma, Spain
Prevention of TB recurrence	VPM1002	Recombinant BCG with deletion of urease C and expression of listeriolysin O to promote Class 1 presentation	Phase 2/3	Serum Institute of India Pvt. Ltd.

Table 32. Candidate tuberculosis vaccines in clinical development.

Abbreviations: BCG, bacillus Calmette-Guérin; MVA, modified vaccinia virus Ankara; TB, tuberculosis; TLR, Toll-like receptor.

3747 Some candidate vaccines are designed to replace BCG for vaccinating infants. VPM1002, originally
3748 developed by the Max Plank Institute, Berlin and now licensed to Serum Institute of India, is a BCG
3749 strain that has been redesigned to elicit better $CD8^+$ T-cell responses. It was safe and immunogenic in
3750 Phase 1 and 2a trials in Germany and South Africa in adults and new born infants (773, 774). A Phase 2b
3751 trial in both HIV-exposed and unexposed South African infants has recently been completed and data is
3752 awaiting public released (ClinicalTrials.gov Identifier NCT02391415) (775) and multinational Phase 3
3753 study is planned (ClinicalTrials.gov Identifier NCT04351685) (776). An additional Phase 2/3 trial is
3754 underway in India to test VPM1002 for prevention of recurrence in adults previously treated for
3755 pulmonary TB (ClinicalTrials.gov Identifier NCT03152903) (777).

3756 A live-attenuated, double-deletion mutant of *Mtb*, called MTBVAC, originated at University of Zaragoza,
3757 Spain and has undergone Phase 1 and 2a trials (778–781). The vaccine candidate was licensed to Biofabri
3758 (Spain) and is now being developed by the South African Tuberculosis Vaccine Initiative. It was recently
3759 tested in previously BCG-vaccinated adults, as well as infants that had not received BCG. MTBVAC was
3760 well tolerated and induced long-lasting $CD4^+$ T-cell responses in infants. An efficacy trial of MTBVAC
3761 in newborn infants is expected to start in South Africa, Madagascar and Senegal in 2022 (782).

3762 Another approach to an improved TB vaccine is to design a booster that can be used following BCG
3763 vaccination of infants. MVA85A is a candidate vaccine developed by Oxford University and Aeras, in
3764 which an antigen from *Mtb* is expressed in the modified vaccinia virus Ankara (MVA). The MVA vector
3765 is a strain of vaccinia virus that has been stably attenuated by serial passage in cell culture. The MVA85A
3766 vaccine was evaluated in a Phase 2b trial in more than 4,000 infants in South Africa that were previously
3767 vaccinated with BCG, but showed little efficacy against infection or disease (783).

3768 A whole new class of candidate vaccines is directed toward the control or eradication of latent TB in
3769 adults with prior BCG vaccination. This group is composed of both vectored and subunit vaccines, the
3770 latter consisting of fusion proteins delivered with an adjuvant. A Phase 2b trial of the MVA85A vectored
3771 vaccine in South African and Senegalese adults with HIV has been completed and demonstrated safety
3772 and immunogenicity (784). There was no evidence of MVA85A efficacy in preventing TB infection or
3773 disease, although with only 650 participants, this study was not well-powered for these outcomes. DAR-
3774 901, an inactivated whole-cell vaccine based on *Mycobacterium obuense* (785), is being tested for
3775 prevention of infection in an efficacy trial using a three-dose regimen among Tanzanian adolescents
3776 (ClinicalTrials.gov Identifier NCT02712424) (786). Initial results from this trial suggest DAR-901 was
3777 not efficacious at preventing infection, however the trial has generated data on the predictive value of
3778 complete blood count-derived metrics for TB infection risk (787).

3779 A third candidate, ID93, is a subunit TB vaccine candidate comprised of four antigens representing
3780 different families of *Mtb* proteins [Rv1813, Rv2608 (PPE42), Rv3619 (EsxV), and Rv3620]. All four
3781 proteins are recognized by *Mtb*-exposed individuals (788). ID93 is combined with the Th1-inducing
3782 synthetic TLR-4 (Toll-like receptor 4) agonist adjuvant Glucopyranosyl Lipid Adjuvant (GLA). In a
3783 recently completed Phase 1 study that showed humoral and T-cell responses to all four antigens in the

3784 vaccine, GLA had a significant enhancement effect on the polyfunctionality of the induced $CD4^+$ T cells
3785 (789).

3786 The H4:IC31 vaccine consists of the fusion of two antigens, Ag85 B and TB10.4, and the IC31® adjuvant,
3787 a mixture of KLK, a leucine-rich peptide (KLKL5KLK), and the oligodeoxynucleotide ODN1a, a TLR-9
3788 ligand. After initial Phase 1 demonstration of safety and immunogenicity (790), the vaccine was tested in
3789 990 TB-free adolescents that had received BCG at birth and were randomized to receive H4:IC31, BCG
3790 revaccination, or placebo (791). Primary outcomes were safety and *M. tuberculosis* infection, as assessed
3791 by QuantiFERON®-TB (QFT; Quest Diagnostics) conversion (TB diagnosis is based on immunologic
3792 sensitization to *M. tuberculosis* antigens, as assessed by the tuberculin and/or IFN-γ release assays,
3793 typically done with the QFT). Recent infection, as diagnosed by means of the tuberculin skin test or QFT
3794 conversion, is associated with a higher risk of disease than is non-conversion or remote conversion (i.e., at
3795 least two years earlier). An important secondary outcome in the study was sustained QFT conversion to a
3796 positive test without reversion to negative status at three months and six months after conversion. Neither
3797 vaccine nor the BCG vaccine prevented initial QFT conversion, but BCG reduced the rate of sustained
3798 QFT conversion by 45.4 percent ($p = 0.03$), while H4:IC31 reduced it by 30.5 percent ($p = 0.16$), a lead
3799 finding that may have a potential impact in further development.

3800 The GlaxoSmithKline $M72/AS01_E$ vaccine is derived from the M72 recombinant fusion protein from
3801 *M. tuberculosis* antigens (Mtb32A and Mtb39A). In a Phase 2b trial conducted in Kenya, South Africa,
3802 and Zambia, approximately 3,500 18- to 50-year-old adults with latent *M. tuberculosis* infection (by
3803 IFN-γ release assay) were given either $M72/AS01_E$ or placebo twice. Most participants had previously
3804 received BCG. Efficacy against progression to bacteriologically confirmed active pulmonary TB disease
3805 was evaluated by clinical suspicion of TB confirmed with sputum by means of a polymerase chain
3806 reaction (PCR) test, culture, or both. Ten participants in the $M72/AS01_E$ group and 22 in the placebo
3807 group presented bacteriologically confirmed active pulmonary TB (primary endpoint) for a vaccine
3808 efficacy of 54 percent (95 percent confidence interval [CI], 2.9 to 78.2; $p = 0.04$) (792). The vaccine was
3809 not associated with higher rates of serious adverse events; however, unsolicited reports of adverse events
3810 in the $M72/AS01_E$ group (67.4 percent) were higher than in the placebo group (45.4 percent); these were
3811 mainly injection site reactions and influenza-like symptoms. Final results of this trial after three years of
3812 follow-up showed 13 cases in the vaccine arm and 26 cases in the placebo arm giving 50 percent vaccine
3813 efficacy (95 percent CI, 2 to 74) (793). Additional support for the M72 study for confirmation and
3814 expansion of the findings to other populations will be necessary for broad implementation (772).

3815 Aerosol administration of the MVA85A vaccine in BCG-vaccinated adults was recently reported. A
3816 priming immunization with the vaccine was well tolerated and highly immunogenic; however, prior
3817 intradermal administration of the same vaccine followed by aerosolized MVA85A boosting led to
3818 moderate-to-severe respiratory and systemic adverse events and resulted in modest, significant boosting
3819 of the cell-mediated immune response to Ag85. Serum antibodies to Ag85A and MVA were only induced
3820 after intradermal vaccination. Aerosolized MVA85A induced significantly higher levels of Ag85A lung
3821 mucosal $CD4^+$ and $CD8^+$ T-cell cytokines compared to intradermal vaccination (794).

3822 Therapeutic vaccines are also in development and being designed for use in adults with active TB. Two of
3823 these use the strategy of vaccinating with whole, killed preparations of non-TB mycobacteria. A leprosy
3824 vaccine (795) and one based on an atypical *Mycobacterium* from cows (796) are in clinical development

3825 in India and China, respectively. A therapeutic vaccine comprised of detoxified liposomal fragments of
3826 *Mtb* is also under development (797).

3827 **A human challenge model for tuberculosis**

3828 A human challenge model that faithfully reproduces human infection with *Mtb* is not available now and
3829 unlikely to be developed in the near future. The key limitation is that wild-type *Mtb* cannot be used for
3830 challenge, since infection leads to the establishment of latency. Upon reactivation, latent TB can cause
3831 extensive lung damage and can disseminate to other organs. Treatment is available but must be continued
3832 for months, and treatment is not a guarantee of cure. Two approaches using BCG as a surrogate for
3833 infectious *Mtb* using different routes of inoculation have been explored, as described below.

3834 An early attempt to develop a TB human challenge model using BCG focused on intradermal inoculation
3835 (798). BCG differs in important respects from *Mtb*. BCG is not a human strain of *Mtb*; rather, it is an
3836 attenuated strain of *M. bovis*, which infects cows. BCG has large deletions in its genome that arose
3837 through the 230 serial passages in culture that Calmette and Guérin used to attenuate BCG in the 1920s.
3838 During the further attenuation process that followed the work of Calmette and Guérin, more than 14 sub-
3839 strains of BCG arose, and many of these sub-strains have been used in the worldwide production of the
3840 BCG vaccines (770). Complete genome sequencing of BCG sub-strains has shown that they contain
3841 different subsets of at least 18 “regions of difference” between BCG and *Mtb*. A region of difference is a
3842 deletion of a block of genes, and the regions of difference between BCG and *Mtb* are numbered RD1
3843 through RD18.

3844 Many of the candidate vaccines in development today are based on recombinant BCG strains that have
3845 been modified to improve the levels of protection and immune memory, as compared to BCG. Different
3846 sub-strains of BCG are being used as the “backbone” into which these modifications are being introduced
3847 (770). When a particular sub-strain of BCG is used to evaluate these candidate vaccines in a human
3848 challenge model, the vaccine strain will differ from the challenge strain. Another important difference
3849 between *Mtb* and BCG is that BCG does not establish latency in humans (762). For this reason, the
3850 intradermal BCG challenge model can only evaluate the capacity of vaccines to limit the replication of
3851 *Mtb* during the initial stage of infection.

3852 The study endpoint for the human TB challenge model using intradermal BCG is to limit mycobacterial
3853 growth at the injection site. Parameters for the model were first developed using mice that were
3854 inoculated with BCG in the skin of the ear (799). The differences between mouse skin and human skin
3855 make extrapolating the experimental results from mice to humans more difficult. The assays developed in
3856 mice included bacterial culture from the site of intradermal inoculation and quantitative PCR. In the first
3857 human challenge study, healthy adult volunteers (some of whom had undergone prior BCG vaccination)
3858 were challenged with BCG by the intradermal route (798). No systemic complications occurred in study
3859 volunteers, but all of the BCG-vaccinated and some of the BCG-naïve volunteers developed a purulent
3860 discharge at the site of inoculation that resolved within four weeks. To gain sufficient material for
3861 evaluating the extent of BCG replication after challenge, a punch biopsy of the injection site was
3862 performed one, two, or four weeks after challenge in BCG-naïve subjects and two weeks after challenge
3863 in the BCG-vaccinated subjects. Though unclear in the text of the published study, the figures appear to
3864 show that subsets of the group of 28 volunteers that were BGC naïve prior to challenge were biopsied at
3865 different times. The heterogeneity of the study results at different time points after challenge may stem

3866 from the fact that the data came from different volunteers at each time point. Little correlation appeared
3867 between the quantification of BCG replication by PCR and by bacterial culture. Overall, the enumeration
3868 of bacteria by PCR gave counts that were 10-fold higher than culture.

3869 The human TB challenge model is also designed to permit the evaluation of immune responses after
3870 vaccination and challenge. To enable the characterization of local immune responses, the investigators
3871 collected cells infiltrating a suction blister generated near the site of inoculation. They also collected
3872 blood samples as a source of peripheral blood mononuclear cells (PBMCs) with which to evaluate
3873 systemic immune responses. The quantity of cells recovered from blisters was very limited, which
3874 precluded an extensive evaluation of immune responses. The investigators measured the fraction of
3875 PBMCs secreting IFN-γ after re-stimulation in culture with the immunodominant *Mtb* antigens 85A and
3876 TB10.3. The magnitude of the IFN-γ responses prior to challenge did not correlate with the number of
3877 live BCG bacterial colonies from skin biopsies after challenge.

3878 In another recent study, five volunteers were challenged with BCG intradermally (800). Swab specimens
3879 to quantitate shedding and mycobacterial immunity were collected from the site of vaccination. A
3880 comparison of three methods to identify the bacteria (PCR, culture, and time to positivity of
3881 mycobacterial growth indicator tubes was made to evaluate and compare their sensitivity. BCG was
3882 detected in swab specimens from all five volunteers by at least one method.

3883 The intradermal BCG challenge model is clearly a significant advance. In its early stages of development,
3884 the community of TB researchers received it with enthusiasm, tempered by some of the limitations of the
3885 model (801). These include the choice of evaluation method for the primary study endpoint (it is unclear
3886 whether this should be PCR, bacterial culture, or both) and difficulty in standardizing the challenge dose
3887 (different lots of BCG can vary up to 10-fold with respect to the numbers of infectious bacteria). The
3888 model would be improved if invasive procedures such as skin biopsies were not required. Finally, this
3889 model will not have the capacity to evaluate the many vaccines in development that include antigens
3890 unique to *Mtb*, including the antigens uniquely expressed during latency.

3891 Some of these limitations are being addressed. A growth-inhibition assay for *Mtb* using cryopreserved
3892 PMBCs has been developed, which could replace skin biopsy if it proves to be a valid measure of
3893 antimicrobial immunity generated by vaccines. In the initial study, the capacity of PBMCs from BCG-
3894 vaccinated individuals to inhibit *Mtb* growth was correlated with their IFN-γ enzyme-linked immunospot
3895 assay response to a purified protein derivative (PPD) skin test, suggesting that this approach may be
3896 useful. Another possible approach would be to develop new readouts, such as luminescence, to measure
3897 BCG replication directly at the injection site. A study is underway to evaluate the dose response for the
3898 human BCG challenge model, which may permit further standardization of the assay over a meaningful
3899 dose range.

3900 Until a well-standardized, broadly applicable human challenge model for TB comes into widespread use,
3901 animal models will continue to be used as a principal means of evaluating candidate vaccines. In light of
3902 the lack of efficacy in the Phase 2b trial in infants of MVA85A (see Table 32), a reassessment of the
3903 preclinical animal data that supported the advancement of this candidate vaccine to efficacy trials has
3904 occurred (802). The recommendations from McShane et al. include increasing the use of clinical isolates
3905 of *Mtb* in animal models, powering preclinical studies for the desired level of efficacy in human clinical
3906 trials, conducting preclinical studies in the same age groups targeted for human vaccine trials, evaluating

protection from infection and from disease, and developing specific animal models for the prevention of latency.

An additional limitation of the animal models for TB is the difficulty of replicating natural infection by the aerosol route in laboratory studies. A study in which guinea pigs were infected, not by intranasal instillation as is done in the laboratory, but by the exhaust air from a hospital TB ward, highlights this difficulty (803). Seventy-five percent of 362 guinea pigs exposed to the exhaust air from the TB ward converted to PPD skin test reactivity, but only 12 percent developed histopathologic evidence of disease. One-fifth of PPD-positive tests subsequently reverted, but later reappeared—possibly the result of re-exposure. These complex dynamics, observed in a setting that more closely resembles natural TB infection, may not be possible to reproduce in animal and human challenge models. Additionally, in some of the currently used animal models, *Mtb* fails to establish latency (802, 804, 805). In light of the pipeline of candidate vaccines that address latent stages of TB, this is a significant limitation.

An alternative approach using bronchoscopically instilled BCG and *Mtb* PPD as challenge agents has recently been reported (806). This study was conducted in 106 healthy South African adult volunteers with a range of prior *Mtb* exposure from asymptomatic household contacts to subjects previous treated and recovered from microbiologically confirmed TB disease. Importantly, the challenge was safe: reported adverse events were mild. Using broncheoalveolar lavage fluid collections before and after challenge to examine the immune responses to challenge, the investigators demonstrated several notable changes at the levels of cellular profiles as well as gene expression and regulation. The bronchioscopic instillation BCG challenge may play an important role in future evaluations of TB vaccine candidates.

A key limitation of the TB human challenge models described above is that neither intradermal inoculation nor bronchioscopic instillation mimic the natural route of aerosol infection. Two clinical studies in healthy BCG-naïve adults are on-going to explore aerosol BCG challenge at University of Oxford. The first will compare aerosol with intradermal BCG administration (ClinicalTrials.gov Identifier NCT02709278) (807) and the second will involve bronchioscopic evaluations up to 56 days post-challenge (ClinicalTrials.gov Identifier NCT03912207) (808). The objective of these challenge studies is to obtain a body of data indicating aerosol BCG can be safely administered to healthy adult volunteers. In parallel, an attenuated strain of *Mtb* is being developed, to be administered by the aerosol route if BCG proves to be safe by this route of administration. Some sort of reporter molecule is envisioned as a readout, as the current attenuated *Mtb* is largely, if not completely, replication incompetent. It is not entirely clear how the data would be collected to establish whether the attenuated *Mtb* strain could nevertheless establish latency, which may represent an additional concern. These are high hurdles to be overcome, particularly the inclusion of a reporter strain in a challenge study, which has no precedent among regulatory authorities. In any case, these are important efforts, should they prove fruitful, to improve the relevance of the human challenge model for TB.

Summary

Current drugs and BCG vaccine alone will clearly not be enough to bring TB under control. In the two decades since WHO declared TB a global emergency, the infusion of resources has made a more complete understanding of the immunology and cell biology of TB infection possible, and has led to a robust and diverse pipeline of candidate vaccines. Despite recent disappointments in some TB vaccine clinical trials, reason exists for cautious optimism. On the one hand, the scientific community has a better

3948 understanding of why the protection afforded by BCG is so limited and variable. Also, significant
3949 discoveries may enable the re-engineering of the world's only TB vaccine for greater efficacy across all
3950 age groups. Boosting BCG with subunit vaccines that supply antigens against multiple stages of *Mtb*
3951 infection has taken hold as a concept and vaccines of this type are in early clinical development together
3952 with vaccines that target only the active replication of *Mtb*. Further study of individuals that are latently
3953 infected, yet retain immune control of their infection over many years, is clearly warranted. The immune
3954 responses in these individuals may hold the key to the development of more effective TB vaccines.

3955 Several human challenge models for evaluating candidate TB vaccines are under development. The
3956 challenge strain is BCG, administered by either intradermal inoculation, bronchioscopic instillation, or
3957 aerosol in healthy adult volunteers with or without prior vaccination with BCG. To regard the models as
3958 tools for down-selecting candidate vaccines, accepting the hypothesis that a reduction in the replication of
3959 BCG predicts the efficacy of vaccines against *Mtb* in humans is necessary. The capacity of animal models
3960 to fully support the development of TB vaccines is also limited.

3961 Some experts in the field regard the next ten to 15 years as a critical time for TB vaccines to demonstrate
3962 improved efficacy while maintaining safety (809). They highlight an upcoming bottleneck in the
3963 availability of clinical trial sites and capacity. Moreover, unless a strategy to prevent reactivation of
3964 disease in the overwhelming numbers of humans that may already be latently infected with TB is
3965 established, attaining the goals for TB elimination is likely to be further delayed (761). At the present
3966 time, we have no means to identify those people with latent TB that are most likely to progress to active
3967 infection, nor do we have a practical way to prevent reactivation short of a new and effective multi-stage
3968 vaccine. One possible roadblock ahead is the potential to cause harm with vaccines that stimulate immune
3969 responses against latent TB infection. Part of the damage to the lungs in TB infection may be caused by a
3970 vigorous immune attack on granulomas in the lungs, which can damage host cells in the process of
3971 attacking the pathogen. Photographs of cavities in the lungs of the TB-infected are a vivid reminder of
3972 this possibility.

3973

Pertussis

Public health impact of *Bordetella pertussis* and the evolution of pertussis vaccines

Pertussis, also called whooping cough, is a serious childhood respiratory disease mainly caused by the bacterium *Bordetella pertussis*. Before the widespread use of whole-cell pertussis vaccines, 115,000 to 270,000 cases of pertussis occurred per year in the United States (810). Whole-cell pertussis vaccines were licensed in the United States in 1914 and came into widespread use after their incorporation into the diphtheria, tetanus toxoid, whole-cell pertussis (DTP) vaccine in 1948. By 1960, DTP vaccines were fully utilized and the rates of pertussis had fallen to 1,200 to 4,000 cases per year in the United States—representing a 99 percent reduction in the rate of pertussis after vaccine introduction. Pertussis was regarded as a vaccine-preventable disease that was under control; however, the control of pertussis has gradually eroded over the last 30 years in the United States (811) and internationally (812). In 2018, more than 15,000 cases were reported to the US Centers for Disease Control and Prevention. A similar resurgence of pertussis is occurring throughout much of the developed world, in spite of high rates of vaccination with acellular pertussis (aP) vaccines (813–815). The World Health Organization estimated that there were 24.1 million cases of pertussis and 160,700 deaths globally due to pertussis among children under 5 years of age in 2014 (816).

Although pertussis vaccination was accepted as standard practice in the United States from 1960 onward, the initial positive perception of pertussis vaccines had changed by 1990. Whole-cell pertussis vaccines are very reactogenic due to the presence of bacterial endotoxin. Most infants experienced injection site reactions after vaccination, about half developed fever, and a few serious systemic reactions to the vaccine occurred, including isolated cases of panencephalitis that lead to serious concerns (817). A few of the licensed DTP vaccines apparently had manufacturing issues and were found to be associated with poor protection, leading to new outbreaks of pertussis. Concerns about safety and effectiveness, coupled with a number of lawsuits that stemmed from injuries believed to be vaccine associated, caused some manufacturers of DTP vaccines to withdraw their products from the market. With widespread use of the DTP vaccine, generations of parents lacked the experience of witnessing an actual case of pertussis, one more factor influencing public perception about the vaccine. Pertussis vaccination programs in many countries were discontinued and vaccination programs were less accepted in others (810). The basic illness does not provoke an inflammatory immune response and usually occurs without a significant fever. What follows is a severe illness with periods of severe coughing, followed by periods without respiratory symptoms. The cough has a distinctive inspiratory whoop and post-tussive emesis. These severe and protracted spasms of coughing up mucus may last for months (818).

The growing reluctance to accept the reactogenic whole-cell pertussis vaccines in the 1970s and 1980s spurred a greater effort to develop acellular vaccines that would not contain endotoxin. Acellular vaccines are based on specific antigens of *B. pertussis* rather than on the whole bacterium. Multiple candidate antigens were tested in various combinations in the 1980s. The outcome of testing was to select the following antigens for inclusion in acellular vaccines: filamentous hemagglutinin (FHA), pertussis toxin, pertactin, and a combination of fimbriae 2 and 3. By 2002, acellular pertussis vaccines from GlaxoSmithKline and Sanofi Pasteur had been licensed for use in infants in the United States, and the US Advisory Committee on Immunization Practices had recommended that acellular pertussis vaccines

4014 completely replace whole-cell pertussis vaccines in the US childhood immunization series. Table 33
4015 describes the currently licensed *B. pertussis* vaccines in the United States.

Table 33. Currently licensed *Bordetella pertussis* vaccines.

Trade name	Valency	Components	Developer	Licensure
Infanrix™	$DTaP_{(3)}$	Pertussis toxin, filamentous hemagglutinin, pertactin, diphtheria toxoid, tetanus toxoid	GlaxoSmithKline	1997
Daptacel™	$DTaP_{(5)}$	Pertussis toxin, filamentous hemagglutinin, pertactin, fimbriae types 2 and 3, diphtheria toxoid, tetanus toxoid	Sanofi Pasteur	2002

Note: GlaxoSmithKline has licensed additional formulations of the same three pertussis antigens combined with inactivated poliovirus and hepatitis B virus. Sanofi Pasteur has licensed additional formulations of the same five pertussis antigens in different combinations with inactivated poliovirus, *Haemophilus influenzae* type B, and hepatitis B under the names Tetraxim®, Pentaxim®, and Hexaxim®.

4016 In the decades since their introduction, the acellular vaccines have been safe. They have provided good
4017 short-term protection from disease, but the duration of protection is not comparable to that provided by
4018 whole-cell pertussis vaccines. The duration of protection has been evaluated in observational studies and
4019 during pertussis outbreaks. Both types of studies indicate that the disease-free interval after vaccination
4020 has been decreasing over the past two decades in the era of acellular pertussis vaccines.

4021 In 2010/2011, the US state of California experienced the largest outbreak of pertussis in a half-century.
4022 The majority of cases were in school-aged children that had only received the acellular vaccines. The
4023 outbreak saw 7,200 cases of pertussis and ten infant deaths. The US states of Michigan, Ohio, and
4024 Oklahoma, as well as Australia and Ireland, all reported similar outbreaks during the same general time
4025 period.

4026 The poor durability of protection with acellular vaccines has prompted the development of new candidate
4027 vaccines (819–821), however difficult it may be to replace a currently used vaccine (820). At the same
4028 time, research intended to uncover the basis for the waning efficacy of acellular pertussis vaccines is
4029 gaining momentum. Several hypotheses have advanced to explain the poor durability, and possibly lower
4030 efficacy, of acellular vaccines as compared to whole-cell vaccines. These hypotheses include a different
4031 quality of immune response generated by the different vaccines (822), escape of bacterial strains from
4032 vaccine-induced immunity (818, 823), and undiagnosed increases in infections with *Bordetella*
4033 *parapertussis* (a related strain of *Bordetella* that causes a similar, but milder, respiratory disease in
4034 humans) (821, 824). The current pertussis vaccines do not protect against *B. parapertussis* (824).

4035 A new recombinant acellular pertussis vaccine developed by BioNet-Asia that contains genetically
4036 inactivated pertussis toxin and FHA (825) has been tested in adolescents as a monovalent vaccine
4037 ($aP_{[PTgen/FHA]}$) and in combination with tetanus and reduced-dose diphtheria vaccines ($TdaP_{[PTgen/FHA]}$).
4038 Most significant is that one year after vaccination, more than 70 subjects still maintained
4039 seroconversion, while only 4 percent of those that had received the TdaP control vaccine had
4040 maintained seroconversion (826). Ongoing studies have tested the vaccine in women of child-bearing
4041 age and more recently in pregnant women (827).

4042 **Immunity to *Bordetella pertussis***

4043 Immunity to *B. pertussis* has been the subject of a comprehensive review (822). The current data on
4044 protective immunity come from studies of humans that have received whole-cell and acellular pertussis
4045 vaccines, and from a murine respiratory challenge model of pertussis.

4046 Higgs et al. used a combination of human and murine data to provide an informative description of the
4047 different mechanisms of immunity that whole-cell and acellular pertussis vaccines may induce (Figure
4048 15) (822). The killed bacteria in the whole-cell vaccine are engulfed and degraded by phagocytic cells of
4049 the immune system which present the bacterial antigens to naïve T cells and produce cytokines that drive
4050 the differentiation of two types of T cells, Th17 cells which produce an IL-17 that activates neutrophils to
4051 take up and degrade intracellular bacteria, and Th1 cells produce IFN-γ that promotes the development of
4052 opsonizing and complement-fixing antibodies. These antibodies bind to antigens on the surface of the
4053 bacteria and promote their uptake and degradation by activated macrophages and can also directly kill
4054 bacteria in the presence of complement. This Th17/Th1-biased immune response is thought to be
4055 responsible for the efficacy of whole-cell pertussis vaccines.

4056 **Figure 15. Distinct mechanisms of immunity induced with whole-cell and acellular pertussis vaccines.**

4057 In contrast, the cytokines produced in response to acellular vaccines are less driven by pathogen-
4058 associated molecular patterns but are influenced by the aluminum hydroxide (alum) adjuvant that is
4059 included in the vaccines. Alum drives the production of IL-1, which fosters the development of Th17
4060 cells. Phagocytic cells that take up the acellular vaccine produce IL-4 by an unknown mechanism. IL-4
4061 drives differentiation Th2 cells. Th2 cells produce cytokines that drive antibody production, but the
4062 antibodies are of a different subclass that does not opsonize or fix complement. Therefore, the immune
4063 responses would not result in the efficient elimination of extracellular bacteria, as occurs with the whole-
4064 cell vaccine.

4065 Additional data on the immunity generated by acellular pertussis vaccines have been gathered using a
4066 nonhuman primate model of infection with *B. pertussis* (828). Warfel et al. vaccinated infant baboons
4067 with acellular or whole-cell pertussis vaccines and challenged them at 7 months of age with *B. pertussis.*
4068 They measured symptoms of disease and rates of colonization using nasopharyngeal washes after
4069 challenge. The acellular pertussis vaccine prevented the most severe symptoms of disease, but did not
4070 prevent colonization, did not clear colonization any faster than in unvaccinated animals, and did not
4071 prevent transmission to unvaccinated contacts. The animals vaccinated with the whole-cell pertussis
4072 vaccine had a more rapid clearance of colonization as compared to acellular vaccinated animals and
4073 unvaccinated controls. Previously infected animals were not colonized upon re-challenge. The T-cell
4074 responses to the acellular vaccine differed from the T-cell responses to the whole-cell vaccine and to
4075 natural infection. Naturally infected and whole-cell vaccinated animals had *B. pertussis*-specific Th17 and
4076 Th1 memory T cells, whereas animals that received the acellular vaccine had a Th1/Th2 memory
4077 response.

4078 A factor that can limit the effectiveness of pertussis vaccines is the increased circulation of bacterial
4079 strains that are less susceptible to the vaccine-induced immunity (818, 823).

4080 **Development of pertussis vaccines**

4081 Data from the baboon model of pertussis and from human infections indicate that acellular pertussis
4082 vaccines provide substantial protection against the most severe symptoms of infection with *B. pertussis*
4083 (828). Therefore, efforts to improve acellular vaccines with respect to the level and durability of
4084 protection they afford should proceed in parallel with efforts to develop new vaccines against pertussis.
4085 Table 34 describes the *B. pertussis* vaccines under development. The data were compiled from a number
4086 of published articles (821, 829–834).

Table 34. *Bordetella pertussis* vaccines under development.

Vaccine type	Vaccine details	Development phase	Developer
Live-attenuated	*B. pertussis* strain BPZE1	Phase 1	Public Health Agency of Sweden and Institut Pasteur de Lille
	B. pertussis strain BPZE1f3 expressing serotype 3 fimbriae Fim3	Preclinical	Institut Pasteur de Lille
	B. pertussis strain GamLPV	Phase 1/2	Gamaleya Research Institute, Russia
	aroQ mutant *B. pertussis* (ATCC 9340 strain)	Preclinical	University of Southern Queensland, Australia
Whole-cell	DTP low, chemical extraction to reduce lipooligosaccharide from the outer membrane of a whole-cell *B. pertussis*	Preclinical	Instituto Butantan, Brazil, and Netherlands Vaccine Institute
DNA vaccines	DNA plasmid expressing the N-terminal 180-amino-acid fragment (C180) of pertussis toxin S1 subunit	Preclinical	National Institute of Infectious Diseases, Japan
	DNA plasmid expressing pertussis toxin subunit 1, fragments of pertactin, and filamentous hemagglutinin	Preclinical	Peking Union Medical College and Chinese Academy of Medical Sciences
	DNA plasmid encoding a genetically inactivated S1 domain of pertussis toxin	Preclinical	University of Southern Queensland, Australia
Nanoparticle/ microparticle	Pertussis toxoid and filamentous hemagglutinin encapsulated in poly-lactide-co-glycolide	Preclinical	National University of Ireland
	Microparticle-based vaccine consisting of pertussis toxoid, polyphosphazene, CpG ODN 10101, and synthetic cationic innate defense regulator peptide 1002	Preclinical	University of Saskatchewan, Canada
	Chitosan–dextran sulfate nanoparticle formulation of pertussis toxoid with IgA adjuvant	Preclinical	Curtin University, Australia

Table 34. *Bordetella pertussis* vaccines under development.

Vaccine type	Vaccine details	Development phase	Developer
Protein-based	Expression of iron-repressible protein-3 pertussis antigen	Preclinical	Universidad Nacional de La Plata, Argentina
Acellular	Outer membrane vesicles expressing lipid adenylate cyclase PagL	Preclinical	Universidad Nacional de La Plata, Argentina

Abbreviations: *B. pertussis*, *Bordetella pertussis*; DTP, diphtheria-tetanus-pertussis vaccine; IgA, immunoglobulin A; IgG, immunoglobulin G.

4087 Current acellular pertussis vaccines induce strong antibody and Th2 responses but fail to protect against
4088 nasal colonization and transmission of *B. pertussis*. Furthermore, immunity wanes rapidly after
4089 immunization. To improve on their performance several avenues are being pursued, including the
4090 introduction of novel adjuvants and intranasal immunization with the same acellular pertussis vaccine
4091 (835, 836). In addition, strategies to incorporate additional virulence factors of *B. pertussis* (e.g.,
4092 adenylate cyclase toxin or iron regulated protein), and to include adjuvants like Toll-like receptor agonists
4093 that drive a stronger Th1 response (820, 821) are being pursued. Another approach under consideration is
4094 to present some of the antigens found in current acellular vaccines as DNA-vectored vaccines, or as
4095 particle or subunit vaccines. Table 34 describes three DNA vaccines and three particle vaccines that
4096 include some of the current acellular vaccine antigens, or modified forms of them. An acellular vaccine
4097 based on the outer membrane vesicles that Gram-negative bacteria spontaneously secrete is in
4098 development (821). All of these candidate vaccines are at the preclinical stage of development.

4099 On the other hand, live-attenuated pertussis vaccines have entered clinical trials. Thus, BPZE1, in which
4100 the genes for three toxins have been inactivated or removed, has been shown to protect mice and
4101 nonhuman primates. BPZE1 was evaluated in a Phase 1 trial and found to be safe, immunogenic, and
4102 capable of colonizing the upper respiratory tract in humans (837). Another live-attenuated vaccine,
4103 GamLPV, was reported to have completed a Phase 1 clinical trial (ClinialTrials.gov Identifier
4104 NCT03137927) (838) and entered a second Phase 1/2 trial (ClinialTrials.gov Identifier NCT04036526)
4105 (839) at the Gamaleya Research Institute in Russia, but no results have been reported.

4106 **Challenge models for pertussis**

4107 Small animal models of *B. pertussis* infection do not replicate the spectrum of disease seen in humans.
4108 Nonhuman primate models of pertussis are under development (828, 840, 841). In their initial study,
4109 Warfel et al. compared the spectrum of disease developed after inoculating Rhesus macaques or baboons
4110 with either of two strains of *B. pertussis* described in Table 35. They found that seven out of seven
4111 Rhesus macaques were persistently infected with *B. pertussis*, but only one developed clinical signs of
4112 disease. Nine of nine inoculated weanling baboons developed classical symptoms of pertussis. After six
4113 months, four of the infected baboons and two naïve controls were re-challenged with *B. pertussis*. Both of
4114 the controls developed disease, but none of the four convalescent baboons developed disease.

4115 Table 35 provides further details on the route of administration of the challenge strains. The baboons were
4116 anesthetized and intubated to deliver part of the inoculum to the top of the trachea, and the remainder of
4117 the inoculum was delivered intranasally. As described in the section on immunity to *B. pertussis,* the
4118 baboon model has been used to evaluate immune responses to whole-cell and acellular pertussis vaccines,
4119 and to demonstrate that natural infection with *B. pertussis* provides protection against subsequent

4120 colonization and disease. Therefore, the baboon model has demonstrated capacity to evaluate correlates of
4121 protection afforded by infection and vaccination. Moreover, a correlation is apparent between the age of
4122 the infected baboons and the severity of disease, as observed in humans (840). Airborne transmission of
4123 *B. pertussis* has also been demonstrated in the baboon model (841). Infected baboons could transmit *B.*
4124 *pertussis* to uninfected cage mates and to uninfected animals housed in cages more than two meters away.

Table 35. Baboon and human challenge models for *Bordetella pertussis.*

Host species	Challenge organism	Strains	Method of production and administration	Location
Baboon	*B. pertussis*	Tohama I, obtained from the US Food and Drug Administration D425, a clinical isolate provided by the US Centers for Disease Control and Prevention	*B. pertussis* was grown on Bordet-Gengou agar, resuspended in phosphate-buffered saline to a density of 10^9 to 10^{10} cfu per ml. Baboons were anesthetized and intubated to deliver 1 ml of the inoculum to the top of the trachea and a catheter was used to deliver 0.5 ml of inoculum to the back of each naris.	US Food and Drug Administration, Center for Biologics Evaluation and Research
Human	*B. pertussis*	B1917, isolated from a Dutch patient and prepared under GMP by Q Biologicals (Belgium)	Frozen vials of *B. pertussis* were thawed, diluted to the appropriate dose, and directly administered intranasally with 0.5 ml in each naris at 10^3 to 10^5 cfu per ml.	National Health Institute for Health Research CRF, Southampton, United Kingdom

Abbreviations: *B. pertussis*, *Bordetella pertussis*; CRF, clinical research facility; cfu, colony forming units; GMP, Good Manufacturing Practice.

4125 The baboon model of *B. pertussis* infection has several limitations. No established, large-scale breeding
4126 program exists to ensure an adequate supply of animals for research, and access to infant or juvenile
4127 baboons may be even more limited. With respect to evaluating immune responses, reagents that cross-
4128 react with baboon antigens are highly limited, particularly those used for separating cells by flow
4129 cytometry. Developing such reagents may be a lengthy process.

4130 A human challenge model for pertussis has been discussed for several years (840). One reason to consider
4131 this option is that no widely available animal model of pertussis currently exists. In practical terms, the
4132 baboon model cannot be expected to provide the capacity needed to support multiple vaccine
4133 development programs, or to provide the numbers of animals that would be needed to conclusively
4134 establish correlates of protective immunity, at least in the near term. A more important limitation for the
4135 field of pertussis vaccine development is the lack of adequate field sites with sufficient incidence of
4136 pertussis to permit the evaluation of candidate vaccines. Moreover, placebo controls will not be ethically
4137 acceptable in field trials; whereas, they may be acceptable in the controlled inpatient setting of
4138 experimental human infection where prompt treatment is assured. New vaccines will need to demonstrate
4139 at least equivalence, if not superiority of the licensed vaccines for preventing disease after infection with
4140 *B. pertussis*, and the required field trials would be large, lengthy, and costly. Smaller comparison trials in

4141 a human challenge model could potentially provide some of the data that would support licensure of new
4142 pertussis vaccines.

4143 On the other hand, human challenge models for *B. pertussis* would have significant limitations. Studies
4144 would need to be conducted in specialized inpatient facilities with a high level of containment since
4145 pertussis is highly contagious by the aerosol route of infection. The delivery of a challenge dose to the
4146 lower respiratory tract, as has been done with the baboon model, may not be feasible in humans. Highly
4147 effective treatment will be a requirement as soon as infection is established. This limitation is imposed by
4148 the fact that antibiotics are effective against *B. pertussis* early in infection, but there is a "point of no
4149 return" beyond which antibiotics are ineffective (818). There is a risk of serious disease, which may not
4150 be completely abrogated by early treatment in every case. Since the development of disease will not occur
4151 with early treatment, the human challenge model would be limited to the evaluation of vaccines for their
4152 capacity to prevent infection, rather than to prevent disease.

4153 A protocol for a pertussis human challenge model that addresses some of the issues noted above has been
4154 proposed by de Graff et al. (842) and recently implemented (843, 844). The methods of the protocol are
4155 summarized in Table 35. Subjects with anti-pertussis toxin IgG concentrations less than 20 IU/ml were
4156 inoculated intranasally with wild-type *B. pertussis* strain B1917 in an inpatient unit. Only minor
4157 symptoms were observed. Colonization, assessed by culture and quantitative PCR was detected in 80
4158 percent of subjects that received a dose of 10^5 colony forming units the organism. Azithromycin
4159 eradicated colonization within 48 hours in 88 percent of colonized individuals. Anti-pertussis toxin IgG
4160 seroconversion was observed in nine of 19 colonized participants and none of those not colonized. The
4161 organism was not detected in environmental samples.

4162 **Summary**

4163 Acellular pertussis vaccines may need reformulation or replacement if we are to maintain control of
4164 *B. pertussis* in human populations. Given the actual and perceived safety concerns that arose with the use
4165 of whole-cell pertussis vaccines, reformulated whole-cell vaccines or live-attenuated vaccines seem
4166 unlikely to gain public acceptance. One potentially useful measure would be to remove pertussis vaccines
4167 from their current formulations with other vaccines to create a pertussis-only vaccine (820). Such a
4168 vaccine could be delivered on its own schedule, with repeated boosting if required. A change in antigen
4169 content of the vaccine could be considered (e.g., by adding additional antigens thought to be protective or
4170 changing the method for detoxifying pertussis toxin). More substantive changes could include modifying
4171 adjuvants or changing the delivery system (e.g., to the intranasal route) (820).

4172 These redesign options for acellular pertussis vaccines must be weighed against the potential to develop
4173 new vaccines of clearly superior levels or duration of protection. As shown in the baboon model of
4174 *B. pertussis* infection, preventing lengthy carriage of *B. pertussis* will be particularly important to prevent
4175 transmission to others. To expect that any of the new candidate vaccines would replace the current
4176 acellular vaccines in the coming decade would seem to be unrealistic.

4177 The baboon model for pertussis could be significantly expanded, but nature is against us because the
4178 establishment of large breeding colonies takes time. Reagents need to be developed for the model to reach
4179 its potential for evaluating protective immunity. A recently developed human challenge model may be
4180 useful in the evaluation of future vaccines, but would present logistical challenges and would be limited

4181 to evaluating asymptomatic colonization. The increased severity of disease seen with *B. pertussis*
4182 infection in infants and young children as compared to adults is replicated in the baboon model of
4183 infection, but would not be replicated in a human challenge model with adult volunteers.

4184

Severe acute respiratory syndrome coronavirus 2

Assessment on the development of a controlled human infection model for COVID-19 due to SARS-CoV-2 virus infection

A novel coronavirus was identified toward the end of 2019 as the cause of a cluster of pneumonia cases in Wuhan, China. The virus is designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is the causative agent of the disease COVID-19. SARS-CoV-2 spread rapidly around the world, and the outbreak was declared a “Public Health Emergency of International Concern” by the World Health Organization (WHO) on January 30, 2020, and the outbreak of COVID-19 was officially declared a pandemic on March 11, 2020 (845). As of September 30, 2021, more than 236 million cases and more than 4.8 million deaths from COVID-19 had been reported (846). Person-to-person spread of SARS-CoV-2 occurs primarily through respiratory droplets. Infection may also occur by touching an infected surface and then one’s own eyes, nose, or mouth. The detection of viral RNA does not necessarily indicate the presence of infectious virus and there may be a threshold of viral RNA level below which infectivity is unlikely (847). Several reports have described detection of SARS-CoV-2 RNA and infectious virus in stool specimens (848, 849), but this does not appear to be a significant factor in the spread of infection.

The interval during which an individual with COVID-19 is infectious is uncertain, although SARS-CoV-2 can be transmitted prior to the development of symptoms and throughout the course of illness, with the highest virus levels soon after symptom onset (847, 850–852). The duration of viral shedding is highly variable and may depend on the severity of the illness (847, 849, 853–855). The risk of transmission from an individual with SARS-CoV-2 infection varies by the type and duration of exposure, factors such as the amount of virus in respiratory secretions, and the use of preventive measures.

Due to the immense and urgent need for interventions to stop the pandemic, several clinicians and bioethicists have proposed the development of a SARS-CoV-2 controlled human infection model (CHIM) to accelerate the development of new vaccines and therapeutics despite the unprecedented speed in bringing both conventional (inactivated SARS-CoV-2) and novel (mRNA, viral vector) technologies to the market saving millions of lives. The advancement of a human infection model of SARS-CoV-2 in low-risk volunteer subjects (856–861) provides a unique perspective that is novel and potentially ground-breaking but is not without controversy. WHO has provided criteria for ethical acceptability of human challenge models using live SARS-CoV-2 challenge virus (862).

First, there needs to be scientific rationale. It is assumed that the application of a SARS-CoV-2 human challenge model would be faster than the conventional clinical trial pathway for testing vaccine candidates and experimental therapeutics. With the COVID-19 pandemic (as of October 2021), well into its second year, considerable progress has been achieved in delivering therapeutics (remdesivir and monoclonal antibodies such as casirivimab and imdevimab, and bamlanivimab and etesevimab) and vaccines (Pfizer, Moderna, Johnson & Johnson, AstraZeneca) without the availability of a standardized and validated human infection challenge model. Nevertheless, a successful human infection model will depend on factors such as (i) availability of Good Manufacturing Practice stocks of virus (863); (ii) advanced knowledge of minimal infectious dose; (iii) secure clinical trial facilities with rigorous infection control procedures for prolonged periods of time; (iv) specific treatment(s) for complications; (v) consensus among policymakers; and (vi) *a priori* regulatory approval.

Assessment of risk and benefit depends on how comfortable regulatory agencies are with accepting the clinical endpoint in human challenge trials to approve a vaccine without a pivotal trial under natural exposure. If not, then the product sponsor may use challenge studies to either advance or stop further development of that vaccine, to spare the cost of developing a vaccine that is unlikely to provide an acceptable level of protection. In either case, the risk to any subject in the control group may outweigh any potential benefit, especially when there is no therapeutic intervention that would prevent a serious adverse event related to the challenge, in contrast to challenge with other pathogens for which there are highly-effective rescue therapeutics available. Furthermore, the relatively limited understanding of COVID-19 pathogenesis represents an additional risk, in contrast to challenge with pathogens that have been studied for decades and are well characterized. Several authors (856, 857, 862) have argued that because younger-aged persons (i.e., 18 to 49 years) are less at risk for serious complications from COVID-19, this alone justifies consideration of the model. They further argue if rigorous informed consent is obtained, then it would be ethically justifiable to proceed; however, there is not universal agreement that informed consent alone is ethically justifiable.

The first human challenge model using virus stocks of SARS-CoV-2 has been approved by regulators and ethical committees sponsored by the University of Oxford (NCT04864548). This Phase I dose escalation challenge study of up to 64 subjects will be challenged with increasing titers of wild-type SARS-CoV-2 administered intranasally in order to achieve a 50 percent attack rate as determined by quantitative viral detection and/or qPCR detection in nasopharyngeal secretions at 12 hourly time points. Rescue treatment monoclonal antibody cocktail (casirivimab and imdevimab) will be administered to subjects after indication of COVID-19 disease beyond mild symptomatology. A Data Safety Monitoring Board will review safety and viral infection data.

Alternatives to intranasal challenge have also been proposed to include oral administration of SARS-CoV-2 that induces a limited infection in the gut without spread to the respiratory tract (864), and attenuated viral challenge strains that induces a self-limiting infection. Finally, coronaviruses different from SARS-CoV-2 and that are known to cause mild upper respiratory symptoms have been considered.

There are several uses cases for SARS-CoV-2 human challenge model development. First, it would accelerate the Go/No Go decision at the end of each stage gate in the project both for vaccine and therapeutic development. Second, a SARS-CoV-2 CHIM could be used to define correlates of risk or protection that would then justify regulatory approval. A validated challenge model would also permit us to better understand how long naturally-acquired or vaccine-induced immunity lasts, and may also help us separate protection against infection versus protection against ongoing forward transmission especially in asymptomatic infected subjects. Third, a SARS-CoV-2 challenge model could be implemented to test updated vaccines against newly emerging variants during a period when the new variants have been identified but are not yet highly prevalent in the population, thus making traditional clinical trials difficult to conduct or interpret. Fourth, the model could be used to determine re-infection rates by drifted variants in comparison to earlier ones. Finally, it would increase our knowledge and understanding of the early events in viral pathogenesis and immune responses that control replication leading to viral clearance (858).

Notwithstanding the possible advantages offered by a CHIM, a number of limitations, logistical barriers, and critical barriers have been raised (858), including by participants in a recent webinar hosted by the

4266 International Alliance for Biological Standardisation (865). These limitations include the route of
4267 administration, dose-finding studies, the timing vis-à-vis vaccine administration, the generalizability to
4268 at-risk vulnerable populations, the requirement for sufficient biocontainment facilities, the Good
4269 Manufacturing Practice-compliant of virus stocks from at least two different viral isolates, the need for
4270 rescue therapy, and the ethics related to participant selection, premature participant withdrawal
4271 necessitating the need for forced quarantine, the risks to study staff, and potential transmission of escaped
4272 virus into the general population. Nonetheless, the eagerness to pursue such measures by groups that have
4273 never developed, participated in, or led a CHIM trial needs to be balanced by thoughtful considerations of
4274 whether classical Phase 3 trials should continue to be the gold standard for evaluation of COVID-19
4275 candidate vaccines and therapeutics.

4276

Regulatory and ethical considerations for conducting human challenge studies

Three different types of human challenge studies are subject to review by regulatory authorities (see Table 36). These include challenge studies in which a strain of a pathogen is administered to adult volunteers to develop a consistent model and investigate pathogenesis; studies in which a challenge is followed by re-challenge at some later time, with the same or a different challenge strain, to understand the level of protection afforded by prior infection; and studies in which a vaccine is administered prior to challenge to test its protective efficacy. These three types of studies have somewhat different regulatory requirements that depend on the country in which the challenge study is to be conducted.

The requirements from regulatory agencies that have jurisdiction over human challenge studies also vary with the type of study and the country in which the study is conducted (see Table 37). In general, human challenge studies may have a more complex, but perhaps clearer, regulatory requirement in the United States as compared to the United Kingdom—the two countries in which the large majority of challenge studies have been conducted. The main differences are that challenge and re-challenge studies that do not include a vaccine are subject to review at the national level in the United States, but not in the United Kingdom. Another important difference is that in the United States, the challenge strains themselves usually are required to meet a level of compliance with Good Manufacturing Practice (GMP) similar to that required for vaccines entering Phase 1 clinical trials. In the United Kingdom, this condition has not been consistently required.

Table 36. Types of controlled human challenge studies and their regulatory requirements.

<table>
<tr><th>Study type and description</th><th>Examples of study objectives</th><th>Regulatory requirements in the United States</th><th>Regulatory requirements in the United Kingdom</th></tr>
<tr><td>Challenge study: A challenge strain of a pathogen is administered to healthy adult volunteers.</td><td>Establish dose-response curve for infection, time to onset of disease, range of disease symptoms, time to resolution of symptoms if no specific treatment is available, effect of prior natural exposure to the pathogen. Evaluate immune response to infection. Establish or optimize model.</td><td rowspan="2">Study must be conducted under an IND application that has been approved by the US Food and Drug Administration. Challenge strains are ideally manufactured in compliance with GMP at a level similar to that required for a product to be studied in a Phase 1 clinical trial. Once allowed to proceed, IND applications (or drug master files) for challenge strains may be cross-referenced in subsequent studies. In addition, the study must undergo review and approval by an IRB, or multiple IRBs if the study is to be conducted at multiple sites. Other review agencies may also have jurisdiction under certain circumstances (see Table 37).</td><td rowspan="2">Study does not require review by the MHRA if it does not involve a vaccine. The challenge strains do not necessarily need to meet GMP requirements. Review and approval by an IRB is a requirement, and by other agencies in select circumstances (see Table 37).</td></tr>
<tr><td>Re-challenge study: A challenge strain of a pathogen is administered to healthy adult volunteers previously challenged with the same strain or a heterologous strain.</td><td>Determine whether prior infection protects against re-infection with a homologous or heterologous strain of the pathogen. Identify breadth and/or correlates of protective immunity.</td></tr>
<tr><td>Vaccine trial using a challenge model: A candidate vaccine is administered to healthy adult volunteers that are later (or in some cases, previously) challenged with one or more strains of the pathogen against which the vaccine (or drug[a]) is directed.</td><td>Evaluate the capacity of a candidate vaccine to prevent infection, colonization/carriage, or transmission of the pathogen; or limit severity of or prevent disease symptoms. Compare different vaccine formulations and/or regimens for the immune responses elicited and level of protection. A candidate vaccine may be directly compared to a licensed vaccine that is suboptimal in some important respect.</td><td>Candidate vaccine must be administered under an approved IND application and must undergo ethical review and approval by the relevant IRB(s), in addition to the approvals for the challenge strain and study procedures as described above.</td><td>Candidate vaccine must be administered under an MHRA-approved application. The protocol must also undergo ethical review and approval by the relevant IRB(s).</td></tr>
</table>

Abbreviations: IND, Investigational New Drug; GMP, Good Manufacturing Practice; IRB, institutional review board; MHRA, Medicines and Healthcare products Regulatory Agency.

[a] The fourth type of challenge study that might be conducted is one in which the challenge agent is used to infect trial subjects that are then treated with investigational drugs for the purposes of drug development. This type of study is not described herein, as the focus is on vaccine development.

4297

Table 37. Regulatory agencies and committees with jurisdiction over human challenge studies.

United States	Indication for review	United Kingdom	Indication for review
FDA, Office of Vaccines Research and Review	A human challenge study is proposed, with or without a vaccine.	MHRA	A vaccine is included in the human challenge study.
IRB	A human challenge study is proposed, with or without a vaccine.	IRB	A human challenge study is proposed, with or without a vaccine.
Institutional biosafety committee	The challenge strain or vaccine is considered to be a GMO, and the institution where the research is conducted receives US government funding.	Advisory Committee on Releases to the Environment of the Department for Environment, Food & Rural Affairs	The challenge strain or vaccine is considered to be a GMO.
US Vaccines and Related Biological Products Advisory Committee to the FDA	The proposed study using a challenge model is a vaccine efficacy trial, particularly if proposed to be a pivotal efficacy trial to support vaccine licensure.		

Abbreviations: FDA, US Food and Drug Administration; IRB, institutional review board; GMO, genetically modified organism; MHRA, Medicines and Healthcare products Regulatory Agency.

4298 Human challenge studies may or may not be regulated by a national regulatory authority (NRA); this
4299 depends on the country in which the research will take place. In all cases, clinical trials should be
4300 reviewed by ethics committees, which are referred to variously depending on the country and location of
4301 the clinical study. Terms for these institutions include institutional review board (IRB), research ethics
4302 committee, independent ethics committee, or, simply, ethics committee. For ease of readability in this
4303 document, we will use the term IRB to denote the institutional ethics committee responsible for
4304 overseeing clinical studies.

4305 In cases in which the vaccine or challenge organism under study is considered to be a genetically
4306 modified organism (GMO), additional oversight may include a governmental regulatory agency separate
4307 from the NRA or may be performed by the NRA itself. Aspects of the clinical study involving a GMO
4308 may also be reviewed at the institute where the research is being conducted, or by an institutional
4309 biosafety committee (IBC). For studies receiving funds from the US government review by an IBC is
4310 always required (see Table 37).

4311 Below we address each of the three aforementioned regulatory processes in turn. The regulatory
4312 procedures and expectations described reflect actual current practices by regulated industry and clinical
4313 trialists conducting studies in which humans are challenged with an infectious organism in the controlled
4314 setting of a human challenge study. In some cases, the ideal is described, but as with all regulated clinical
4315 studies, a case-by-case basis is considered by regulators and their actual requirements in any given case
4316 may not meet the ideal. Instead, their decisions are driven by scientific and ethical considerations, in
4317 addition to applicable regulations.

4318 **United States procedures for regulatory authority applications**

4319 Applications, filings, submissions, or dossiers (the term varies depending on the country) must be
4320 submitted to the various regulatory bodies in order to gain approval to conduct a human challenge study.
4321 The contents and format of the regulatory filing may also vary depending on to whom it is being
4322 submitted. The succeeding sub-sections will provide summaries on the terminology, contents, and format
4323 expected by regulators.

4324 In the United States, human challenge studies are regulated by the NRA (the US Food and Drug
4325 Administration [FDA]) and an IRB (or more than one IRB if multiple clinical sites are involved in the
4326 clinical study). Furthermore, if the challenge organism or the vaccine is deemed to be a GMO, an IBC
4327 will review the trial. In practice, at most US institutions, the IBC will review all studies involving
4328 infectious organisms, whether or not they are GMO.

4329 **United States Food and Drug Administration**
4330 The FDA is the NRA that regulates human challenge studies, whether or not they involve a vaccine. The
4331 same FDA review office that has jurisdiction for vaccines, the Office of Vaccines Research and Review,
4332 is also responsible for challenge organisms. A sponsor of a clinical trial must submit an Investigational
4333 New Drug (IND) application to the FDA, which has 30 days to review and approve the proposed clinical
4334 trial(s) or place on clinical hold. The FDA prefers submission of separate IND applications for the
4335 challenge organism and the vaccine, even if they are to be used in the same clinical trial, at least initially.
4336 The rationale for this is that the challenge organism may be used to test other vaccine candidates,
4337 particularly if the original vaccine candidate fails (for reasons of safety, immunogenicity, or efficacy). In
4338 such a case, the sponsor of the new clinical trial may cross-reference the IND application describing the
4339 challenge organism without prejudice due to the failed vaccine candidate. Particularly, if the sponsors are
4340 different, this eases the regulatory burden on the new sponsor (and the FDA) to avoid reiterating
4341 information the FDA has already reviewed. The IND regulations are promulgated in Title 21 of the US
4342 Code of Federal Regulations (CFR) part 312 (866) and sub-sections. Additional parts of 21 CFR may also
4343 apply in regard to product manufacture (867, 868), nonclinical research (869), and human subjects
4344 protections (870, 871).

4345 In addition to review by FDA staff, the FDA may seek the advice of its expert advisory committee at its
4346 discretion. For vaccines and related products, this would be the Vaccines and Related Biological Products
4347 Advisory Committee. Information submitted to the FDA in an IND application is held confidential,
4348 protected by their regulations (872). The FDA's advisory committees are generally held as open, public
4349 meetings. Phase 1 clinical trials are not typically the subject of an advisory committee meeting, although
4350 they can be. Efficacy trials, particularly pivotal efficacy trials to support product licensure, are generally
4351 reviewed by an FDA advisory committee. A human challenge study may fit in this category depending on
4352 the claims the sponsor may want to make, though this would be a rare and exceptional situation. The only
4353 guidance the FDA provides on human challenge studies specifically and their role in regulatory
4354 evaluation is in the 2011 *Guidance for Industry: General Principles for the Development of Vaccines to*
4355 *Protect Against Global Infectious Diseases* (873).

4356 The contents and format of an IND application are promulgated in 21 CFR 312.23 (874). An alternative
4357 format, however, may be used, specifically in the form of a common technical document, an
4358 internationally accepted format for regulatory dossiers (875). In either format, the contents expected by

4359 the FDA will cover information about the product (in the case of a challenge organism, the manufacture
4360 and control of the challenge organism itself), nonclinical research and development, and proposed clinical
4361 trial(s). The information about the product or challenge organism includes the microbiology,
4362 manufacturing, and controls (referred to as chemistry, manufacturing, and controls), reflecting that the
4363 majority of FDA IND reviews are for chemical drugs rather than for vaccines or challenge organisms.
4364 The nonclinical information includes pharmacology and toxicology, which for vaccines would be the
4365 immunogenicity and proof of concept (e.g., protection from challenge in an animal model), as well as
4366 other laboratory-determined characterizations, and the safety studies (in animals and/or *in vitro*). Finally,
4367 clinical information not only includes the protocol for the proposed trial(s), but also several other
4368 documents. These include a sample informed consent form, signed and completed FDA Forms 1572 and
4369 3674, the investigators' *curriculum vitae* and financial disclosures (FDA Forms 3454 and 3455), a
4370 summary of prior human experience, and an Investigator's Brochure if the trial will be conducted at
4371 multiple sites or by an investigator(s) who was not intimately involved in the product development. The
4372 Investigator's Brochure will be particularly important for human challenge studies conducted at multiple
4373 sites, as it will provide necessary information to ensure that all investigators are fully informed about
4374 potential risks to the human subjects they will enroll in the study. Another document that may be required
4375 is a general investigational plan that describes what the sponsor plans for the overall development of the
4376 product, with greater focus on the coming year (876).

4377 Depending on the phase of the clinical trial, the FDA has a sliding scale of expectations for compliance
4378 with GMP regulations (868): Phase 1 studies do not require full compliance with the GMP regulations,
4379 but products used in Phase 2 or 3 studies are required to be manufactured and controlled in full
4380 compliance with GMP regulations. The FDA recognizes that challenge organisms are not a true product
4381 in development, but a tool for vaccine development and for other scientific experiments, such as
4382 experimental medicine and human challenge studies. Therefore, even if used in Phase 2 or 3 efficacy
4383 studies, the FDA will likely have the same expectations for challenge organisms as for Phase 1 products.
4384 This may not be strictly the case if the challenge organism will be used widely in a relatively large
4385 number of Phase 2 and 3 human challenge studies or given to larger numbers of individuals (e.g.,
4386 exceeding 100 to 200 people). But, since the scale of human challenge studies usually involves numbers
4387 of subjects more in line with Phase 1 vaccine trials, the FDA will not likely expect full compliance with
4388 GMP (877, 878). Nonetheless, aspects of GMP will apply to the manufacture and testing of human
4389 challenge organisms. For this reason, consulting the FDA's *Guidance for Industry: CGMP for Phase 1
4390 Investigational Drugs* (877) is recommended for sponsors conducting a human challenge study.
4391 Particularly, aspects of production in a multi-use facility, such as stringent change-over procedures and
4392 the need for cleaning validation, will be important given the infectious and potentially pathogenic nature
4393 of the challenge stock being made. While other processes might not be validated, validation of the
4394 cleaning (and disinfecting/sterilizing) procedures will likely be expected.

4395 The FDA has 30 days in which to review the original submission of the IND application. At the end of the
4396 30 calendar-day period, the sponsor may initiate the clinical trial unless they have heard from the FDA
4397 that the IND or the protocol has been placed on clinical hold. The grounds for clinical hold are
4398 promulgated in 21 CFR 312.42 (879); they primarily focus on the safety of any phase trial or, for Phase 2
4399 or 3 studies, the study design must be adequate to meet stated aims. If the IND application is placed on
4400 hold, the sponsor must not initiate or must halt the clinical trial (if ongoing already). The FDA has 30
4401 days to provide, in writing, the grounds on which the application was placed on clinical hold and what

4402 must be done for the clinical hold to be lifted. When the sponsor provides a complete response to the
4403 clinical hold letter (as deemed complete by the FDA), the FDA has another 30 calendar days to review the
4404 complete response and provide, in writing, to the sponsor whether the clinical hold has been lifted, and if
4405 not, why not, and what further must be done for the hold to be lifted. The FDA may also request
4406 modifications to the proposed trial without placing a clinical hold. The sponsor may initiate the clinical
4407 trial in that case, but should respond to the requested modifications. If a sponsor fails to address the
4408 requested modifications, the FDA may not accept the data from the completed clinical trial to support
4409 product development. Therefore, making the requested modifications as soon as possible is in the
4410 sponsor's interest, ideally before initiating the trial.

4411 If the investigational product is to be imported into the United States for use in US clinical trial sites, the
4412 FDA has jurisdiction to permit the importation once the IND application is in effect (i.e., after the 30-day
4413 review and when no clinical hold is in place). US Customs will determine the status of the IND
4414 application before allowing the shipment of the investigational product to pass US borders. Shipments of
4415 investigational products for an IND application that is on hold or is not yet in effect will be held.

4416 If a US manufacturer wishes to export a product for a clinical trial outside the United States, then the
4417 FDA also has jurisdiction if the trial site is in a country that is not on a list of countries with NRAs the
4418 United States recognizes as having equivalent responsibility and capability to adequately regulate the
4419 product exported from the United States. The import and export regulations are promulgated in 21 CFR
4420 312.110 (880). If the product is being shipped to a country with a competent NRA recognized by the FDA
4421 (i.e., in the European Union and European Economic Community, Australia, Canada, Japan, Israel, New
4422 Zealand, Switzerland, or South Africa), then the NRA in that country has jurisdiction over the product
4423 from the United States that is imported into their country.

4424 Safety reports must also be provided to the FDA for their review. The safety reporting expectations for
4425 any clinical trial under an IND application are promulgated in 21 CFR 312.32 (881). Expedited safety
4426 reporting is required for serious and unexpected adverse events (within 15 calendar days of the sponsor
4427 being informed about the adverse event). Other adverse events must also be reported to the FDA at a
4428 frequency determined by the agency, but at least annually. Discussing with the FDA what their reporting
4429 expectations would be for expected adverse events from the challenge organism, if a pathogenic one is
4430 used, might be appropriate. Primarily, expedited reporting is for serious and unexpected events; however,
4431 a clear plan should be described in any protocol submitted for FDA review.

4432 **United States institutional review boards**

4433 Human challenge studies will be held to the same ethical review standards as all research involving
4434 human subjects, so the human subjects protection standards, including the need to gain approval from an
4435 IRB, should apply. This process will be no different than it would for any investigational medical
4436 interventional research. Essentially, each clinical trial site and institution participating in the research will
4437 require their institution's IRB approval, unless agreement for review by a central IRB can be obtained.
4438 The principal investigator or investigator at the particular trial site will submit to their IRB the proposed
4439 clinical protocol, the informed consent documents to be used at that site, the investigators' and sub-
4440 investigators' *curriculum vitae* and training records (e.g., Collaborative Institutional Training Initiative
4441 training records), and an Investigator's Brochure if there is one. Also, any recruitment material to be used
4442 should be provided for IRB review. The IRB will review and either approve the research, approve the

research conditional upon addressing the IRB's stipulations, or disapprove the research. The clinical trial site must not proceed with a trial until the IRB has approved it and must use the IRB-approved informed consent form and protocol. The IRB will also require updates on the progress of the clinical trial, at least annually. Adverse events occurring during the study must be reported to the IRB for their review and oversight according to the guidelines of the IRB.

United States regulations for genetically modified organisms

If the challenge organism or vaccine is a recombinant organism generated by recombinant DNA technology (i.e., genetic engineering), then it could be considered to be a GMO. GMOs are subject to procedures beyond those required for non-GMOs (i.e., natural organisms or organisms that are the result of manipulated breeding or hybridization). In the United States, the FDA has the authority to review and approve clinical trials conducted with GMOs. The US National Institutes of Health (NIH) also has some oversight responsibilities, if the institution that will perform the research receives US government funding. The NIH Office of Science Policy issued a document explaining the procedures, which are quite complex depending on a number of factors. This document, *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules* (882), describes the three separate, but overlapping, regulatory procedures of the FDA, IRBs, and IBCs. The Office of Science Policy also provides information governing IBCs. IBCs are responsible for overseeing the research to ensure that the risks to the environment and public health are managed through adequate biological containment, investigator qualifications and training, appropriateness of standard operating procedures (SOPs) and protocols for the research, and compliance (e.g., review of adverse event reports). An IBC may approve or disapprove the research.

United Kingdom procedures for regulatory authority applications

The European Medicines Agency (EMA) does not approve controlled human infection model (CHIM) study protocols, leaving to the NRAs to decide whether they wish to regulate CHIM studies. Nonetheless, EMA can provide scientific advice to developers on the use of CHIM approaches. The Medicines and Healthcare products Regulatory Agency (MHRA) is the UK's NRA. The filing to the MHRA should be in the format of the forms for EudraCT or the Integrated Research Application System (883). Similar to the FDA, information on the product and the nonclinical and proposed clinical trials are required for MHRA review. A major difference in the submission is the requirement for a Qualified Person with quality assurance responsibilities for the product in the United Kingdom. In the United States, these responsibilities fall to a quality unit, rather than an individual with the specific credentials. Only the Qualified Person may release a clinical trial lot of material for clinical research. Another major difference between the MHRA and the FDA is that the MHRA does not necessarily have an expectation for challenge organisms to meet GMP requirements because the challenge organism is not considered to be a medicinal product (more below). While IRBs have responsibility for reviewing research involving the challenge organism, if there is not a vaccine (or other medical product) used in the study with the challenge, then the MHRA will not review such protocols.

The MHRA does not regulate the development of CHIM agents and has no plans to take up this role. However, if a CHIM study is used to test the efficacy of an intervention (e.g., a vaccine), the study requires approval by MHRA. But even though there is no formal requirement, MHRA guidance states that GMP standards should be considered to ensure the safety of study subjects and quality of the

4484 challenge material. For challenge organisms that are GMOs, the UK Department for Environment, Food
4485 & Rural Affairs' Advisory Committee on Releases to the Environment (ACRE) will assess the risk to the
4486 environment and subjects not participating in the study and must give approval before the GMO challenge
4487 agent can be administered as part of a CHIM study.

4488 Guidance has been introduced by the European Union Health and Consumers Directorate-General
4489 recommending more formal assessment of Non Investigational Medicinal Products, such as challenge
4490 agents, and specifies the appropriate GMP requirements for them (884). Under the new European Union
4491 Clinical Trials Regulation, challenge agents fall under a new classification as Auxiliary Medicinal
4492 Products (AxMP): a medicinal product used for the needs of a clinical trial as described in the protocol,
4493 but not as an investigational medicinal product. Such products shall be manufactured according to GMP
4494 or to at least an equivalent standard, in order to ensure appropriate quality. Appropriate GMP
4495 requirements foreseen for the safety of the patients should still be applied and the sponsor should ensure
4496 that AxMPs are of appropriate quality for the purposes of the trial, taking into account, among other
4497 things, the source of the raw materials and any repackaging. It is clearer on the other hand that Good
4498 Clinical Practice guidelines must be followed for the use of such AxMP in human trials.

4499 Following Brexit, the United Kingdom withdrew from the European Union on January 31, 2020;
4500 however, there was a transition period through December 31, 2020, while the United Kingdom and the
4501 European Union negotiated additional arrangements until new rules took effect. During the transition
4502 period, the United Kingdom continued to follow the rules of the European Union and MHRA related to
4503 clinical research. From January 1, 2021, the MRHA is the UK's standalone medicines and medical
4504 devices regulator.

4505 The World Health Organization's (WHO) GMP standards are a set of international standards designed to
4506 ensure that biological products, including human challenge agents, are manufactured to minimum
4507 standards. As a result, the decision of whether to manufacture challenge pathogens to GMP for use in
4508 CHIM studies is an important consideration when conducting such a study. While the research
4509 community recognizes the need for high quality in the manufacture of human challenge agents, it has
4510 been suggested that these standards could be at least "GMP-like," meaning they would fulfill GMP
4511 requirements to as much as is practically possible without being GMP certified. Such a step would enable
4512 the manufacture of challenge agents outside of GMP-certified settings, such as in academic laboratories
4513 or in countries where GMP facilities might not be as practical. While GMP-like standards would allow
4514 this flexibility, it should not be an excuse for adopting low standards of manufacturing, as GMP standards
4515 provide reassurance of the safety of agents and the reproducibility of data generated, and GMP-like would
4516 need to be justified in each specific case. Consistent with the MHRA position, the requirement for a
4517 CHIM agent to be GMP certified, rather than just GMP-like, is the suggestion that the requirement should
4518 apply to the cases in which a CHIM study contributes directly to the licensing of a vaccine. In many
4519 cases, manufacture of an agent under GMP may not be technically feasible (e.g., helminth challenge
4520 agents and *Cryptosporidium*). In other cases, challenge agents that were developed long ago and found
4521 safe in humans were commonly used in trials before GMP guidelines were adopted. Like the United
4522 States, however, there is an expectation that IRBs, and potentially IBCs, would review the proposed
4523 research. The ethics review procedures in the United Kingdom are similar to those for the US IRB and for
4524 this reason will not be reiterated in this section.

4525 **United Kingdom regulations for genetically modified organisms**
4526 The Department for Environment, Food & Rural Affairs and ACRE have responsibility for oversight of
4527 GMOs. ACRE's advice is forwarded to regulators for consideration in evaluating clinical trials (or
4528 marketing authorization) for GMOs. If the vaccine or challenge organism could be considered to be a
4529 GMO, then the need for consultation with or filing to these bodies should be determined.

4530 **Efforts toward regulatory convergence on human challenge studies for vaccine development**

4531 Despite varying regulatory procedures in different countries, WHO has provided a considerations paper to
4532 guide regulatory convergence on the subject of human challenge studies: *Human Challenge Trials for*
4533 *Vaccine Development: Regulatory Considerations* (885). In addition to this guidance, the International
4534 Alliance for Biological Standardization has hosted four conferences on the subject of human challenge
4535 studies for vaccine development in concert with various co-sponsors over the past six years: in
4536 Strasbourg, France, in 2014 (886); in Rockville, Maryland, United States, in 2017 (887); in Langen,
4537 Germany, in 2019 (888); and in Oxford, United Kingdom, in 2020 (889). The meeting reports from these
4538 conferences provide recommendations, conclusions, and summaries of the current state-of-the-field in
4539 terms of regulatory, ethics, and scientific thinking on this subject.

4540 **Regulatory and ethical considerations for the various applications of human challenge studies to**
4541 **vaccine development**

4542 Clinical studies in which subjects are intentionally given a virulent or attenuated challenge organism in a
4543 controlled manner may be performed for different purposes and, thus, with differing study designs. These
4544 studies may be exploratory in nature. They may also support the development of a challenge model and
4545 the establishment of its parameters. Other studies may be similar to conventional clinical trials of
4546 candidate vaccines in many respects. Vaccine clinical trials that incorporate a challenge model may be
4547 considered Phase 1, 2, or 3 and might even be performed as Phase 4 studies in certain circumstances.

4548 A developer may conduct human challenge trials to accomplish one or more of a number of aims. The
4549 aims of the study determine in what clinical phase the study may be considered to be. Human challenge
4550 trials are often a type of efficacy study, but not all would be considered a Phase 3 pivotal study. Human
4551 challenge trials can have multiple purposes. These purposes could include, but may not be limited to
4552 characterization of the challenge stock and model system (titration, symptoms, kinetics, shedding,
4553 transmissibility, etc.); gaining a clearer understanding of the pathogenesis of and immunity to the
4554 organism in order to guide decisions on what (type and/or quantity of) immune responses a vaccine might
4555 need to accomplish to protect against that disease (i.e., to gain insight for vaccine design); and
4556 identification of potential immune correlates of protection (which would then require validation in a
4557 traditional efficacy study). The use of human challenge models to explore vaccine efficacy can identify an
4558 optimal trial design for Phase 3 traditional efficacy trial(s) (e.g., case definitions, endpoints, study design
4559 aspects); generate appropriate hypotheses to be formally tested in traditional efficacy trials; and establish
4560 proof of concept that a particular vaccine candidate might be capable of providing protection. They can be
4561 used for de-risking or "left-shifting"[a] risk of failure in a vaccine development program, as well as down-

a. In a theoretical vaccine development timeline with early-stage development depicted on the left-hand side and late-stage or advanced development depicted on the right-hand side, shifting the risk of failure earlier (or to the left) in the timeline could result in significant cost (and resource) savings and could minimize opportunity costs by abandoning an unpromising candidate prior to assuming greater expenditures of later-phase clinical trials. Doing so would also minimize the risk to human subjects by avoiding large efficacy studies of non-efficacious vaccines.

4562 or up-selecting among various potential lead vaccine candidates to advance only the best to large Phase
4563 2b or 3 efficacy trials and to eliminate those that are unworthy of advancement. Additionally, human
4564 challenge trials can be used for comparison purposes, as when comparing vaccine performance in
4565 endemic settings versus in efficacy trial populations,[b] including evaluating impact of prior immunity or
4566 comparing investigational vaccine performance against a licensed vaccine that is suboptimally efficacious
4567 (e.g., suboptimal durability, inadequate strain coverage, low efficacy) when a head-to-head trial might be
4568 infeasible (e.g., sample size, attack rate, etc.). Finally, human challenge trials can be used to support
4569 emergency use of an investigational vaccine (e.g., in a pandemic); establish a basis for licensure (this
4570 purpose would generally be an exception rather than the rule); and to explore post-licensure, whether or
4571 not immunity to vaccination wanes and if or when booster doses might be required for durable
4572 protection.[c]

4573 Not all challenge studies or models would support accomplishing each of the aims above. For example, if
4574 the human challenge model system does not adequately mimic the wild-type disease and/or the
4575 populations the vaccine would need to protect, then a human challenge trial would likely not be usable as
4576 a primary basis for licensure. It might, however, still serve one or more of the other purposes above or
4577 could be considered by regulators as supporting evidence for licensure.

4578 In designing any human challenge study, consideration must be given to the study's aim and its role in
4579 vaccine development. Furthermore, the overall regulatory strategy for developing a given vaccine
4580 candidate or regimen should reflect the role the model might play in that development. In planning an
4581 overall regulatory strategy for development, consideration should be given to whether the time and
4582 resources required to develop and validate the human challenge model (if not already available) need to
4583 be offset by the degree of left-shifting that could occur in the vaccine's development timeline. Also, some
4584 vaccine companies may be concerned that a human challenge model that is not particularly predictive or
4585 relevant from the perspective of testing vaccine efficacy could inaccurately indicate vaccine failure,
4586 which would stifle further development of a vaccine candidate that still has promise. Therefore, the task
4587 of developing human challenge models generally falls to public interests, since private enterprises often
4588 fail to see benefit when comparing to the model's development costs and resource intensiveness and may
4589 balk at the perceived risks to vaccine development. The development of a human challenge model, by a
4590 public organization that may be willing and able to share the model with multiple industrial vaccine
4591 developers, could significantly encourage the development of vaccines against diseases for which they
4592 currently do not exist or are difficult to develop and license. Of particular note are diseases for which
4593 many vaccine candidates have been tried and failed, those considered too difficult to be addressed by
4594 vaccination, or those for which development was determined to be unprofitable. Further, in a setting of an
4595 emerging disease for which a large number of vaccines are being developed simultaneously, it may be
4596 impossible for all candidates to perform large efficacy studies, so down-selection to the most promising
4597 few through use of a challenge model might be the most efficient and speediest path to vaccine
4598 development.

b. Target population(s) in endemic areas may differ in important respects to volunteers in human challenge studies, which include prior exposure to the pathogen that might impact the immune response elicited by a vaccine, different nutritional status and gastrointestinal flora, and human genetic markers that could influence vaccine performance. Therefore, the results of a human challenge study may not reproduce results that would otherwise be observed in field trials in endemic regions.

c. Post-licensure exploration might entail a human challenge study in adults to extrapolate when children might need booster doses.

4599 **Manufacture, characterization, and maintenance of the challenge organism**

4600 Since the challenge organism will be the subject of regulation through an IND application in the United
4601 States, expectations are that the challenge organism will be manufactured and characterized as would any
4602 medicinal product for human use (current Good Manufacturing Practice [cGMP] expectations will likely
4603 be very similar to those for Phase 1 products). Although the challenge organism is not a medicinal
4604 product, it will be given to humans and, therefore, should be relatively safe and pure, of correct identity,
4605 and of suitable quality for use in humans. This means that many, though not all, considerations for
4606 manufacturing, testing, and characterizing a vaccine product apply to the challenge organism. These
4607 considerations entail some aspects of manufacturing according to cGMP and documenting the
4608 manufacturing and testing so that a chemistry, manufacturing, and controls section of an IND application
4609 may be prepared for regulatory review. While the challenge organism may cause an acceptable level of
4610 disease symptoms in the study volunteers, harm resulting from some impurity or unsuitable contents
4611 would be unacceptable (risks should be minimized, to be ethically used in humans).

4612 Ideally, a sufficient quantity of the challenge organism stock could be prepared to permit its use in a
4613 number of challenge studies. Such studies could be designed to characterize the stock in humans, for the
4614 trials for which it was prepared and for use by multiple developers if the stock is to be a standardized
4615 challenge for testing multiple developers' vaccine candidates. In some situations, propagating a human
4616 challenge organism in culture or using it directly from a frozen stock may not yet be possible. Some
4617 challenge inoculates require fresh propagation immediately preceding their use as a challenge. In these
4618 cases, cGMP compliance may be quite difficult. For some bacterial challenges, for example, material
4619 scraped freshly from an agar plate might be used. For other challenges, material prepared from infected
4620 humans may need to be the source of the challenge material. Whatever the case, regulators should be
4621 consulted and agreement gained on the planned manufacture and testing in advance of its use in a human
4622 challenge study.

4623 The FDA's *Guidance for Industry: CGMP for Phase 1 Investigational Drugs* (877) contains guidance on
4624 challenge organism manufacturing. While the guidance permits a Phase 1 product to be generated in a
4625 laboratory rather than a cGMP-compliant manufacturing facility, the intent of this portion of the guide is
4626 really aimed at autologous cell therapies (which are always made in very small batch sizes for a single
4627 individual). In some situations, when challenge material is made fresh in a small batch for a given study,
4628 discussion should be held with the FDA in advance about whether or not this part of the guidance could
4629 apply to a challenge organism. To prepare a sufficient batch size for the purposes of a standardized
4630 challenge stock and with suitable containment, an appropriately designed manufacturing facility will most
4631 likely be needed. Since establishing a dedicated facility for producing a particular challenge stock is
4632 unlikely, considerations in FDA guidance about multi-use facilities should be addressed. In fact,
4633 regulatory expectations for control (including cleaning procedures and validation, environmental and
4634 personnel monitoring, and change-over procedures) of a multi-use facility are higher than for a dedicated
4635 facility where the risk of cross-contamination of the product would be substantially lower. Therefore,
4636 although process validation is not expected for Phase 1 products, certain validations would be required for
4637 a multi-use facility, even if used to produce material for Phase 1 investigations (890).

4638 The challenge organism, once developed, will require banking so the challenge used in each trial is
4639 homogenous and consistent, just as would be required of a vaccine. Although using a two-tiered banking

4640 scheme may be unnecessary, as would be done for cells or vaccine seeds, a “master” seed should be
4641 generated at a minimum and banked by storage in appropriate containers and temperature conditions (e.g.,
4642 frozen in the vapor phase of liquid nitrogen). This master seed may be used as the challenge stock for
4643 each human challenge study itself or it may be used to seed the production of each lot of challenge stock
4644 made (if fresh material is required each time). Periodic re-titrating of the seed material may be required,
4645 preferably *in vitro* if possible, to ensure the seed is stable under storage conditions. Ideally, a stability
4646 program or something akin could be planned for the challenge organism seed stock. Monitoring the
4647 viability and titer each time a vial(s) of seed is removed for the purpose of generating a fresh lot of
4648 challenge material may be suitable. Trending of these data can aid in monitoring storage stability.

4649 In some cases, the challenge organism may have been genetically altered either by attenuation (growth of
4650 a wild-type organism in cell culture, passage through a non-host species, or some other manner) or by
4651 recombinant DNA techniques. In other cases, a wild-type organism might be used. In either case, there
4652 may be concern about the organism’s genetic stability. Particularly for genetically altered organisms,
4653 assurance that the characteristics determined for that organism are retained when used in a human
4654 challenge study requires assessment of genetic stability. Whole genome sequencing as evidence of genetic
4655 stability may be encouraged or required by regulatory agencies. Passaging the organism several times
4656 under conditions like those used in production is desirable, as is characterizing the genetic stability of any
4657 organisms recovered from humans that have been challenged should they shed the organism. While this
4658 latter issue is not strictly one of GMP, genetic stability during production of both the material to be used
4659 in the human challenge study and the organism in humans are critical attributes to characterize in order to
4660 ensure subject safety and potentially for environmental containment.

4661 Characteristics upon which a manufacturer would release a lot of medicinal product to the clinic (or the
4662 market) include safety, purity, potency, identity, and quality. While these characteristics do not translate
4663 directly one-to-one between a vaccine and a challenge organism, some control over attributes that would
4664 lead to the use of a challenge organism in the clinic is needed.

4665 While “safety” may seem an inappropriate attribute for a virulent challenge organism, in fact safety and
4666 purity are intertwined. An impure material could be unsafe. Particularly, one needs to address if the
4667 challenge organism could be contaminated with other materials that would render it more unsafe or
4668 unsafe in unanticipated ways beyond the expected virulence of the challenge organism. Testing should be
4669 performed to know whether other micro-organisms could be present in the challenge material or if
4670 endotoxins are present. Testing for process residuals may be important if the challenge organism is grown
4671 in the presence of antibiotics, antibodies, inducers, or other unwanted substances in the final material.
4672 This kind of testing is also important if downstream processing steps are used to purify the challenge
4673 organism, which could introduce substances undesired in the final material (e.g., cesium from a cesium
4674 chloride gradient used to purify a viral preparation).

4675 While the concept of “potency” may not directly apply to the challenge organism, a measure of content or
4676 titer of the challenge organism needs to be known for dosing purposes. While the human titer of the
4677 challenge organism requires characterizing the material in the human challenge model, an *in vitro* (or
4678 animal) titer should be determined for each lot as a criterion for releasing the material to the clinic. For
4679 example, for viruses, a determination of plaque forming units or a 50 percent tissue culture-infectious
4680 dose should be made. For bacteria, colony forming units may be a useful measure. In other cases,

4681 organisms may be enumerated by microscopic examination, often in conjunction with reagents that
4682 increase the sensitivity of detection.

4683 The ability to identify the challenge organism is crucial for confirming the vial (or other container type)
4684 of material to be used for a human subject actually contains the challenge organism and not some other
4685 product or organism produced in the same facility. For these reasons, an identity test of some type is
4686 needed. Quality measures that might be important might include, but not be limited to, appearance, pH,
4687 osmolality, or viscosity.

4688 **Pathogen strain selection and clinical assessment following challenge**

4689 Many human challenge models include the development of disease symptoms after challenge. In some
4690 cases, no specific treatment for the disease symptoms exists and symptoms resolve naturally due to the
4691 human immune response. In many cases, however, treatment for preventing or ameliorating disease
4692 symptoms is an integral component of the challenge model. In the latter case, determining *in vitro* or in
4693 animals if and to which antimicrobial drugs the challenge organism is susceptible and/or resistant is an
4694 important and current practice. For example, a bacterium used for challenge should be susceptible to well-
4695 tolerated and frequently used antibiotics and not require an antibiotic to which allergies are frequent. For
4696 challenge with a virus-like influenza, using a strain that is sensitive to oseltamivir or baloxavir could be
4697 important. Susceptibility to the antimicrobial should be normal and not require higher than normal doses
4698 or longer treatment times to cure the subject of the challenge organism.

4699 Clinical case ascertainment may be important to demonstrate that symptoms of illness are due to the
4700 challenge organism and not some concomitant infection. Trial subjects could have been exposed to either
4701 the same or a different disease organism before enrolling in the trial or during the trial itself if not
4702 confined to inpatient settings. Specimens should be taken not only to define kinetics or assess shedding
4703 and cure, but also to determine whether the challenge organism and no other organism is present in the
4704 relevant anatomical compartment. For example, if a subject has diarrhea, then stool specimens should be
4705 collected and analyzed. For respiratory symptoms, collecting and analyzing sputum samples or nasal
4706 washes may be necessary.

4707 A severity scale for the disease endpoints that will be key to defining the impact of vaccination to
4708 ameliorate disease in the human challenge model should be defined. The range of severity seen without
4709 intervention in the initial characterization of the challenge model may need to be compared to the range of
4710 severity seen following vaccination for those vaccines that do not prevent all disease symptoms. Of
4711 course, a balance between the severity of the disease caused by the challenge and the safety of the human
4712 volunteers enrolled must be met while also maintaining scientific integrity of the study. In human
4713 challenge studies, the development of disease symptoms is often limited to the mild end of the spectrum
4714 of disease that the pathogen causes in natural infection and should generally avoid severe disease
4715 symptoms. For these reasons, in many cases human challenge studies of candidate vaccines will be
4716 limited to an assessment of the vaccine to ameliorate mild symptoms, with the hope that limiting mild
4717 disease will extrapolate to more severe disease in natural infection. Ideally, human subjects should be
4718 treated to cure the disease as soon as they express reliable disease symptoms or markers on which to
4719 assess impact of vaccination. Again, the ethical precept of minimizing risk applies.

4720 **Clinical site health and safety requirements**

4721 In addition to manufacturing under an appropriate biosafety level (BSL), the clinical trial may need to be
4722 conducted with biosafety in mind. If the challenge organism or the vaccine is a recombinant organism and
4723 if the challenge organism is virulent or not endemic in the clinical trial location, subjects may require
4724 housing in a quarantine unit during the trial and for the duration of shedding, if any. Waste generated in
4725 the quarantine unit, including excretions that might normally flow into the sewer system, will likely need
4726 to be decontaminated or incinerated to prevent the spread of a virulent challenge organism or a
4727 recombinant organism. Using a quarantine unit with negative air pressure to contain respiratory or
4728 airborne pathogens may be necessary.

4729 Defined laboratory BSLs range from 1 to 4, with 1 being rudimentary and standard laboratory practices
4730 and 4 being full containment, including wearing personal protective gear and respirators that prevent
4731 contact between the laboratory scientist and the disease organism. For details on these BSL procedures,
4732 consult the WHO *Laboratory Biosafety Manual* (891). Most organisms that might be used for a human
4733 challenge study would likely be BSL2, which entails but is not limited to restricted access with posting of
4734 biohazard signage; standard laboratory personal protective gear such as a laboratory coat, safety glasses,
4735 and gloves; availability and use of biosafety cabinets; and access to an autoclave for decontamination
4736 purposes.

4737 Laboratory BSL guidelines do not strictly translate to a clinical trial setting. Instead, consideration must
4738 be given to whether the trial should be conducted as an outpatient trial with no additional precautions, an
4739 outpatient trial with additional precautions (e.g., bandaging to cover a site of inoculation from which an
4740 organism might shed), or as an inpatient trial in a quarantine unit. Most vaccine trials are conducted on an
4741 outpatient basis with no specific additional precautions beyond informing the trial subject to possibly
4742 avoid contact (for live vaccines) with children, pregnant women, or immunosuppressed people. In some
4743 cases (such as when investigating a recombinant poxvirus), the site of inoculation or scarification might
4744 be bandaged, and careful instructions given for changing the bandaging and returning the bandaging
4745 materials to the trial site for decontamination and destruction. However, human challenge studies may
4746 require more stringent biosafety controls.

4747 Many human challenge studies are performed in quarantine to ensure that volunteers are closely
4748 monitored, clinical data are accurately collected, and care and treatment is administered during the course
4749 of the study. Quarantine also reduces (and hopefully eliminates) the possibility of spreading the virulent
4750 or recombinant organism, which could be excreted, respired, or secreted by trial subjects. However, not
4751 all human challenge studies require such containment. Closely monitoring the trial subjects on an
4752 outpatient basis and providing them with clear instructions and a means of rapid communication (e.g.,
4753 mobile phone) could be possible. In particular, if the challenge organism is a vaccine (like bacillus
4754 Calmette-Guérin for tuberculosis) or a vaccine candidate that was perhaps under-attenuated and the
4755 organism itself is endemic or substantially similar to an endemic organism, then outpatient trials may be
4756 performed. Also, if the challenge organism is endemic, an outpatient trial may be considered, even if the
4757 organism is virulent. If the challenge organism cannot be spread person to person readily, consideration
4758 may also be given to an outpatient setting with precautions even if the disease is not endemic. Inpatient
4759 quarantine would be expected if the challenge organism is not endemic, is substantially different from
4760 endemic strains, causes substantial symptoms of illness that require round-the-clock monitoring, and/or is

a recombinant organism that would be considered a GMO that could be released into the environment inappropriately. Challenged individuals might be required to remain at the clinical trial site following challenge, not necessarily for containment but for frequent trial endpoint monitoring and specimen collection.

Furthermore, conducting the human challenge study in a facility that uses negative air pressure might be necessary so that air flows into the unit from outside, containing pathogenic respiratory or airborne challenge organisms inside the quarantine unit and preventing their escape into the outside environment. Consideration must be given to the ability of the challenge organism to be excreted or secreted from the trial subject into their environment, including onto or into other people. If the challenge organism might be spread person to person by close or casual contact, the potential for transmissibility might be high. In characterizing the human challenge model, this possibility should be explored, as some individuals in an inpatient trial may not get challenged but may become infected from interacting with challenged individuals within the inpatient setting. Although this scenario does not take place in every human challenge study, the possibility of it occurring makes it worth addressing. Overall, transmissibility must be presumed and accounted for in the study design whenever a live organism is used. A clear exception is if the disease organism cannot be spread person to person. If the vector for spread (e.g., mosquitos) is present in the locale of the trial site (i.e., the catchment area from where the trial subjects would be selected), then transmissibility should still be presumed.

In the event that materials used in both inpatient and outpatient studies become contaminated with the challenge organism, such biohazardous waste must be handled with precautions and decontaminated before disposal into municipal waste. An organism that may be spread through urine or feces into the municipal sewer system must be collected and decontaminated before sewer system or standard waste disposal if the organism is not endemic. Challenge organisms such as enteric pathogens that can cause diarrhea must be contained by ensuring that all feces and diarrheal fluids are collected in some type of container rather than allowed to flow into the sewer. These excretions must be decontaminated prior to disposal. Such precautions are in current practice.

Clinical trial staff and anyone working at the clinical trial site (especially a quarantine unit) could be at risk of exposure to infection with the challenge organism. Consideration must be given to staff health and safety regardless of the staff member's role (e.g., a clinical trialist, a janitor responsible for cleaning and removal of waste materials, or someone bringing meals to subjects contained in-house). Appropriate precautions should be taken to prevent spreading the challenge organism to anyone susceptible to infection with it. Such precautions may include, but not be limited to, wearing personal protective gear, regular health screenings, and vaccination if a licensed vaccine is available.

Risk assessments and standard operating procedures for human challenge studies

Informal or formal risk assessments that identify potential hazards, characterize risks, and plan for and develop risk management strategies should be considered for both manufacturing and clinical studies. The International Organization for Standardization or the International Council for Harmonisation describe useful tools for conducting formal risk assessments. Commonly used risk assessment tools for manufacturing include Failure Mode Effects Analysis and Hazard Analysis and Critical Control Points, which could lend themselves to either quantitative analysis or semi-quantitative analysis (i.e., giving relative weights to ranks such as high, medium, and low). One such semi-quantitative tool gives ranks of

4802 1, 2, and 3 and multiplies scores assigned to the probability of a given event's occurrence, the probability
4803 of promptly and actionably detecting the event if it occurs, and the severity of the event's impact. Higher
4804 scores require more attention in the risk management plan. Whatever tool is used, the purpose of the risk
4805 assessment is to consider various scenarios in which a hazard may occur and to make decisions about how
4806 best to mitigate or manage such risks. Planning overlapping fail-safes whenever feasible is prudent.

4807 With any regulated environment, operating according to standardized and documented procedures that
4808 have been approved by an oversight group (e.g., a quality assurance unit) is imperative. SOPs are
4809 expected not only for manufacturing the challenge organism (essential components of GMP), but also for
4810 clinical practices, including, but not limited to, the challenge procedure itself. To ensure reproducible
4811 effects in challenges, a documented standardized procedure should be put in place as part of the
4812 development of the human challenge model. Personnel involved in such procedures must be trained on
4813 the SOPs and the training documented for regulatory review and oversight. A human challenge model
4814 may involve dozens of SOPs to cover all facets of the model. These SOPs need to be prospectively
4815 written and approved, complied with, and documented to prove that either no SOP deviations occurred or
4816 that deviations that did occur were held subject to investigation and corrective/preventive action. Once the
4817 human challenge model is established, future challenges will be governed by the SOPs written during the
4818 development process.

4819 **Ethical considerations**

4820 By their very nature, human challenge studies have unique ethical considerations and confront our ethical
4821 precepts about trial participation. In general, human challenge studies are undertaken for generalizable
4822 knowledge rather than for direct benefit to the participants. If the challenge study includes a vaccine, the
4823 knowledge gained may directly increase the probability that an efficacious vaccine will be developed; yet,
4824 this benefit is not likely to be directly linked to participation in a human challenge study, since many
4825 diseases for which human challenge models have been developed are not endemic in the countries where
4826 challenge studies are typically performed or are not relevant to the populations participating in the studies
4827 (e.g., pediatric infections). Due to the ethical considerations associated with human challenge models, the
4828 risk to participating volunteers must be kept to the absolute minimum required for such models to have
4829 any utility in advancing vaccine development. Since the risks of participating in human challenge models
4830 are highly pathogen specific, any assessment of how to control these risks must be specific to the
4831 challenge model in question.

4832 One of the most important considerations is whether the utility of the challenge model depends on the
4833 development of disease symptoms. This is generally the case for the human challenge models in current
4834 use, with some notable exceptions, like the sporozoite challenge model for malaria. A human challenge
4835 model should not be acceptable if it uses a challenge strain of a pathogen that causes serious symptoms or
4836 conditions that do not resolve naturally and cannot be cured with a therapeutic intervention. Human
4837 challenge studies cannot use an organism that has significant potential to lead to chronic symptoms or
4838 other long-term sequelae, nor can they use subjects considered to be vulnerable (such as children;
4839 pregnant or lactating women; immunosuppressed or autoimmune individuals; individuals with diminished
4840 capacity to provide informed consent; or other such persons with abnormal risks, like asthmatics and
4841 transplant recipients). Therefore, the current practice is that only healthy adults fully capable of giving
4842 informed consent can be considered for enrollment in a human challenge study and that practice should

4843 continue. A notable exception is pediatric studies using licensed live-attenuated vaccines with well-
4844 established safety profiles (e.g., rotavirus or poliovirus) as challenge agents.

4845 Worth acknowledging is that different individuals are willing to accept risk to differing degrees. Some
4846 people are risk averse and others are risk-takers. While a human challenge study should not expose
4847 subjects to undue risks, the level of risk is higher than in a typical vaccine trial. Individuals such as
4848 firefighters, military personnel, extreme sports enthusiasts, skydivers, and police officers may accept
4849 more risk in life than others—sometimes for the greater good of humanity or sometimes for their own
4850 personal gain or enjoyment. Given that a human challenge study should be designed with the goal of
4851 obtaining knowledge that will aid the greater good, many individuals would likely accept the risks of such
4852 a trial. Therefore, stating *a priori* what level of risk is acceptable in a human challenge study can be
4853 difficult.

4854 Nonetheless, the ethical precept of beneficence requires that researchers minimize risks and maximize
4855 benefits. While the risks required in a human challenge study are generally anticipated, the study should
4856 be developed to minimize those risks as much as possible while maintaining scientific integrity.
4857 Consideration must be given to potential individual benefits and risks, as well as to potential societal
4858 benefits and risks (such as release into the environment of a pathogen that might not otherwise be
4859 present). In a clinical trial, ethics provisions are made for situations in which there may be greater than
4860 minimal risk but little (or no) potential for individual benefit. In these cases, knowledge may be gained
4861 that will be of benefit to the larger societal population with whom the potential trial participant shares
4862 significant characteristics. Asking trial participants to accept the risk from a challenge can be compared to
4863 the justifications that support inclusion of placebos in controlled clinical trials.

4864 While all clinical trials require thorough informed consent procedures, human challenge studies require an
4865 informed consent process that ensures that individuals enrolling in the trial understand they will be
4866 intentionally infected with an infectious disease-causing organism and that they are likely to get ill and
4867 suffer a predictable level of disease symptoms. While all trials must minimize risk and maximize benefits,
4868 these studies may require individuals to experience higher risks than other trials. Participation in human
4869 challenge studies cannot be considered an everyday risk even though the infectious diseases the
4870 investigators are attempting to model are circulating in the human population. Despite the highly
4871 controlled environment and high standard of care provided for volunteers, intentional inoculation with a
4872 pathogen carries a potentially greater risk to a participant than that expected from natural pathogen
4873 exposure. Participants must understand this. The recommendation is that some means of assessing
4874 participant understanding of the information conveyed during the informed consent process be utilized in
4875 a human challenge study (e.g., a pre-study quiz that must be passed with a very high percentage of correct
4876 answers).

4877 Ensuring the study sponsor has a way of insuring trial participants that experience study-related injuries is
4878 important. Although every precaution should be taken in a human challenge study to ensure that
4879 individuals suffer no more harm than is scientifically necessary to gain a credible result from the study,
4880 eventualities may occur in which a trial participant experiences more harm than foreseen and that study
4881 procedures could not prevent. While this should be a rare exception, the possibility nonetheless exists and
4882 necessitates insurance. This would be true whether or not the trial uses an investigational vaccine
4883 candidate. The vaccine itself would put trial participants at risk of unforeseen adverse events. In a human

4884 challenge study that uses an investigational vaccine candidate, the separate risks inherent in the
4885 administration of the candidate vaccine and in the challenge with the pathogen should be separately
4886 communicated and distinguished in the information provided to study volunteers.

4887 In most cases, a human challenge study should involve treatment to either cure or, at a minimum,
4888 ameliorate disease symptoms. Bacterial diseases can generally be treated with antibiotics. For some
4889 parasites and viruses, anti-infectives and antiviral drugs, respectively, are available. For diseases that have
4890 no available treatment, palliative therapy to minimize severity and relieve symptoms should be available
4891 to trial participants as part of the study. For example, with diarrheal diseases, fluid and electrolyte
4892 replacement must be available for use as needed. For febrile illnesses, antipyretics should be available for
4893 use. This is consistent with current practices.

4894 **Practical and ethical considerations of controlled human infection model studies in endemic regions**

4895 While the vast majority of controlled human infection model (CHIM) studies have been conducted in
4896 high-income countries (HICs), in recent years there has been increasing interest in conducting such
4897 studies in endemic regions, including low- and middle-income countries (LMICs). While such studies
4898 heighten sensitivity to ethical concerns, there are also important benefits to be obtained, and with proper
4899 oversight and precautions, it is possible to conduct them ethically. One benefit is that in the context of
4900 vaccine development, residents of endemic regions may have different immunological responses to
4901 vaccines compared to residents of HICs, who are typically immunologically naïve (892). This could occur
4902 because of either increased exposure to the corresponding pathogens due to living in an endemic region,
4903 or alternatively because of genetic differences. Another emerging factor is that the microbiota of
4904 endemic-region residents differs substantially from residents of HICs (893), and these differences may
4905 result in differential susceptibility to disease as well as differential vaccine responses (894, 895). Already,
4906 CHIM studies have been conducted in endemic regions, including with malaria (896–899), cholera (900,
4907 901), and shigellosis (902, 903). Additional studies are in progress or will be initiated within the next few
4908 years (904).

4909 One of the major concerns about conducting challenges in LMICs is the needed availability of state-of-the
4910 art expertise, equipment, and facilities to diagnose and be able to treat any unexpected safety outcomes to
4911 the highest possible standards. This has been partially addressed by the fact that currently used challenge
4912 agents have been tested before in HICs, where all potential unexpected outcomes and complications can
4913 be rapidly addressed.

4914 Current experience has indicated that early and robust engagement with local communities, public health
4915 officials, and regulatory authorities is crucial for acceptance and success (905). Ideally, the principal
4916 investigator for such a study should be a respected local investigator, rather than someone from outside
4917 the community. Ideally, these studies should also be approved by ethical review committees from both the
4918 local region as well as the sponsor's or developer's home country (assuming this is an HIC). In some
4919 cases, informed consent may involve not only individual volunteers for the study, but also local
4920 authorities or the members of the local community (906). Frequent communications about risks and
4921 benefits of the study should be delivered to local community members through multiple channels to
4922 ensure the utmost transparency. Financial compensation and the potential for inducement must be
4923 considered in LMIC settings even more carefully than in HICs (907). CHIM studies in endemic settings

have the potential to accelerate the development of efficacious vaccines and drugs that benefit these communities, but they must be approached cautiously and with a high sensitivity to local conditions.

Summary

The regulatory and ethical considerations for human challenge studies are numerous. While some are common to any clinical study of a candidate vaccine against an infectious disease, some are unique due to the specific nature of human challenge studies. International regulatory authorities lack harmonization as to whether human challenge studies that do not involve an investigational medicinal product (such as a vaccine candidate or new drug) require submitting a request for clinical trial authorization from an NRA. The Unites States has a requirement to file an IND application, which brings with it many considerations, such as the appropriate application of some level of compliance with GMP to the manufacture and control of the human challenge stock. In addition, scientific considerations for the validity of the human challenge model and the reliability and credibility of the data to be derived during its use invoke additional regulatory expectations. Standardized procedures are imperative for this purpose.

Special facilities and potential requirements of containment (e.g., inpatient quarantine units) may be necessary. A risk assessment may aid in considering what potential risks a human challenge study might pose so that researchers can strive to minimize risks. Ethical considerations are particularly key in a human challenge study, and only fully informed and consenting healthy adults should be considered for enrollment. These expectations are reflected in current human challenge study practices and should continue. Ultimately, the scientific integrity and design of the human challenge study is what drives decisions, but this must be balanced with the need to minimize risks and maximize benefits to study participants. Only data derived with credibility and reliability will be acceptable for regulatory decision-making. Studies performed with scientific rigor, while reflecting the pragmatic needs of studies performed in humans, should provide the necessary credibility and reliability.

Conclusion and recommendations

The fundamental processes by which vaccines are being developed have come under scrutiny. With the exception of the extraordinary success achieved by COVID-19 vaccines, over the years candidate vaccines entering advanced clinical development offer a low probability of success. In contrast, the success of COVID-19 vaccines was driven by the urgency of a global pandemic, unprecedented resources allocated to their development, unprecedented sharing of data between scientists prior to publication, and the decades-long foundation of basic research understanding mRNA and other vaccine platforms (adenovirus, protein subunits, inactivated viruses) and coronaviruses on which the clinical development was built. Further, taking vaccines through several development steps is a lengthy process even if some of the studies can take place in tandem. We have considered in this report the possibility that the expanded development and use of human challenge models could provide, in a shorter time than required for efficacy trials, data that are more predictive of the efficacy of candidate vaccines in large field trials. Our overall conclusion is that human challenge studies can clearly play a more pivotal role in vaccine development than they do today. In the following sections, the recommended steps that may be taken to realize the full potential of human challenge studies to support and advance vaccine development are described.

Overarching needs for the field of human challenge studies

Human challenge models can be improved in the following ways virtually across the board. These measures could be implemented individually for each different challenge model. Whenever it is possible to identify groups of challenge models with similar needs, efficiencies will be gained by involving a range of experts across different diseases to maximize opportunities for shared learning.

These improvements include the increased standardization and validation of study procedures, data collection, and data analysis, as well as the enhanced availability of more numerous, updated, and relevant challenge strains. Clinical trial registries such as ClinicalTrials.gov could play an important role in standardizing procedures for data collection and reporting. Human challenge models would benefit from better defined clinical endpoints for vaccine studies, and increased use of endpoints that can be directly applied in subsequent field trials. Standardized endpoints are critical for challenge studies with naïve volunteers, as they can facilitate translation to real-world applications in vaccine recipients with some immunity from prior natural infection. More focused and comprehensive studies to identify correlates of protective immunity using conventional immune assays and new technologies will improve the development of vaccines overall. It is critical to plan for longer-term follow-up of volunteers for challenge studies in which there is any known potential for post-infection sequelae or precedence for failure to clear the challenge strain from volunteers at study conclusion. Better integration of vaccine studies conducted with human challenge models into the overall development plans for candidate vaccines, including criteria to specify which results will curtail further development will result in significant savings in time and development costs. One pitfall is challenging with a dose of pathogen that is higher than physiologically relevant and thus setting an unreasonably high bar, resulting in termination of a vaccine that would provide protection against natural exposure. Finally, it is necessary to develop systematic approaches to efficiently address the anticipated regulatory and ethical concerns that may accompany the proliferation of human challenge studies.

4988 **Critical evaluation and recommendations to increase the capacity of human challenge models to**
4989 **support vaccine development**

4990 Each of the human challenge models described in this report has a unique set of characteristics that must
4991 be considered to determine what role the model can play in vaccine development. These characteristics
4992 include the depth of experience with the model, the availability of appropriate challenge strains, their
4993 ability to mimic natural infection, the capacity to adequately manage the risk to volunteers, and the
4994 potential to gather data with the model that can clearly advance vaccine development for the disease in
4995 question. Some models clearly have inherent obstacles to further development, and in these instances, it
4996 may not be technically feasible to substantially improve the model. Such models have limited relevance to
4997 natural infection and disease and consequently may have a smaller role to play in the development of
4998 vaccines, compared to conventional clinical trials. The requirements for a challenge model may vary
4999 according to the stage of vaccine development to which it will be applied. A lot depends on the number of
5000 volunteers that will participate in challenge studies with the model, and on the quality of data the model
5001 will need to provide in order to fulfill its purpose for a given study. A case-by-case assessment is offered
5002 for each challenge model, along with concerns and recommendations for further development, if any, that
5003 have arisen from document review and expert consultations conducted for this report. The basis for the
5004 summary recommendations provided in Table 38 is further described in the subsequent paragraphs.

Table 38. Summary recommendations for the continued development and utilization of human challenge models for 17 infectious diseases.

Diseases	Challenge agents	Recommendation from the literature review
Malaria	*Plasmodium falciparum*	Proceed. Few concerns identified, if any.
Cholera	*Vibrio cholerae*	
Pneumococcus	*Streptococcus pneumoniae*	
Rotavirus	Live oral rotavirus vaccine	
Poliomyelitis	Oral poliovirus vaccine	
Influenza	Influenza strains pertinent to the development of universal influenza vaccines	
Typhoid/Paratyphoid	*Salmonella* Typhi and *Salmonella* Paratyphi serovars	
ETEC	ETEC strains expressing selected toxins and colonization factors	Proceed with caution. Minor concerns identified.
Shigellosis	*Shigella flexneri* serotypes and *Shigella sonnei*	
Norovirus	Norovirus genogroups and genotypes	
RSV	RSV strains of Genogroups A and B	
Dengue	Attenuated dengue virus serotypes 1 through 4	
Malaria	*Plasmodium vivax*	Address major concerns before proceeding.
Campylobacteriosis	*Campylobacter jejuni* serotypes	
Tuberculosis	Bacillus Calmette-Guérin	
Pertussis	*Bordetella pertussis*	
Cryptosporidiosis	*Cryptosporidium parvum* and *Cryptosporidium hominis*	
COVID-19	SARS-CoV-2	

Abbreviations: COVID-19, Coronavirus disease 2019; ETEC, enterotoxigenic *Escherichia coli*; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

5005 We identified no significant concerns with the malaria challenge model using *Plasmodium falciparum*
5006 challenge. Our findings recommend the continued use of the sporozoite model, increased utilization of the
5007 blood-stage model, and development of a transmission-blocking model to enable development of new
5008 malaria vaccines against multiple stages of the parasite life cycle. However, major concerns with the
5009 malaria challenge model using *Plasmodium vivax* need to be addressed before proceeding. A
5010 recommendation for this model is to decrease risk for challenge participants by replacing field strains
5011 with a cloned challenge strain of known susceptibility to antimalarial drugs, even though doing so could
5012 be more difficult than what has been done for *P. falciparum* due to the lack of a cell culture system for
5013 growth of the parasite. Additional steps are also recommended to minimize or eliminate the potential for
5014 recrudescence of liver-stage *P. vivax* in study volunteers, either by genetically modifying a cloned
5015 challenge strain to eliminate potential for dormant liver-stage parasites or by verifying the capacity of
5016 drug regimens to clear liver-stage parasites at study completion, or both.

5017 We identified minor concerns with the challenge models for dengue that should be monitored as these
5018 studies move forward. A recommendation is to limit the use of challenge strains attenuated through serial
5019 passage in tissue culture until a fuller understanding is gained of their potential to cause severe disease in
5020 adult volunteers without pre-existing immunity to the pathogen. The genetically attenuated challenge
5021 strains do not raise significant concerns for severe disease, and their continued use is recommended. The
5022 potential for severe disease in natural infection with a heterologous serotype following primary infection
5023 is well documented. Re-challenge studies or simultaneous challenge with heterologous serotypes of
5024 dengue viruses should be avoided with both genetically and tissue culture-attenuated strains until
5025 additional data indicate that this has no potential to induce serious disease outcomes in study participants.

5026 We identified no significant concerns with the challenge model for cholera. Cholera bacteria are non-
5027 invasive for the human gastrointestinal tract and supportive care in the context of a challenge study and
5028 antibiotic treatment can effectively minimize any significant risk of severe disease. The cholera challenge
5029 model should continue to be fully utilized and continue to focus on the serogroup O1 El Tor biotype,
5030 since it is responsible for the majority of cholera outbreaks worldwide (including the recent severe and
5031 protracted outbreaks in Haiti and Yemen).

5032 There are only minor elements of concern with the human challenge model for enterotoxigenic
5033 *Escherichia coli* (ETEC), which relate to the need for additional reagents and the types of vaccines that
5034 may be evaluated, rather than to the challenge model itself. Like cholera, ETEC are non-invasive for the
5035 human gastrointestinal tract. There is need for further characterization of recently developed challenge
5036 strains expressing heat-stable toxin (ST) but not heat-labile toxin, to support development of vaccines that
5037 target ST. ST is not immunogenic in humans because it mimics two human peptide hormones. Ensuring
5038 that candidate vaccines that seek to render ST more immunogenic have no potential to invoke an
5039 autoimmune response in human volunteers is important.

5040 There is one element of concern with the *Shigella* human challenge model that warrants two
5041 recommendations for improvement. *Shigella* is invasive for the human gastrointestinal tract, which raises
5042 the level of vigilance required to maintain the safety of volunteers. The concern relates to the potential for
5043 serious post-infection sequelae, which can occur at low frequency in natural infection. These sequelae

5044 include hemolytic uremic syndrome, irritable bowel syndrome, and reactive arthritis. Human challenge
5045 studies use antibiotic treatment early in the course of disease development to mitigate this risk, but the
5046 residual potential for post-infection sequelae in human challenge studies is not known. Longer-term
5047 follow-up of volunteers should be considered and the data should be reviewed by experts not directly
5048 involved in the studies. The *Shigella* human challenge model could be improved by (i) gaining a fuller
5049 understanding of the relationship between challenge dose and attack rate for disease development and
5050 (ii) developing new challenge strains that represent more of the prevalent *Shigella flexneri* serotypes.

5051 We identified several major concerns with the human challenge model for *Campylobacter*. The most
5052 important is the clear, but incompletely defined, potential for serious post-infection sequelae in adult
5053 volunteers. These include Guillain-Barré syndrome, irritable bowel syndrome, and reactive arthritis.
5054 Antibiotic treatment of study volunteers undoubtedly mitigates this risk to some extent, but the
5055 observation that some volunteers experienced recurrent disease episodes after study completion despite
5056 treatment raises concerns. The selection and evaluation of *Campylobacter* challenge strains is another
5057 area of concern. The potential for Guillain-Barré syndrome arises from ganglioside mimicry, in which the
5058 bacteria produce a carbohydrate capsule that invokes an immune response that cross-reacts with human
5059 nerve gangliosides. Strains with this potential have been disqualified as challenge strains, but these
5060 bacteria may still have the potential to mimic other human carbohydrates. One strain of *Campylobacter*
5061 that mimics human blood group antigens has been described. Phase variation is another challenge facing
5062 the human challenge model for *Campylobacter*. The range of carbohydrates expressed in the
5063 *Campylobacter* capsule depends on growth conditions. That additional carbohydrates that mimic human
5064 antigens are expressed upon infection of humans, but not under bacterial culture conditions, is possible.
5065 Therefore, *in vitro* screening assays applied to the carbohydrates that *Campylobacter* produces cannot be
5066 sufficiently comprehensive to fully rule out the possibility of mimicry for the full range of human
5067 carbohydrate antigens. The current challenge strains have apparently been safe, but they do not represent
5068 the globally prevalent strains. If additional human challenge studies with *Campylobacter* are performed,
5069 continuing the long-term follow-up of volunteers will be essential.

5070 *Salmonella* Typhi and *Salmonella* Paratyphi serovars are invasive for the human gastrointestinal tract, and
5071 they have the potential to establish a long-term carrier state. In human challenge studies with these
5072 organisms, prompt antibiotic treatment has been successful in preventing both severe gastroenteritis as
5073 well as establishment of a carrier state in volunteers; therefore, we have no significant concerns about this
5074 model. Severe disease and carriage should continue to be closely monitored in future studies with the
5075 *S.* Typhi and *S.* Paratyphi challenge models. The potential for *Salmonella* Typhimurium and *Salmonella*
5076 Enteritidis serovars, the causative agents of invasive non-typhoidal *Salmonella* disease in infants and
5077 immunocompromised individuals in Africa, to cause severe disease in immunocompetent adults is
5078 incompletely understood. This lack of information regarding disease pathogenesis precludes the
5079 establishment of challenge models for these organisms at the present time. Improvements are needed with
5080 respect to the *S.* Typhi challenge strain, which is decades old and should be replaced with a contemporary
5081 isolate. Experience with the newly developed *S.* Paratyphi challenge strain is limited and use of the
5082 human challenge model with this strain should be expanded cautiously.

5083 We identified minor concerns with the human challenge model for norovirus, related to challenge pools
5084 that are purified directly from infected humans. Recent successes with growing norovirus in tissue culture
5085 should permit derivation of cloned viruses that can be manufactured under Good Manufacturing Practice

5086 (GMP). The focus on the GII.4 genotype strains is appropriate and should be expanded, since this
5087 serotype is responsible for the majority of infections. The use of the GI.1 serotype strains should also
5088 continue.

5089 We identified major concerns with the human challenge model for *Cryptosporidium*. The most significant
5090 is the recent decision from the US Food and Drug Administration that *Cryptosporidium* oocysts for use in
5091 a controlled human infection model study must be produced in compliance with GMP. No such source
5092 currently exists and establishment of this manufacturing capacity will require substantial effort and
5093 resources. Another key factor is the importance of gaining a better understanding of the potential for
5094 pulmonary infection by airborne transmission of these organisms. If so, knowing under what
5095 circumstances this can occur is important because it would impose new requirements on human challenge
5096 studies with respect to quarantine and physical containment. A variable duration of oocyst shedding
5097 following challenge occurs, and the time period during which this takes place may need further definition.
5098 However, no transmission to household contacts was documented in the studies conducted to date. Efforts
5099 to grow *Cryptosporidium* in routine, large-scale tissue culture should be re-doubled to avoid the need for
5100 deriving challenge organisms directly from infected humans and animals. The experience with the
5101 *Cryptosporidium hominis* human challenge model is limited and should be expanded cautiously.

5102 We identified no significant concerns with the challenge models for rotavirus and poliovirus. Both use
5103 licensed live oral vaccines that have been administered in multimillions of doses. Challenge studies for
5104 both have been conducted in the target population of infants without any serious safety concerns. For both
5105 diseases, these challenge studies have the potential to advance important next generation vaccines that
5106 could significantly impact disease burden: subunit or inactivated vaccines for rotavirus and genetically
5107 stabilized novel oral poliovirus vaccines in the case of polio.

5108 There are few, if any, concerns with the human challenge model for influenza virus. The strains used in
5109 this model are seasonal, low-pathogenicity strains that are distinct from the highly-pathogenic avian
5110 influenza strains that have a high case fatality rate. The development of universal influenza vaccines
5111 would be better supported if additional challenge strains were manufactured under GMP to permit a more
5112 widely distributed and concerted effort in the United States and the United Kingdom.

5113 Only minor concerns were identified with the human challenge model for respiratory syncytial virus
5114 (RSV). It would be optimal to re-develop the RSV Genogroup A Memphis-37 challenge strain to gain
5115 approval by the US Food and Drug Administration, and to develop an RSV Genogroup B challenge strain
5116 (Genogroup B strains cause up to one-third of RSV infections worldwide). The human challenge model
5117 for RSV may have limited capacity to be instructive regarding correlates of protective immunity because
5118 (i) infection with RSV does not protect against subsequent challenge with the same strain and (ii)
5119 protection against the mild, upper respiratory disease that develops in adult volunteers may be poorly
5120 predictive of protection against bronchiolitis in infants.

5121 Significant advances have been realized in the development of the human challenge model for
5122 pneumococcus from studies conducted in the United Kingdom. The manufacturing of challenge strains
5123 representing additional prevalent serotypes would increase the capacity of the model to evaluate candidate
5124 vaccines in development, especially if these strains were to be manufactured under GMP for utilization in
5125 the United States and the United Kingdom. The clinical endpoint for the challenge model, prevention of
5126 pneumococcal carriage in the nasopharynx of adult volunteers, may not have predictive value for

5127 evaluating vaccine success in infants and young children that are most affected by severe disease. The
5128 degree to which positive results with adults in the human challenge model will extrapolate to preventing
5129 development of severe disease in infants is incompletely understood.

5130 The development of a tuberculosis (TB) human challenge model that is relevant to human infection with
5131 *Mycobacterium tuberculosis* faces many hurdles. The newly developed models using intradermal or
5132 bronchioscopic administration of bacillus Calmette-Guérin (BCG) as a challenge strain do not represent
5133 the causative organism, route of infection, or disease development as they occur in natural infection.
5134 Whether a model of improved relevance can be developed while maintaining volunteer safety is unclear
5135 at present. Meanwhile, intradermal or bronchioscopic challenge with BCG may support the development
5136 of TB vaccines designed to improve upon the licensed BCG vaccine. The development of TB vaccines
5137 that reduce the establishment of latent TB, however, is not supported by the current models, in which
5138 latency antigens are not expressed.

5139 An improved vaccine against *Bordetella pertussis*, the bacterium that causes whooping cough, is needed
5140 because the acellular pertussis vaccine in current use does not provide durable immunity. Infant baboons
5141 can be infected with *B. pertussis* and develop a disease that closely mimics whooping cough in human
5142 infants. The baboon challenge model can be used to evaluate candidate vaccines, but may be limited in its
5143 capacity to identify correlates of protective immunity because of the difficulty in extrapolating immune
5144 responses from animals to humans. A human challenge model for pertussis has recently been
5145 implemented and has the potential to complement the baboon model. The human challenge model
5146 requires that volunteers receive prompt antibiotic treatment soon after infection is established, and may
5147 therefore be limited to the evaluation of the ability of vaccine candidates to prevent *B. pertussis*
5148 colonization, rather than disease.

5149 The global pandemic of severe acute respiratory syndrome coronavirus 2 has stimulated an important
5150 discussion about the potential for a COVID-19 human challenge model. Such a model could play a role in
5151 accelerating development of vaccines to prevent COVID-19 infection and disease. The model could
5152 additionally answer key questions about the transmissibility and correlates of protection that would be
5153 more difficult to address in a traditional field study. The lack of a reliable rescue treatment has led many
5154 to conclude it would be unethical to conduct such a study at the present time. However, a vocal, growing
5155 minority has argued that the massive, unprecedented scope of the COVID-19 pandemic shifts the risk-
5156 benefit calculation and justifies conducting human challenge studies.

5157 **Forging a new community of experts in human challenge studies under the aegis of a global health**
5158 **experimental medicine network**

5159 The level of enthusiasm for the use of the controlled human infection concept in the scientific community
5160 is substantially increasing. To forge support for the concept, it may be necessary to demonstrate that the
5161 risks can be mitigated and the expected benefits will accrue. This was recently achieved for some
5162 vaccines, leading to better understanding of how they work and acceleration of their development.

5163 The expansion of human challenge studies should be approached with an abundance of caution, and with
5164 clear distinctions made among the available human challenge models according to their strengths and
5165 limitations. The expanded use of a given model is best initiated in experienced hands, and dissemination
5166 of shared learning and leadership can be provided by seasoned veterans in the field.

Considering a phased development plan in which the initial studies are conducted in a loosely constituted network may be useful before establishing an elaborate (and often bureaucratic) structure. In addition, projects in which the benefits of human challenge studies are most likely to be realized in the near term should be targeted. Twelve human challenge models are available now for which we identified no major concerns (see Table 38). From these, several groups actively working with them have joined forces to establish experimental medicine networks to capitalize on the advances made.

The vision of a new approach to vaccine development against many of the diseases of global public health importance is bold and inspiring. Momentum could build quickly as additional vaccines are tested and proven efficacious in the most affected populations, with the help, in large part, of the results of human challenge models. This approach, however, is neither suitable nor feasible for certain important diseases. For these, the traditional approaches to vaccine development must continue to be supported and applied. Where essential scientific knowledge can be gained and the safety of volunteers maintained, human challenge studies can play a greater role in vaccine development. Human immune responses to infection hold the key to vaccine development, and the controlled setting of a human challenge study can provide the means to evaluate these immune responses with a depth and sophistication never dreamed of by the developers of most of the vaccines we use today. Putting the best science behind the quest for new vaccines would be something the founder of experimental medicine, Claude Bernard, would heartily endorse.

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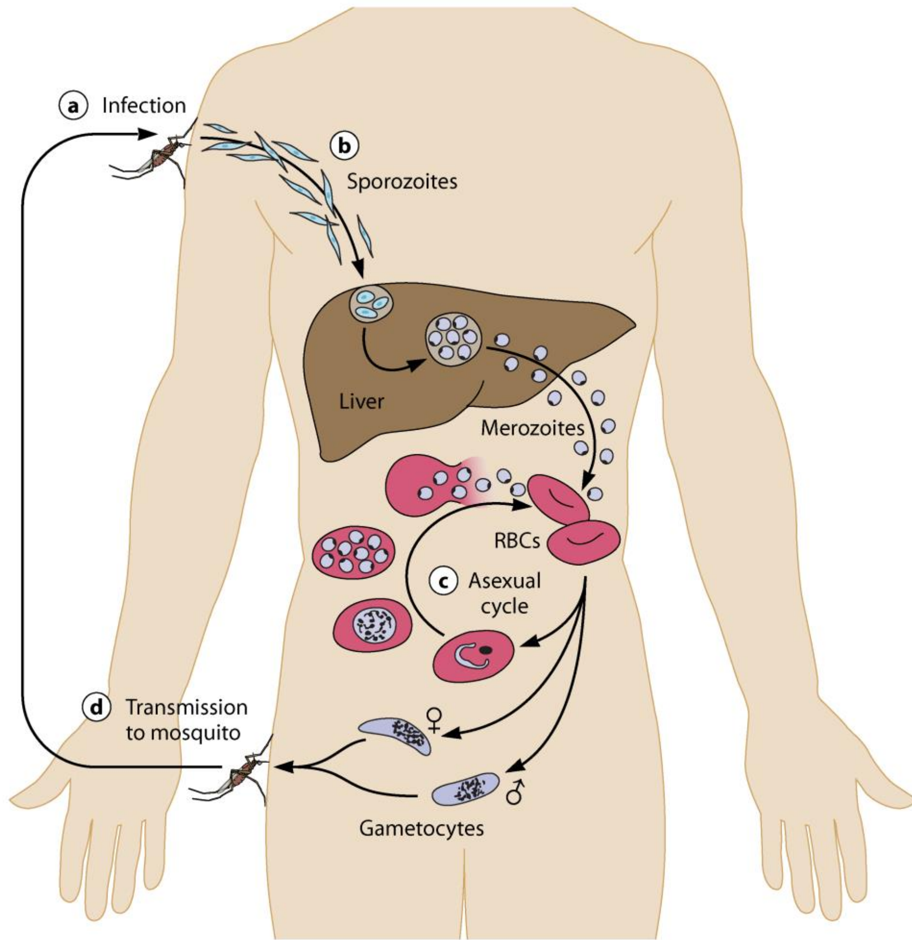

Figure 1. ***Plasmodium*** **life cycle and malaria challenge platforms.**

Plasmodium species	*Plasmodium* strain	*Anopheles* vector	Challenge model
P. falciparum	NF54/3D7 (West Africa)	*An. stephensi; An. freeborni*	Pre-erythrocytic stage sporozoite challenge, blood stage challenge, transmission challenge
	NF135.C10 (Cambodia)	*An. stephensi*	Pre-erythrocytic stage sporozoite challenge
	NF166.C8 (Guinea)	*An. stephensi*	
	7G8 (Brazil)	*An. stephensi*	
P. vivax	Natural isolate (Colombia)	*An. albimanus*	Pre-erythrocytic stage sporozoite challenge, blood stage challenge
	Natural isolate (Thailand)	*An. dirus*	
P. malariae	Natural isolate (Guinea)	–	Blood stage challenge only

Figure 2. Parasite strains and mosquito vectors commonly used in challenge models.

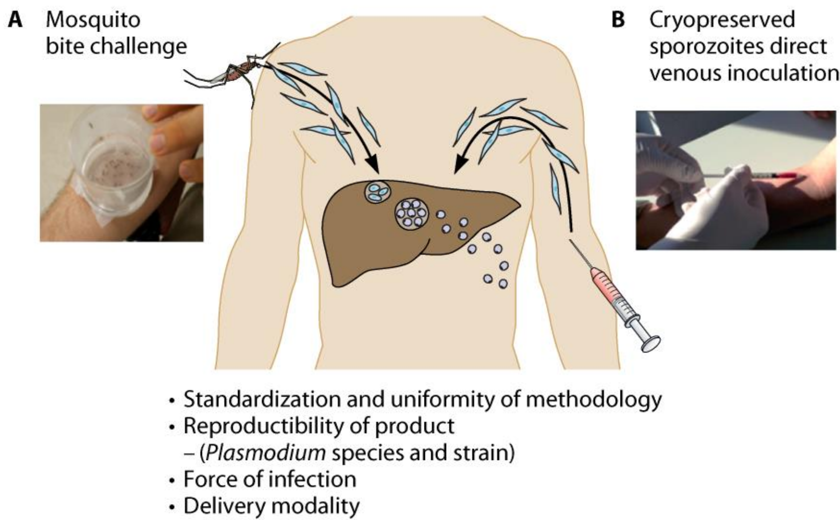

Figure 3. Pre-erythrocytic human challenge models using mosquito bite delivery or direct venous inoculation of infectious sporozoites.

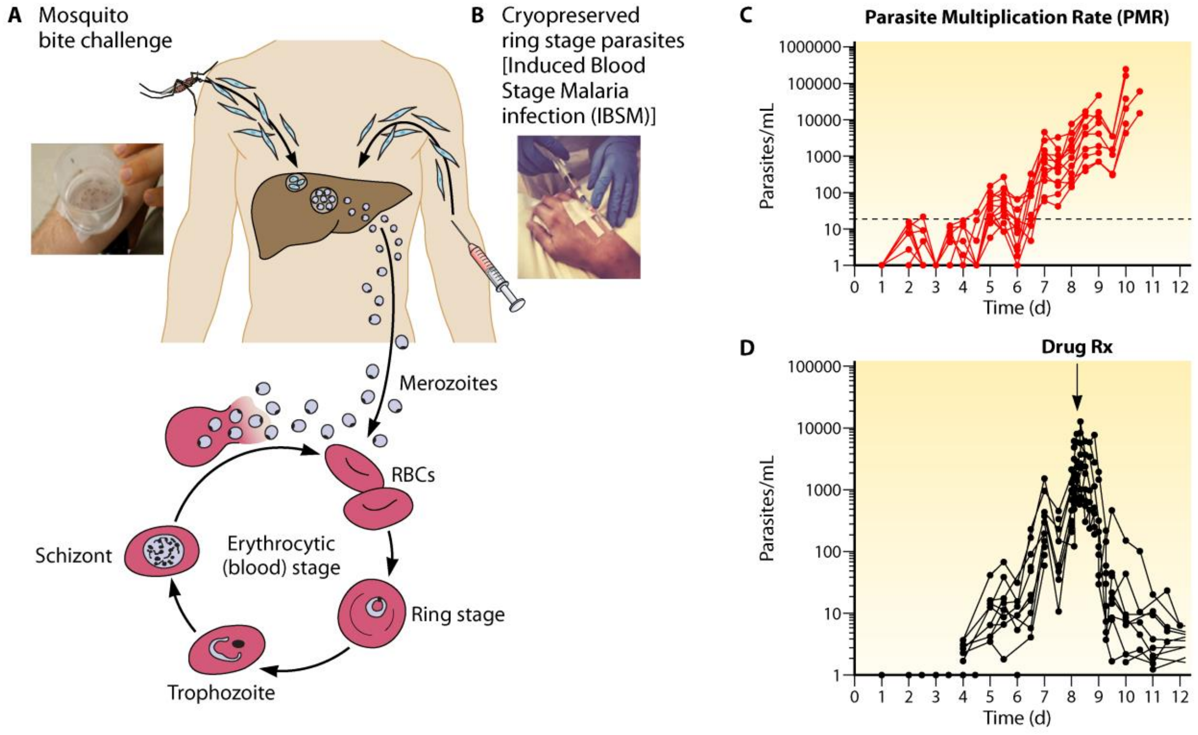

Figure 4. Blood-stage controlled human malaria infection.

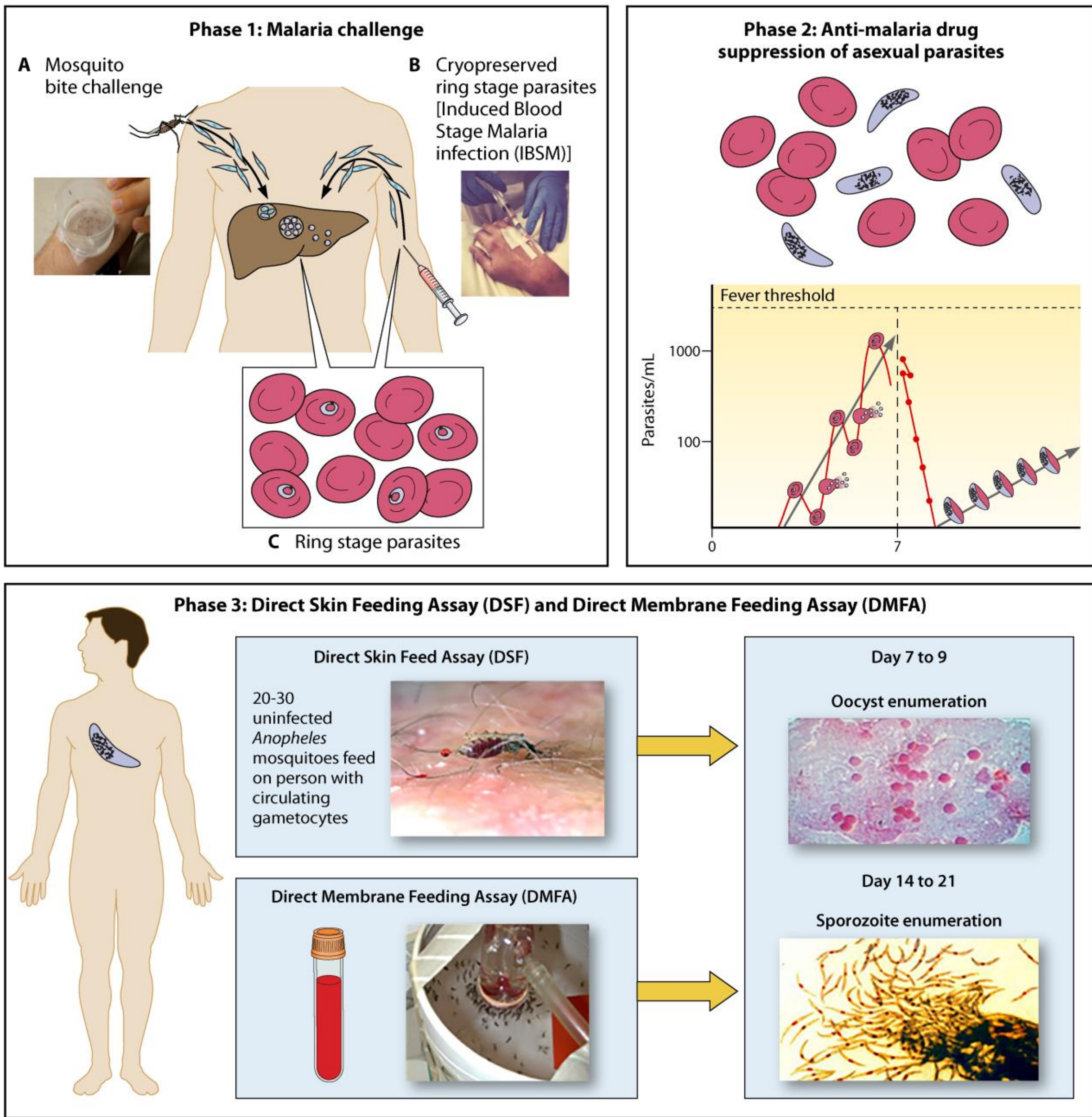

Figure 5. Transmission-stage challenge model.

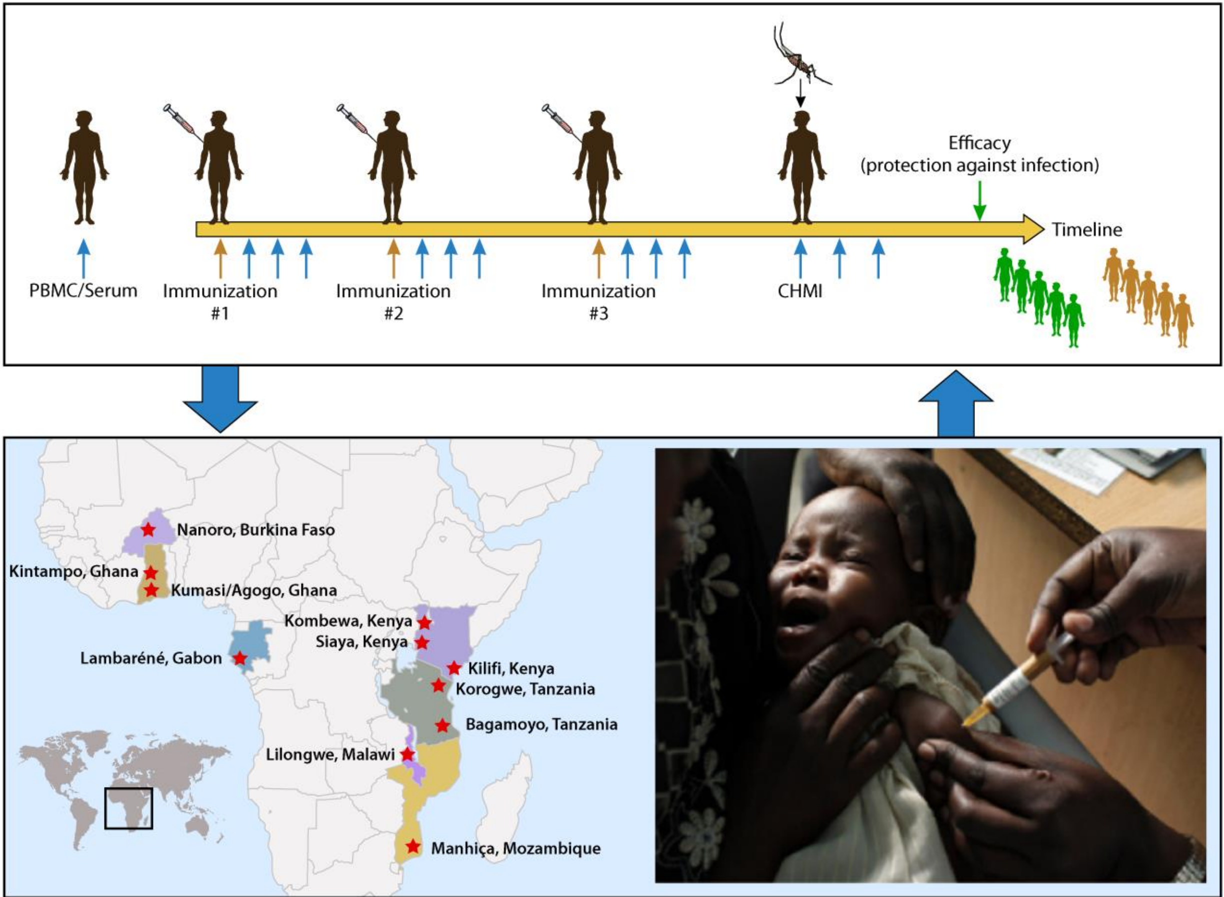

Figure 6. Malaria vaccine development from controlled human malaria infection to the field and back.

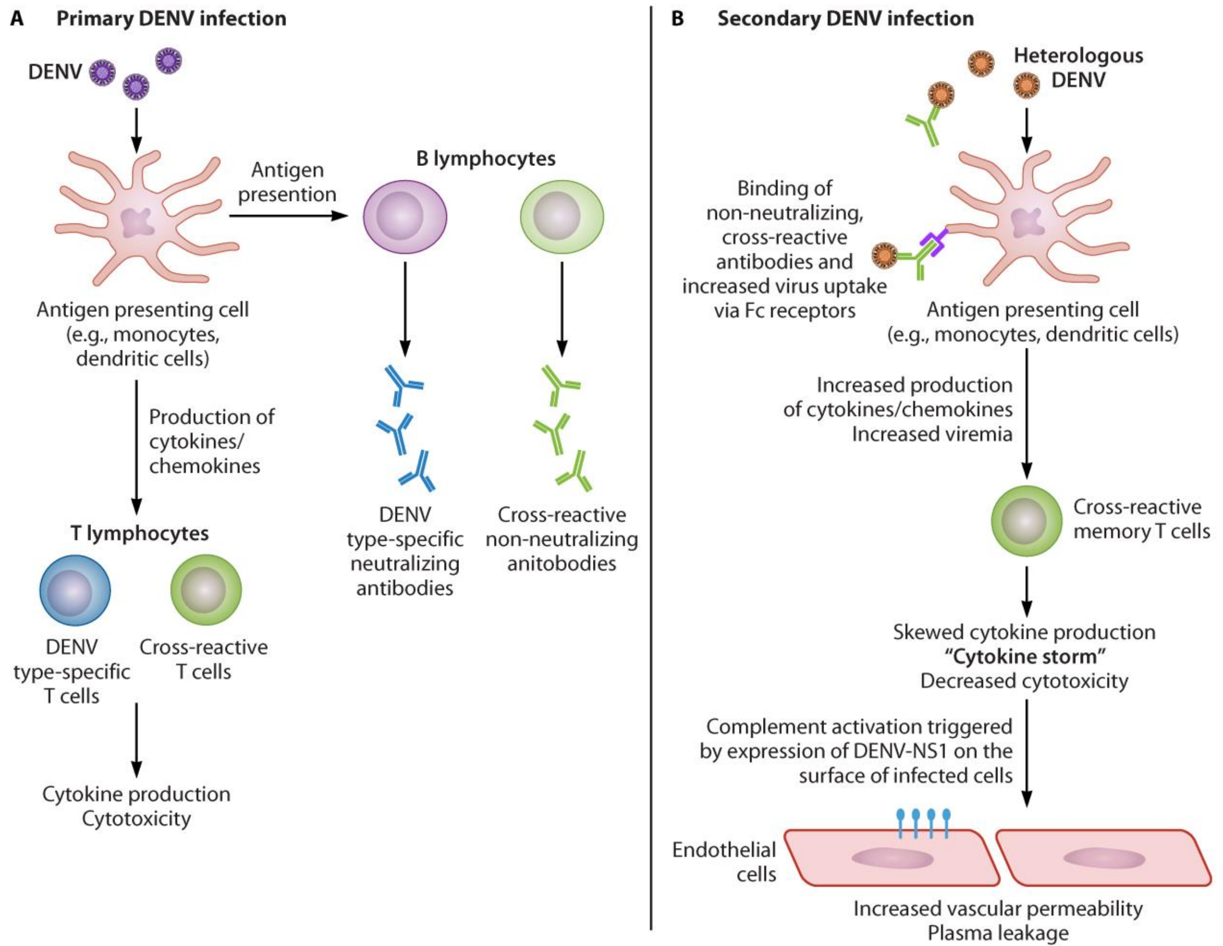

Figure 7. Potential mechanisms for immune-mediated enhancement of dengue virus infection.

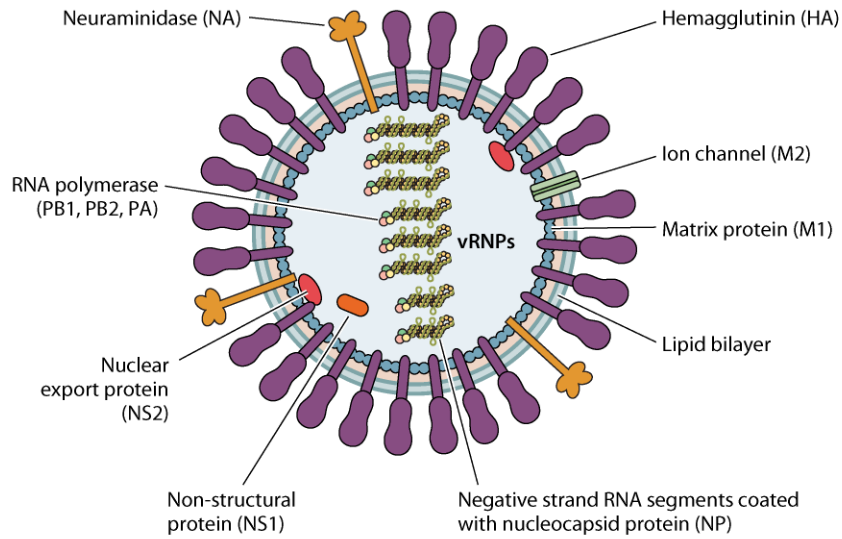

Figure 8. Diagram of influenza virus structural elements.

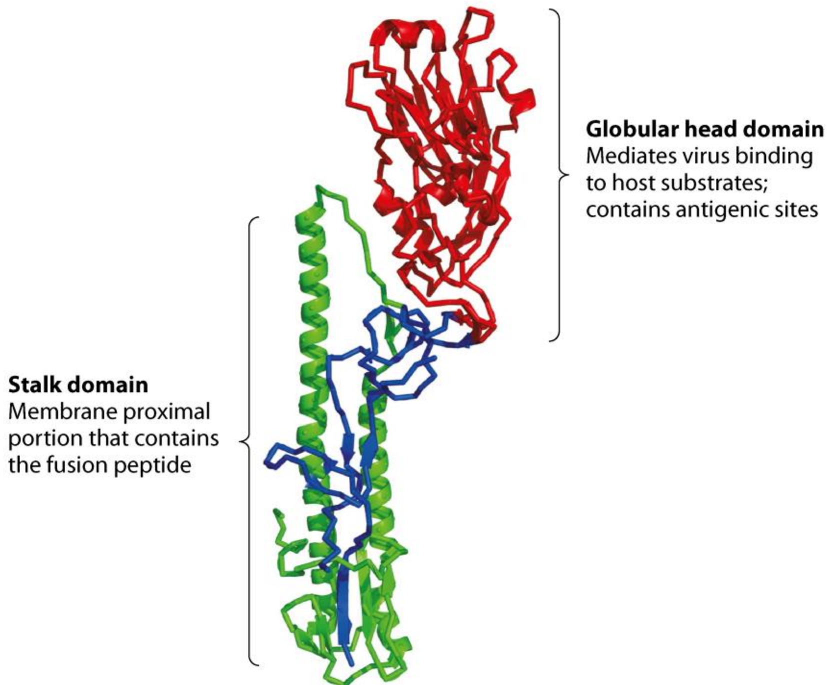

Figure 9. Structure of influenza hemagglutinin with a globular head domain (red) and elongated stalk domain (green).

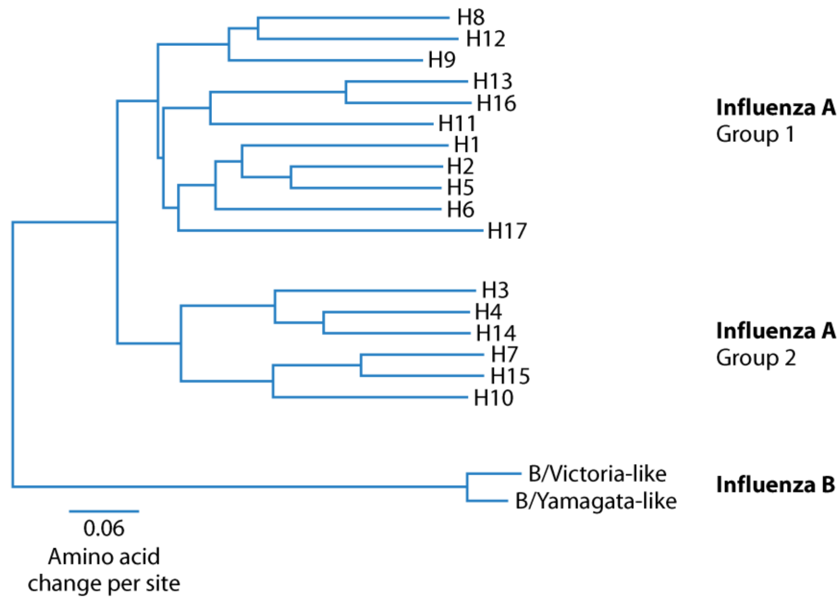

Figure 10. Phylogenetic classification of human influenza A and B viruses according to the hemagglutinin gene.

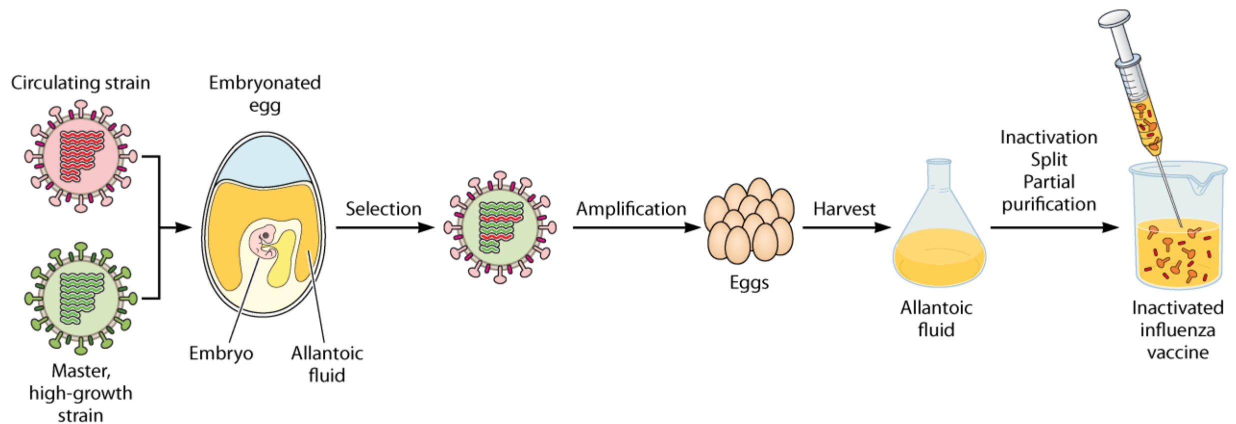

Figure 11. Inactivated influenza A virus vaccine manufacture.

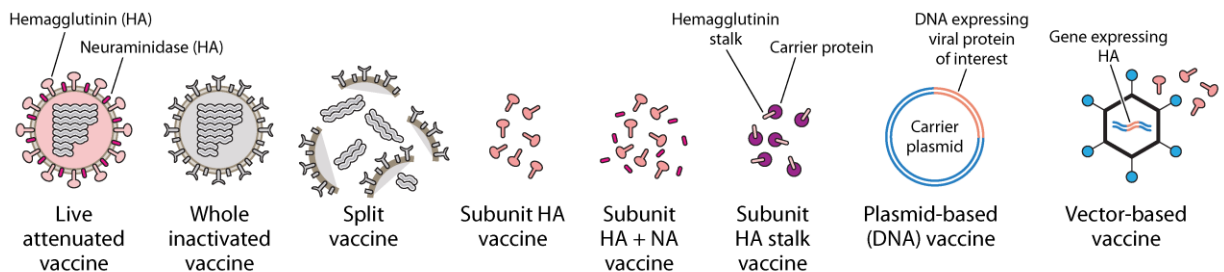

Figure 12. Range of current and new vaccine approaches against influenza A virus.

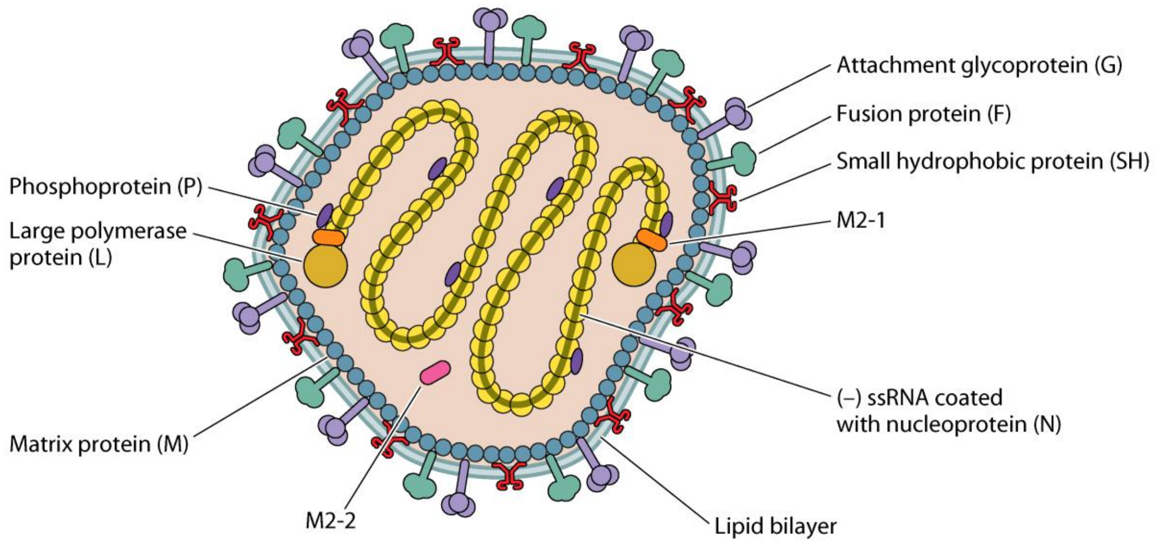

Figure 13. Structural diagram of respiratory syncytial virus.

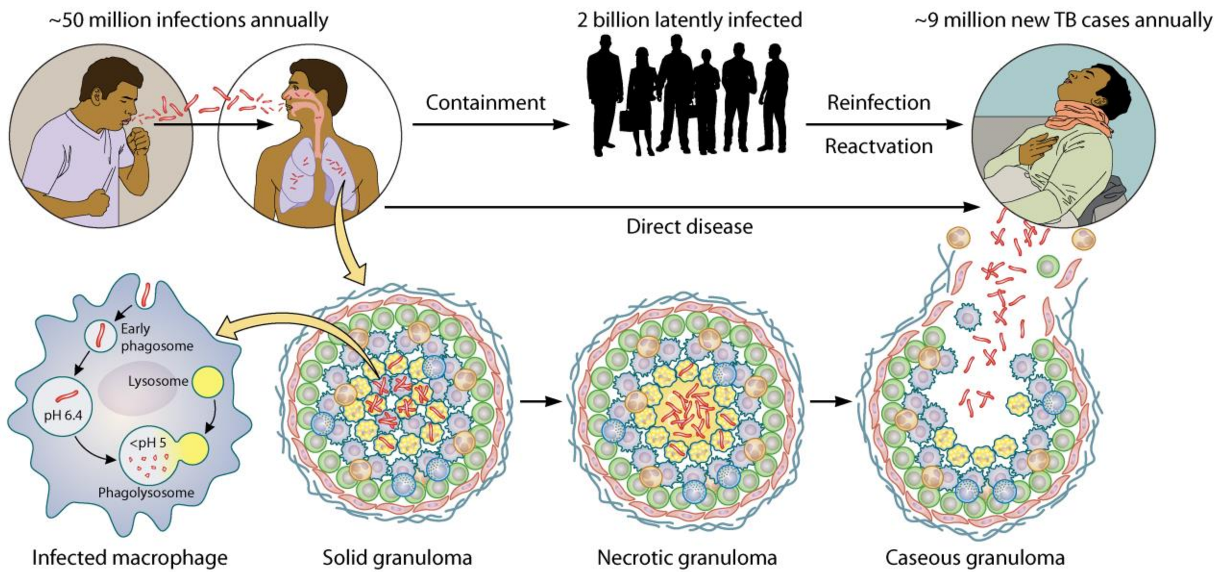

Figure 14. Transmission and pathology of tuberculosis.

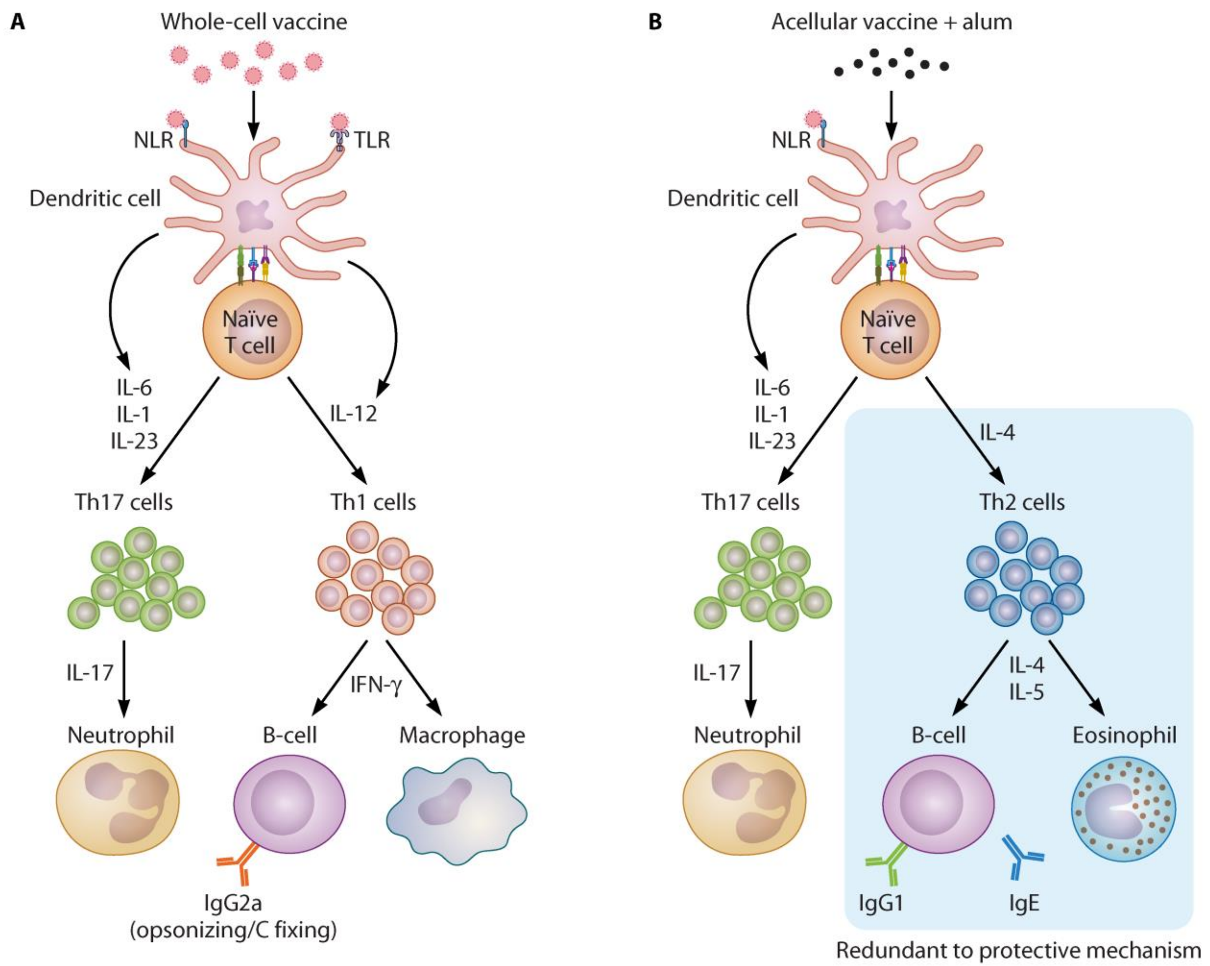

Figure 15. Distinct mechanisms of immunity induced with whole-cell and acellular pertussis vaccines.