EXPERT REVIEWS

Development of Vi conjugate – a new generation of typhoid vaccine

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Shousun Chen Szu

Eunice Kennedy Shriver National Institute of Child Health & Human Development, National Institutes of Health, 9402 Wildoak Drive, Bethesda, Maryland, MD 20814, USA
Tel.: +1 240 606 3601
SzuNIH@gmail.com

Typhoid fever remains to be a serious disease burden worldwide with an estimated annual incidence about 20 million. The licensed vaccines showed moderate protections and have multiple deficiencies. Most important of all, none of the licensed typhoid vaccines demonstrated protection for children under 5 years old. These limitations impeded successful implementation of typhoid vaccination programs. To improve immunogenicity Vi was conjugated to rEPA, a recombinant exoprotein A from *Pseudomonas aeruginosa*. Vi-rEPA showed higher and longer lasting anti-Vi IgG in adults and children than Vi alone in high endemic areas. In school-age children and adults, the immunity persisted more than 8 years. In a double-blind, placebo-controlled and randomized efficacy trial in 2- to 5-year-old children, Vi-rEPA conferred 89% protective efficacy against typhoid fever and the protection lasted at least 4 years. When given concomitantly with infant routine vaccines, Vi-rEPA was safe, immunogenic and showed no interference with the routine vaccines. Vi conjugate vaccine was also attempted and successfully demonstrated by several other laboratories and manufactures. Using either rEPA or different carrier proteins, such as diphtheria or tetanus toxoid, recombinant diphtheria toxin (CRM_{197}), the Vi conjugates synthesized was significantly more immunogenic than Vi alone. Recently, two Vi-tetanus toxoid conjugates were licensed in India for all ages, starts as young as 3 month old. This new generation of typhoid vaccine opens up a new era for typhoid prevention and elimination.

KEYWORDS: infants • typhoid fever • vaccine development • Vi conjugate • young children

The first anti-typhoid inoculation was conducted by Sir Almroth Wright in British soldiers during the Boer war in 1896 and the inactivated vaccine of *Salmonella enterica* serovar Typhi (*S.* Typhi) vaccine was licensed more than 100 years ago [1–3]. But typhoid fever remains to be a major health problem in developing countries [4,5]. Since 1990s, the emergence of antibiotic resistant strains of *S.* Typhi making treatment of typhoid fever more difficult [6–8]. Typhoid fever is both a food-borne and water-borne disease and transmission through asymptomatic carrier is also a source of infection [9–12]. With no near-term solution to clean water and environment in the heaviest typhoid stricken regions, vaccination is the most effective and economic way to control and eliminate this disease [13–14].

The three licensed typhoid vaccines available for global distribution all have limitations. The whole cell killed parenteral vaccine provided ~65% protection, but strong adverse reactions limited its usefulness. In the late 80s, two safer vaccines were licensed: the live, attenuated oral vaccine Ty21a and the parenterally administered Vi polysaccharide vaccine [15–19]. There are major differences between the two vaccines. Vi immunization is single dose and consistently demonstrated 70% efficacy in Asia or African high endemic areas, while the efficacy of Ty21a varies with different multiple dose formulations and geographic regions or even during a typhoid outbreak [19,20]. Both Vi and Ty21a provide limited immunity to children younger than 5 years of age [21].

Typhoid fever in young children was often unrecognized due to atypical clinical symptoms, difficulties in the number or volume of blood drawings and less than optimum culture

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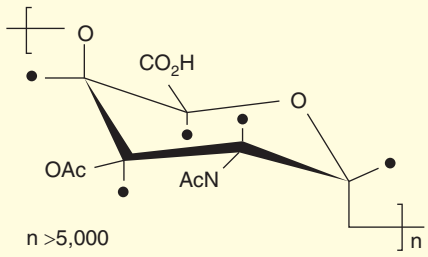

Figure 1. Vi polysaccharide repeating unit (1 → 4) α-D-galacturonic acid, *O*-acetylated 40–80% at C3 position. n > 5000.

media [22–24]. Recently with improved diagnostic techniques, increased awareness of typhoid fever in young children has been reported [24,25]. In developing countries, children <2 years old had the highest attack rate in both community- and hospital-based surveys [26–28]. For example in a hospital-based survey in Bangladesh, more than 60% of typhoid fever cases occurred in children under 4 years of age [29]. Such children would be unprotected under the recommended vaccination schedule.

Vi capsular polysaccharide is the virulence factor and protective antigen of *Salmonella typhi*. Similar to other licensed polysaccharide vaccines, such as pneumococcal, meningococcal polysaccharides and *Haemophilus influenzae* type b (Hib) conjugate, Vi vaccine functions by eliciting critical level of serum IgG at the mucosal sites and lyses pathogens upon contact [30–33]. Polysaccharide vaccines are safe and efficacious and can be administered in multivalent form without interference; for example, pneumococcal polysaccharide vaccine contains 23 and meningococcal vaccine 4 types [34,35]. Further, vaccine-induced polysaccharide antibodies confer type-specific inhibition of colonization that results in 'herd' immunity [36,37]. However, polysaccharide vaccines are T-independent antigens that do not induce booster response upon re-injection, have short duration of immunity (~3 years) and are poor immunogens in young children [21,38]. The demonstration of Hib conjugate vaccines showed that the immunogenicity of polysaccharide antigens was significantly improved by conjugating to carrier proteins [39]. In recent years, the same principle applied successfully to pneumococcal and meningococcal conjugate vaccines [40–42]. This approach has also been successfully applied to antigens from enteric pathogens such as Vi polysaccharide in young children in high endemic areas of typhoid fever [43,44].

The current review will cover the synthesis and clinical studies of Vi conjugates with emphasis on immune response in young children and infants. Detailed manufacture end points and surrogate markers for protection are identified to accelerate future Vi conjugate development, manufacture and licensing. The pre-clinical developments that are still pending clinical proof will not be reviewed here.

Vi conjugate synthesis & pre-clinical development

Physicochemical & immunological properties of Vi

The efficacy of the licensed Vi polysaccharide vaccine, its post-licensure demonstration and impact on typhoid control during the past 2 decades have been thoroughly reviewed and will not be repeated here [5,13,14,16–18].

In order to design an ideal conjugation scheme that preserves immunoepitopes, there is a need to re-examine the Vi structure at the molecular level. Vi is a linear homopolymer of (1 → 4) α-D-galacturonic acid with *N*- and *O*-acetylation at its O2 and O3 positions (Figure 1). The relationship between Vi structure and its immunologic properties had been investigated by chemical modification of the residues surrounding the galactose [45]. One of the most important factors that affect the Vi immunogenicity is the degree of *O*-acetylation that varies in different Vi preparations. For example, partial de-*O*-acetylation of the native Vi from 65 to 45% by acid or base treatment could increase Vi immunogenicity slightly. However, complete de-*O*-acetylation eliminated Vi immunogenicity entirely [46]. The study of Courtauld-Koltun space-filling model of the Vi showed that the bulky hydrophobic *O*-acetyl and *N*-acetyl groups, which protrude on both sides of the molecule, serve as immunodominant epitopes [46]. However a fully *O*-acetylated Vi showed to be rigid and the less flexibility could hinder some of its intermolecular interaction. By contrast, the carboxyl groups on Vi are less exposed and their role in Vi immunogenicity is inconsequential [46]. The carboxyl group was thus chosen as the linking site in Vi conjugation schemes.

There are other properties of Vi polysaccharide that may affect the choice of conjugation scheme: i) Vi being an aminouronic acid is resistant to acid hydrolysis and the conventional carbohydrate colorimetric assays are not applicable for quantitative determination; ii) Vi is a polyeletrolyte with high charge density, which impeded solubility in low salt solvent; iii) most of the Vi purified from *S.* Typhi has high molecular weight (estimated greater than 1 million KD), which makes gel filtration difficult. Lower molecular weight Vi can be achieved by sonication with moderate success or from selected field isolates [47].

Synthesis Vi conjugates

Vi can be conjugated to various proteins suitable for human use by several different schemes [47–50]. Briefly, based on our experience, the immunogenicity of Vi conjugate is more affected by the choice of the scheme, less so by the type of the carrier protein.

Two schemes of conjugation have been studied in clinical settings. In the first method, Vi was conjugated to the recombinant exoprotein A from *Pseudomonas aeruginosa* (rEPA) or to the B-subunit of heat labile toxin (LT-B) from *Escherichia coli* with a heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridldithis) propionate (SPDP) (Figure 2) [47,48]. Briefly, protein was derivatized with SPDP in aqueous buffer such as *N*-(2-hydroxyethyl) piperazin-*N'*-2-ethansulfonsaure (HEPES). This scheme has several advantages: all reactions were conducted near neutral pH, the progress and the completion of conjugation can be followed spectrophotometrically, the level of derivizations can be measured by colorimetic reaction, and for most proteins the reaction does not introduce protein–protein cross-linking. However, since the

final conjugate contains disulfide bridges, and is susceptible to reducing agent *in vivo*. Furthermore, this method requires modification of both the carrier protein and Vi, the extra steps could reduce the efficiency and the final yield.

The second conjugation scheme was simplified by only pre-derivatize protein with homobifunctional linker adipic acid dihydrazide (ADH) [50]. Briefly, rEPA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and the preferred level of derivatization ([AH]/[rEPA]) ranged between 2.5 and 3.1 wt/wt. Higher derivatization tends to cause protein cross-linking and gel formation during subsequent conjugation step (Figure 3). Vi was covalently linked to ADH derivatized rEPA in the presence of EDC. Investigators at the International Vaccine Institute, using Vi of lower molecular weight purified from a field isolate and were able to increase the reaction concentrations and, in term, obtained higher yield [51].

There are several other Vi conjugation methods reported, including to pre-derivatize Vi with ADH before coupling to the naked protein via EDC [40]. But this method was difficult to control due to both the high charge density of Vi and its high molecular weight and often resulted in gel formation. Another attempt was by using cross-linker *N*-hydroxysulfosuccinimide in the presence of EDC to activated Vi and also provided preliminary success when conjugated to proteins from *Streptococcus pneumococci* [52]. The final product of this method differs from the others in its close proximity between Vi and the carrier protein. This method, similar to the SPDP approach, also minimized protein cross-linking.

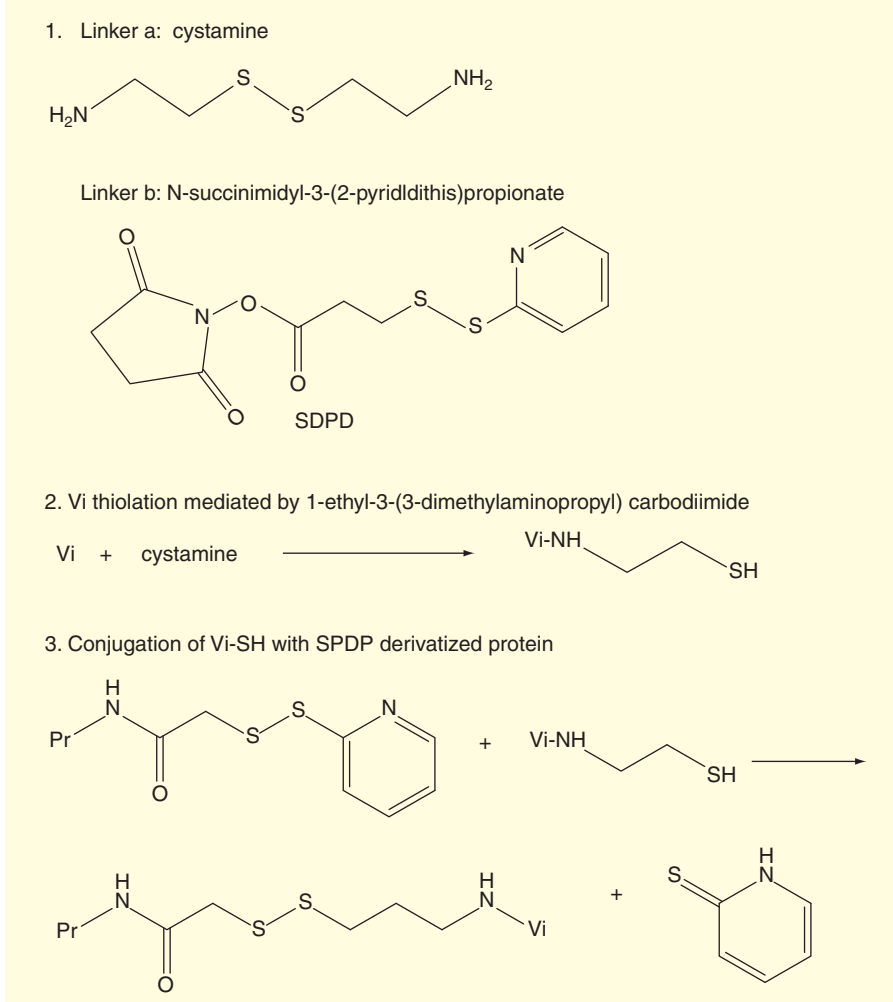

Figure 2. Schematic drawing of Vi conjugate synthesis with linker *N*-succinimidyl-3-(2-pyridldithis) propionate.

Accurate determination of Vi concentration is critical in conjugation preparations and final product formulation. Due to the extremely stable glycosidic linkage of aminouronic acids, traditional colorimetric assay for hexosamine or uronic acid that requires complete hydrolysis are not applicable for Vi. The measurement of *O*-acetyl groups by Hestrin assay has been found to be reproducible, sensitive enough for the diluted concentration in the final container and has little interference from proteins. The Hestrin results could also serve the purpose of monitoring the *O*-acetyl content to reassure the retention of the *O*-acetyl group in the final conjugate (Table 1). There are several other methods and some requires special equipment, including: proton nuclear magnetic resonance (NMR), anion exchange chromatography with amperometric detection or modified resorcinol assay on hydrolyzed Vi [45,53]. All assays, regardless of the choice, need to be close calibrated and validated against a reference Vi to warrant reproducibility.

From clinical trials studied by our laboratory and also demonstrated by other groups (*vide supra*), Vi conjugate prepared by the protein-ADH method consistently elicited higher level of anti-Vi IgG than the conjugate made with the SPDP scheme. For the rest of this review, we'll focus more on clinical studies of Vi-rEPA prepared with the ADH scheme.

Characterization of Vi-rEPA conjugate vaccine

Potency control of conjugate vaccines, unlike whole cell vaccines, can be related to specific antigenic and biochemical properties. Standardization schemes devised by control agencies have followed clinical trials of experimental vaccines as guidelines [55–57]. Here certain critical parameters based on the physicochemical and serological characterization of Vi-rEPA clinical lots are summarized to serve as benchmarks for future development (Table 1).

There were a total of five clinical lots of Vi-rEPA prepared following the same protein-ADH method [44,58–62]. The consistency of production among Vi-rEPA lots, following the model

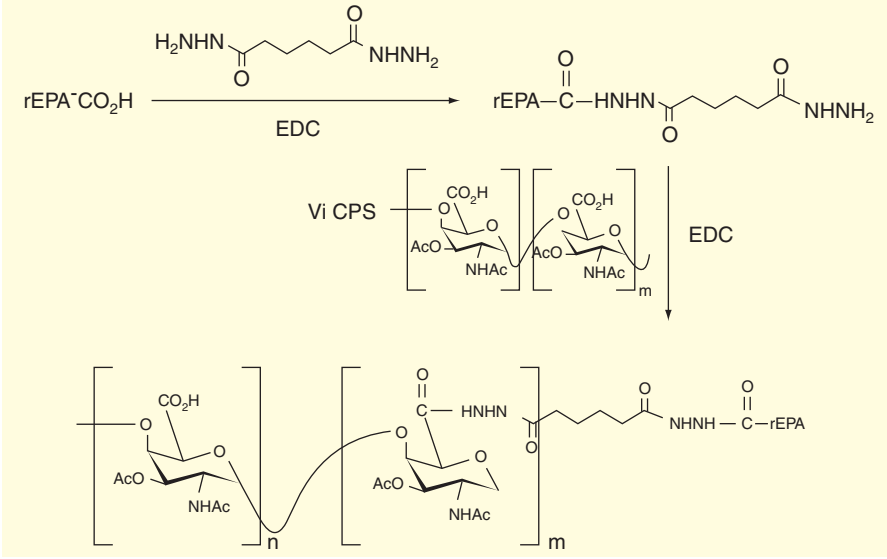

Figure 3. Vi-protein conjugation scheme with adipic acid dihydrazide derivatization proteins.

of licensed polysaccharide conjugate vaccines, was monitored by: the level of AH-derivatization in rEPA-AH, the concentration of *O*-acetyl, the range of molecular size distribution, the retention of antigenicity, the molar or weight ratio of protein-polysaccharide in the final container and the antibody response in laboratory animals. Some of the analytical results are listed in Table 1. It is worth to emphasize that one of the critical elements is the protein-polysaccharide ratio in the final conjugate, all five lots are within a narrow ranged between 0.91 and 1.17 (wt/wt).

In addition to *in vitro* potency tests, the *in vivo* immunogenicity test in suitable animal models is also useful in predicting vaccine potency. Guinea pig was initially chosen by many investigators since Vi is not immunogenic in guinea pigs and the enhanced antibody response from conjugation can be easily recognized. More commonly used animal model for Vi conjugate immunogenicity test is the weaning mouse model. Mice showed low immune response to Vi with no booster effect upon re-injection. By contrast, Vi-rEPA elicited significantly higher level of anti-Vi IgG with booster response, a signature characteristic of T-cell dependence antigen (Table 1) [50].

Each of the Vi-rEPA clinical lots passed pyrogen and general safety tests according to the Code of Federal Regulation for Investigational New Drug, US FDA and were bottled by the Pharmaceutical Development Section (PDS), Pharmacy Department, Clinical Center, NIH. The formulation of Vi-rEPA studied by the NIH group contained approximately 25 μg of Vi and equal amount of rEPA in each injection (0.5 ml in 0.1 M phosphate buffer pH 7.2 without adjuvant or preservatives). This dosage was chosen based on the licensed Vi and other polysaccharide vaccines. Several other investigators have lowered the amount of injection. The effect of dosage was re-examined in two clinical trials and will be discussed in later sections *(vide supra)* [61]. So far, all the Vi conjugates that have been clinical studied are in liquid form and cold chain storage is required. In a stability study, the NIH Vi-rEPA conjugate was stable for at least 5 years based on physicochemical characterization and mouse immunogenicity test (by comparison with the original antibody data, $p = 0.25$).

Vi-rEPA clinical development

Comparison of immune response of Vi-rEPA conjugate & Vi polysaccharide

For the purpose of comparison, Vi-rEPA synthesized by SPDS or by ADH schemes were studied in the US and Vietnam adults, respectively [49,58]. Both conjugates were safe and significantly more immunogenic than the control vaccine Vi polysaccharide. One month after the injection, SPDP-conjugate elicited approximately 23-fold rise in IgG anti-Vi in the US healthy adults and ADH-conjugate 50-fold in Vietnamese adults. The levels remained to be approximately 12-fold higher than the pre-immune levels 6 months later for both groups (Table 2). There were also significant rise in anti-Vi IgM and IgA, in a lesser extent than IgG, by both conjugates.

The persistence of IgG anti-Vi levels elicited by Vi-rEPA (ADH) in adults was examined 10 years later. The GM level of IgG anti-Vi remained significantly higher than that of the pre-immune level and all volunteers had levels above the estimated protective threshold (Table 2). The prolonged duration of antibody elicited by Vi conjugate pass up the need for re-boost every 3 years as required for Vi polysaccharide antigen.

Vi-rEPA in school-age children

A community-based survey showed high incidence of typhoid fever in children in the Cao Lanh District, Mekong Delta region, Vietnam. The highest rates were among children under 15 years of age: with 478/100,000 annually in school-age children and 358/100,000 in 2- to 4-year-old children [62]. Most *S. typhi* isolates from patients in this area were multiple antibiotic resistant. We therefore chose this site for most of the Vi-rEPA clinical evaluation and the target age group are children under 5.

The Phase II studies were conducted progressively in school-age, then in pre-school-age children with slightly different emphasis. The first study was performed in school-age children using Vi as the control, which has been shown to be efficacious (55%) for this age group [17,58]. Children recruited from elementary, middle and high schools in the Cao Lanh District were randomized to receive one injection of either Vi-rEPA or Vi. Pre-injection levels of anti-Vi IgG were significantly lower than those in adults. Six weeks after the injection, all volunteers responded with greater than eightfold rises. The GM antibody level in the Vi-rEPA group was significantly higher than those received Vi alone at 6 and 26 weeks intervals (Table 2) (58).

Table 1. Physicochemical and immunogenicity characterization of various lots of Vi-rEPA.

Clinical trial	*O*-acetyl/Vi (mmol/g)	rEPA derivatization AH/rEPA (wt/wt)	Conjugate rEPA/Vi (wt/wt)	IgG anti-Vi in mice (EU)†		Ref.
				1 inj	*2 inj*	
Phase II	3.19	2.3%	0.91	3.7	79.5	[58]
Phase III	2.97	2.5%	1.02	13.4	109.5	[59]
Crossover	2.95	3.19%	1.17	2.5	62.3	[61]
Dosage	2.95	3.19%	1.17	4.5	71.6	[44]
Infants	2.96	3.09%	1.11	1.7	66.7	[60]

†NIH general purpose female mice, 5–6 weeks old, 10/group, injected subcutaneously twice, 2 weeks apart, with Vi-rEPA contained 2.5 μg/injection of Vi. IgG anti-Vi IgG was expressed in ELISA unit (EU) using a high tittered pooled mice sera as reference. Control group was injected with 2.5 mg Vi alone and had average IgG anti-Vi = 0.45 EU (data not included in table).

Vi-rEPA in pre-school children receiving one or two injections

The most compelling need in typhoid vaccination program is an effective vaccine for children under 5 years old, where Vi polysaccharide elicited low levels of anti-Vi IgG for short duration [Szu, Robbins, Schneerson *et al.*, Unpublished Data]. Recently in a field trial in Pakistan, Vi vaccine showed no protection against typhoid fever for this age group [21].

In the second part of Phase II study, 2- to 4-year olds were randomly divided into two groups receiving either one or two doses of Vi-rEPA [58]. Six weeks after the first injection, 99.5% children had greater than eightfold rise of serum anti-Vi IgG (Table 3). Four weeks after the second injection, the GM antibody level increased from 86.7 to 118.3 μg/ml (p=0.04) and was significantly higher than those received only one injection. However, the antibody gap between one and two injections gradually diminished: at the 26 weeks, the titers were 37.9 versus 25.3 μg/ml (p = 0.2) and there was no difference at the 3 years interval (5.65 vs 5.84) (Table 3) [58].

The better immunogenicity of Vi-rEPA than Vi was again demonstrated in the 2- to 4-year-old children. At 26 weeks post-injection, the antibody levels of 2- to 4-year-old children receiving either one or two doses of Vi-rEPA were higher than those of the school-age children who received one injection of Vi.

Efficacy of Vi-rEPA in 2- to 5-year-old children

The gold standard for vaccine evaluation is a randomized, placebo-controlled, double-blind efficacy trial. Once the efficacy is established, the corresponding serologic response to the antigen can be used to estimate the antibody protective level and serve as benchmark for future reference. The efficacy trial of Vi-

Table 2. Kinetics of anti-Vi IgG levels elicited by one injection of Vi or Vi-rEPA in adults and 5- to 14-year-old children.

Age group	Time post-immunization	Anti-Vi IgG level GM (μg/ml)	
		Vi-rEPA conjugate	*Vi polysaccharide*
Adult (n = 22)	Pre-injection	11.9	NA
	6 weeks	576.6	NA
	26 weeks	147.6	NA
	3 years	114.8	NA
	10 years	84.2†	NA
5–14 year (n = 55)	Pre-injection	0.83	0.54
	6 weeks	200.0	23.4
	26 weeks	37.2	16.6
	3 years	18.4‡	NA
	8 years	19.7§	NA

†N = 20; all >4.3 μg/ml, estimated protective level.
‡N = 18; all >4.3 μg/ml.
§N = 75; from children received cross-immunization in efficacy trial, children were 5–8 years old at the time of immunization; 84% >4.3 μg/ml.
37.2 vs 16.6, p < 0.001; 147.6 vs 114.8 or 84.2, p = NS.

Table 3. Persistence of anti-Vi IgG in children 2–5 years old received one or two injections of Vi-rEPA.

Interval post-first injection	Serum anti-Vi IgG (μg/ml)	
	1 injection (n = 48)	*2 injections (n = 52)*[†]
	0.24	0.22
6 weeks	95.7	86.7
10 weeks	67.3	118.3
26 weeks	25.3	37.9
3 years	5.84	5.65

[†]Children received two injections of Vi-rEPA at 6 weeks apart: 118.3 vs 86.7, p = 0.04; 118.3 vs 67.3, p = 0.01; 37.9 vs 25.3, p = 0.1; 5.84 vs 5.65, p = NS.

rEPA was conducted in 11,091 2- to 5-year-old children in the high endemic region in Vietnam, each child received two injections of either Vi-rEPA or saline at 6 weeks apart. Less than 2% of children had adverse reactions and none of the reactions was considered serious [59]. Typhoid cases, diagnosed by the isolation of *S. typhi* from blood cultures after 3 or more days of fever, were identified by active surveillance over a period of 27 months, and by passive surveillance for an additional 19 months after the vaccine code was opened [44].

At the end of active surveillance, Vi-rEPA demonstrated 91.5% protection and 82.4% during the passive surveillance. Over the entire 46-month period, the vaccine efficacy was 89.0% [44,59]. This is the first time that a typhoid vaccine demonstrated safety and protection in this young age group.

It was interesting to note that there were 771 children who received only one correctly labeled injection. During the active surveillance period, there was one case of typhoid among 388 children in the vaccine group and 8 among 383 in the placebo group and the calculated efficacy with only 1 injection of Vi-rEPA was 87.7%. The comparable efficacy between 1 and 2 injections is consistent with the similar antibody response as shown in the Phase II study (Table 3, *vide infra*).

It is worthwhile to mention that the typhoid cases in the Vi-rEPA group appeared to be milder since none of the sick children in the vaccine group were hospitalized compared with 34% in the placebo group.

Dosage study

Dosage-related immunogenicity has been observed in other polysaccharide conjugates vaccines such as Hib and pneumococcal conjugates [63,64]. Furthermore, the amount of polysaccharide in the current licensed conjugate vaccines ranged from 2 μg/injection for pneumococcal conjugates to 10 μg/ml for Hib conjugate [40,41]. The 25 μg Vi/injection of Vi-rEPA in earlier studies was chosen based on the Vi content in the licensed Vi polysaccharide vaccine. To find out the dosage effect on immunogenicity in young children, Vi-rEPA was evaluated in 2- to 5-year-old children in Phu Tho Province, Vietnam. Children, each received 2 injections 6 weeks apart of 25 μg (full dosage), 12.5 μg and 5 μg of Vi as Vi-rEPA [61]. At 10 weeks after the first injection, all children responded with greater than estimated protective level of anti-Vi IgG (>4.3 μg/ml), with the full dose responded the highest. At 1-year interval, the anti-Vi IgG levels were 16.5, 14.0 and 8.0 μg/ml EU, respectively. The anti-Vi IgG levels in all three dosages remained significantly higher than the pre-immune levels and 96% children had a greater than eightfold rise. On the basis of these data, we recommend a dosage approximately equal to 12.5 μg of Vi per injection for all ages. In a separate study using Vi conjugated to CRM_{197}, a similar dosage-dependent antibody response was also observed in European adults volunteers (vide supra) [65].

Safety, immunogenicity & compatibility with infant routine vaccines

The ideal typhoid fever vaccination program would be to incorporate typhoid vaccine with the routine infant immunization. To investigate the safety, immunogenicity and compatibility of Vi conjugate immunization with EPI vaccines, Vi-rEPA was administered concurrently to infants with routine vaccines in Phu Tho Province, Vietnam. Hib conjugate, which was considered at the time as one of the routine infant vaccines in Vietnam, was used as a second control vaccine. A total of 301 full-term infants were randomly divided into three groups each receiving four injections of Vi-rEPA or Hib-TT or none in addition to the routine vaccines at 2, 4, 6 and 12 months. EPI vaccines administered concomitantly were diphtheria-tetanus-pertussis vaccine (DTP), oral polio vaccine and hepatitis B vaccine at 2 and 4 months and DTP at 6 months [60].

Vi-rEPA was safe in infants after all injections. All three groups responded similarly to diphtheria and pertussis vaccines and also to tetanus toxoid except the Hib-TT group. After immunization, the Hib-TT group had higher TT antibody levels than the other two groups likely due to the extra doses of TT.

The serum IgG anti-Vi levels were measured in cord sera (served as the pre-immune base level). At 7 months, the IgG anti-Vi GM for the Vi conjugate group increased from the cord level of 0.8–21.5 μg/ml, with 90% of infants having levels higher than 4.3 μg/ml, the estimated protective level. At 13 months, 1 month after the fourth injection, there was a booster response and the level of anti-Vi IgG increased to 62 μg/ml EU and 95% of infants had levels greater than the estimated protective level. By contrast, the serum IgG anti-Vi in both the Hib-TT and EPI groups declined from cord levels of 0.5–0.1μg/ml at 7 months and remained at about the same level at 13 months.

Approximately 10% (8 out of 89) of the infants in the Vi-rEPA group had cord anti-Vi IgG levels greater than 4.3/mg/ml. The percentage of infants that achieved a 4.3 μg/ml level was higher among infants of the group having lower cord anti-Vi level compared with that in the high group: 97 versus 75% at 13 months. Even though the sample size was small, but since similar interference from the maternal antibodies were observed in other vaccines such as pertussis and Hib conjugate vaccines [66,67], it is worthwhile for further investigation especially in areas where typhoid is endemic.

Table 4. Cross-comparison of anti-Vi IgG responses in children 2–5 years old (Phase II, III, crossover, dosage) and in infants immunized with separate lots of Vi-rEPA.

Clinical trial	Region of study in Vietnam	Number of children	IgG anti-Vi in children in EU (μg/ml)[†]		Ref.
			Pre-immu.	*Post-inj.*[‡]	
Phase II	Cao Lanh District (high endemic)	52	0.18 (0.22)	95.4 (118.30)	[58]
Phase III	Cao Lanh District (high endemic)	36	0.11 (0.14)	72.9 (94.40)	[59]
Dosage	Phu Tho District	77	0.13 (0.16)	102.3 (126.85)	[61]
Crossover	Cao Lanh District (high endemic)	64	0.13 (0.16)	48.4 (60.02)[§]	[44]
Infants[¶]	Phu Tho District	80	0.73 (0.91)[#]	50.1(62.14)	[60]

[†]All antibodies are in GM. Anti-Vi IgG in ELISA units or converted to μg/ml using a calibrated human reference serum Vi-IgG_{R1}.
[‡]Blood samples taken 4 weeks after the second dose in 2–5 years old; 4 weeks after the booster dose at 12 months for infants.
[§]Representing GM of 2 to 20 weeks post-crossover immunization; children were 5–8 years old.
[¶]Infants were injected at 2, 4, 6 and 12 months. Cord blood anti-Vi levels represent pre-immunization levels.
[#]GM of cord blood antibody levels.
102.3 vs 50.1, p = 0.03, all other p > 0.2.

Persistency & consistency of antibody response of Vi-rEPA

During the efficacy study, the persistence of vaccine-induced IgG anti-Vi was assessed in serum samples collected from each of the 16 communes monthly. At 46 months after vaccination, the GM IgG anti-Vi decreased from 22.5 EU at 6 months to 3.87 EU. There is a tendency of age-related immune response when stratified into 2- to 3- and 4- to 5-year-old groups, but the difference was not statistically significant [44]. Based on these results, the estimated protective level of anti-Vi IgG be 3.5 EU (or 4.3 μg/ml), the GM in the younger group at 46 months, since there was no statistical difference between the efficacy in the two stratified age groups [44,59]. This is likely a conservative estimate, since 42% of the children had antibody levels below this GM level but were still protected [68]. Based on the analysis of antibody kinetics during the 46 months surveillance, the more accurate estimate of the protective threshold is in the range of 1.4–2.0 μg/ml [manuscript in preparation]. This number is higher than the 1 μg/ml anti-Vi (measured by radioimmunoassay) estimated from the Vi polysaccharide study in South Africa [68].

At crossover vaccination of the efficacy trial, children were between 5 and 8 years old and received only one injection of Vi-rEPA. The persistence of the antibody levels of this cohort was re-examined 8 years after the vaccination in 75 randomly selected children. The GM anti-Vi IgG level remained high at 17.7 μg/ml and majority of the children (84%) had greater than the estimated protective level (Table 2). These data demonstrated that, similar to that observed in adults, Vi-rEPA elicited long-lasting protection in school-age children.

In total, there were five separate lots of Vi-rEPA prepared to facilitate the clinical trials. The safety and immunogenicity were consistently demonstrated in both high (Cao Lanh district) and low endemic (Phu Tho district) regions in 2- to 5-year-old children as well as in infants.

The link between immunogenicity and efficacy has traditionally been the strongest with acellular vaccines such as toxoid and polysaccharide vaccine. To examine the consistency of immune response elicited by separate Vi-rEPA lots, the level of IgG anti-Vi elicited in 2- to 5-year-old children 4 weeks after the second injection was used as a benchmark for comparison (Table 4). There was no statistical difference in the level of Vi antibody elicited by various lots when compared with the batch used in the efficacy trial. The consistency observed in the clinical outcome demonstrated that following the pre-clinical quality control benchmarks ensures safety and potency of new Vi conjugates.

Vi conjugates using other proteins as carriers & various dosage range

Although the carrier protein rEPA in most of the studies described here is not a licensed vaccine, but there are multiple clinical studies during the past 25 years to show that rEPA is safe for all ages and serves as a good carrier in several other polysaccharide conjugates [69–72]. It also has the advantage of not over loading routine vaccination with diphtheria or tetanus toxoids [40–42].

Since the demonstration of safety and efficacy of Vi-rEPA in young children, several candidates of Vi conjugate are in the global manufacture pipeline. Lanzhou Institute of Biologics, China synthesized Vi-rEPA and conducted a double-blind, placebo-controlled (Vi polysaccharide), randomized Phase I and II studies in a high typhoid endemic region. The results showed their conjugate was safe and immunogenic, and the levels of anti-Vi IgG in 2- to 5-year-old children (25 mg/ml, 2 doses at 8 weeks apart) are similar to those observed in Vietnamese children (Table 5) (preliminary results presented in [73].

There are several Vi conjugates prepared with licensed toxoids such as tetanus (TT) or diphtheria toxoids (DT) or recombinant DT CRM_{197} (Table 5) [51,53,65]. One Vi-TT conjugate was licensed for local distribution in India in 2008 (PedaTyph, BioMed

Table 5. Current status of Vi conjugate vaccine under clinical development and licensure.

Investigator/producer	Carrier protein/ Vaccine/brand name	Vi antigen dosage (μg/0.5 ml) age, injections	Current status	Ref.
BioMed Pvt. Ltd. India[†]	Tetanus toxoid PedaTyph	5 μg <2 years, 2 doses, boost at 2 years, >2 years, 1 dose	Licensed in India, 2008 (Peda)	[44,58,59,60,61]
National Institutes of Health, USA	Recombinant ExoProtein A *Pseudomonas aeruginosa* (rEPA) Vi-rEPA	25 μg 2–6 months, 3 doses, boost at 12 months 2–5 years, 2 doses >5 years, 1 dose	Phase I, II, III, dosage and infant	[43,65,74]
Novartis Vaccines Institute for Global Health, Italy	Mutant diphtheria toxoid (CRM_{197}) Vi-CRM_{197}	5 μg >6 weeks, 3 doses 9–59 months, 2 doses >59 months, 1 dose	Phase I, II	[73]
Lanzhou Institute of Biological Research and Product, China	rEPA Vi-rEPS (LIBP)	25 μg 2–5 years, 2 doses >5 years, 1 dose	Phase I, II License for production (2013)	
Bharat Serum and Vaccines Limited, India	Tetanus toxoid (TT) Typbar-TCV	25 μg >6 months, 1 dose	Phase I, II, III Launched for production (2013)	

[†]PedaTyph package circular.

Biologicals). The immunogenicity results of PedaTyph based on its package insert, showed that the conjugate Vi-TT elicited similar level of anti-Vi IgG in infants as in older children (2- to 5-year-old), In adults, PedaTyph was significantly more immunogenic than Vi alone. The suggested dosage of PedaTyph is 5 μg/0.5 ml (Table 5). As presented at the 8th International Conference on Typhoid Fever and Other Invasive Salmonelloses (2013), the preliminary results from the Phase I and II clinical trials of Vi-TT produced by Bharat Inc. (Typbar-TCV, 25 μg/0.5 ml, single dose) showed to be safe in 6–month to 2-year-old children (n = 307), 98% had fourfold seroconversion 6 weeks after one injection, the GM anti-Vi IgG level are significantly higher than those in the Vi group (preliminary results presented in [73]). Typbar-TCV was officially launched in 2013 in India.

Recently, a Vi conjugate using the Vi purified from *Citrobacter freundii sensu lato* and linked through ADH to CRM_{197}, a mutant non-toxic diphtheria toxin, was studied [53,65,74]. The Vi used in these conjugates contained >97% *O*-acetylation, much higher than those found in Vi purified from *S.* >Typhi. Vi-CRM_{197} was compared with Vi vaccine in Phase I and dosage studies in European adults [65]. Four weeks after the immunization, the group injected with Vi-CRM_{197} elicited approximately six-times higher anti-Vi IgG levels than those injected with Vi alone (304 vs 52 EU). At 6 months, the difference in the antibody levels was reduced (69 vs 51 EU). The reason for this fast decline of antibody elicited by Vi-CRM_{197} as opposed to the long persistence of Vi-rEPA is still to be investigated. Similar to those observed in the Vi-rEPA dosage study in young children, the immune response induced by Vi-CRM_{197} was also dosage dependent: within the range tested between 1.25 and 25 mg, there was a direct correlation between the dosage and anti-Vi IgG response [65]. As reported at the typhoid conference in 2013, the Vi-CRM_{197} conjugate when injected to 9-month-old infants (5 μg/0.5 ml, 2 injections 8 weeks apart) the level of anti-Vi IgG elicited was significantly higher than those in adults immunized with Vi (25 μg/1 injection) (preliminary results presented in [73].

Laboratory investigation of Vi conjugated to DT was attempted by International Vaccine Institute (IVI) and showed to be successful in mouse immunization study [51]. The Vi polysaccharide purified by IVI had a lower molecular weight and, and in term, the final conjugate Vi-DT could be successfully sterile filtered without blockage or lost. Vi-DT technology was transferred to several pharmaceutical companies in Asia, including India, Indonesia and Korea. Clinical trials of the co-developed Vi-DT by Shanta Biotech are underway and their clinical outcome is much anticipated. If successful, the vaccine will be part of the affordable vaccine program sponsored by Bill Melinda Gates Foundation aimed for the most impoverished populations in the typhoid endemic regions [75].

It is worthwhile to make a note on the wide range of Vi conjugate dosages, from 5 to 25 μg per dose, used in the clinical studies by the four manufactures mentioned above. In addition, the age and number of injections also vary (Table 5). A systematic comparison of the short- and long-term immune responses will be helpful for better assessment on these important issues.

Anti-Vi IgG human reference standard

To evaluate Vi-based vaccines, it is necessary to accurately quantify serum Vi antibodies. ELISA has been the most common

method to determine the level of anti-Vi [44,58,76]. The assignment of ELISA units varies widely among investigators with no common reference to calibrate and that made comparison of clinical results nearly impossible [21,44,58,65,73,77,78]. For manufactures or regulatory agencies to estimate vaccine potency or to compare with existing efficacy trials by immune response, the process also relies on the availability of a standardized human reference and preferably with weight unit assigned [79]. A human reference was prepared and characterized, the level of IgG anti-Vi was determined by quantitative precipitation with a purified Vi, as was done for Hib, *Streptococcus pneumoniae* and *Neisseria meningitidis* [80–82]. Plasmas from volunteers of high levels of anti-Vi were pooled, IgG anti-Vi was purified and the content of this preparation, assayed by precipitin analysis, was 33 μg /ml [83]. Accordingly, the estimated protective level of 3.5 ELISA unit/ml IgG anti-Vi, derived from the efficacy trial of Vi-rEPA in 2- to 5-year-old Vietnamese children, is equivalent to 4.3 μg/ml [44,83]. Similar to the standard reference sera for Hib and pneumococcal polysaccharide vaccines, this reagent and the corresponding coating Vi antigen are available for distribution to investigators at the US FDA [84].

Expert commentary & five-year view

Since Sir Almroth Wright's first inoculation of typhoid vaccine in British soldiers in 1896, there are two more improved typhoid vaccines [85]. The elimination of typhoid fever from disease burden in high endemic regions has been considered as a low hanging fruit due to these existing vaccines [86,87]. However the deficiencies, in particular not suitable for young children vaccination, hindered implementation of typhoid vaccination programs. The new generation of typhoid conjugate vaccine provides higher and longer lasting protections than the Vi polysaccharide vaccine in recipients of all ages based on inclusion of Vi polysaccharide in a single trial during clinical development, and historical data available from Vi polysaccharide trials [44]. In adults, the antibody level persisted at least for 10 years and in school-age children 8, pass up the need for re-injection every 3 years. When given to pre-school-age children in a two-dose regimen, Vi-rEPA conferred 89% protective efficacy and sustained for at least 4 years. Also, for the first time, a typhoid vaccine showed to be safe, immunogenic and compatible when given concurrently with the infant routine vaccines. These results provided the first evidence on the effectiveness of Vi conjugate vaccine when deployed routinely in a typhoid-endemic area and supported the use of Vi vaccine as a public health tool to control typhoid fever.

An estimate protective level of anti-Vi IgG was proposed to be 3.5 EU based on the geometric mean of antibody response of 2- to 3-year-old children in the efficacy trial and, when converted to weight unit, it is equivalent to approximately 4.3 μg/ml. This value is most likely a conservative estimate since about 40% of the children at the end of 46 months observation period did not reach this level but were still protected. In a more in-depth analysis of the antibody kinetics compared with the placebo group in the Vi conjugate efficacy, a more accurate estimation of the protective level for anti-Vi IgG was in the range of 1.4–2.0 μg/ml [83].

Since the demonstration of safety and efficacy of Vi-rEPA, there were global interests in investigation and production of the Vi conjugate vaccines. Measurement of Vi antibody response in clinical trials is an important marker for potency assessment and to bridge with the existing efficacy trial. To establish a global serum reference and to replace the arbitrary ELISA units applied by various laboratories, a human anti-Vi IgG reference standard was prepared and the concentration was determined in an unambiguous weight unit (μg/ml), similar to those for pneumococcal and Hib human reference sera and this product and Vi coating antigen are available for distribution to investigators by the US FDA [84].

There are advantages and issues of using rEPA as the carrier protein [88]. It has been shown to be safe and served as a good carrier for polysaccharide antigens [44,58–61,69–72]. The production of rEPA does not require the extra step of detoxification and the yield is relatively high. Using rEPA as the carrier protein also avoids adding extra doses of TT or DT to the already crowded toxoid regimen in infant vaccines [75]. However up to this day, there is no licensed vaccine containing rEPA. Other options of carrier protein, such as TT, DT or CRM_{197}, also showed to be good carriers for Vi conjugate [51,65,73].

The source of Vi polysaccharide is another potentially important issue and worth further exploration. Using lower molecular weight, Vi selected from field isolates could offer technical advantages such as better solubility, easier sterile filtration and higher yield [51]. But caution should be taken from the experience of other polysaccharide vaccines, also observed in pre-clinical study of Vi conjugates in mice, that lower molecular weight polysaccharides are immunologically less favorable and should be further evaluated in clinical settings [48]. Vi purified from the non-pathogenic bacteria *C. freundii sensu lato,* avert fermentation of the dangerous pathogen *S. typhi*, is another plausible approach [53,65,74]. One of the most essential factor in Vi immunogenicity is the level of *O*-acetylation. While using Vi from various sources, it is important to investigate the optimal level of *O*-acetylation that can provided sufficient antigenic determinants on the one hand but still allow ample conformational flexibility to accommodate close proximity to interact with immune systems [46].

A relevant element of successful vaccine manufacture is the consistency in adequate testing during production to ensure safety and potency from lot-to-lot [56,57,82]. There is a wealth of information about the immunological property of polysaccharide antigens and their protective functions that permits using physicochemical parameters to constitute Vi conjugate product profile [57,89–90]. The five clinical lots of Vi-rEPA reviewed here showed narrow variations in key physicochemical markers and consistent animal immunogenicity response during pre-clinical tests. Adherence to the same standard can help to ensure product potency [56,57]. Recent clinical study of Vi-rEPA (LIBP) by Lanzhou Institute of Biological Product, China showed that in pre-school children the level of anti-Vi IgG responses were similar to those observed in the NIH efficacy trial [73]. New conjugates may surrogate with the existing efficacy data through pre-clinical and serological markers

without awaiting the results of costly and time-consuming efficacy trials [57,73,82]. Adoption of these scientifically based approaches in licensing could hasten the availability and implementation of the new typhoid conjugate vaccine [86,91,92].

Recent findings in Asia and Africa, especially in regions started typhoid vaccination, indicated non-typhoidal infections emerge as the major cause of enteric fever too [93,94]. The surface polysaccharide antigens of non-typhoidal salmonella are the *O*-specific polysaccharide (*O*-antigen) of the lipopolysaccharides. Conjugate of the *O*-antigen from *Salmonella enterica* serovar paratyphi A (*S.* >para A) showed to be safe and immunogenic in adults and pre-school children [95]. Similarly, demonstrated in laboratory animals, group B and D *O*-antigen conjugates were protective against *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis, respectively [96–98]. Once proven to be efficacious, an ideal vaccine to control enteric fever would be a combined regimen of Vi conjugate and the non-typhoidal conjugates.

Additional efforts from non-profit organizations including: Bill Melinda Gates Foundation, International Vaccine Institute, Sabin Vaccine Institute and Wellcome Trust started comprehensive programs of Vi conjugate vaccine development, aimed at affordable vaccines for target populations, and have made considerable progress in production [73,86]. Recently in response to the growing trend of Vi conjugate vaccine, global health organizations together with regulatory agencies from the USA, Korea, China, Indonesia, India, Thailand and the UK initiated guideline preparation for Vi conjugate vaccine licensing [73,86,91]. The new perspective of Vi conjugate vaccination could accelerate typhoid elimination in high endemic regions.

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Key issues

- Typhoid fever remains a health problem worldwide. Safe, effective and affordable typhoid vaccines are currently available. The new generation of Vi conjugate vaccine conferred higher and longer protection against typhoid fever than the current vaccines.
- Single injection of Vi conjugate in school-age children and adults provided >8 years protection, eliminates the need for multiple dose regimens or re-injection every 3 years.
- Efficacy trial showed Vi conjugate protects pre-school age children between 2 and 5 years old with 90% efficacy for 4 years.
- Vi-rEPA demonstrated to be safe and immunogenic when given concomitantly with routine vaccines to infants and can be incorporated into the Expanded Program of Immunization.
- Among various method of Vi conjugate synthesis, adipic dihydrazide scheme showed to be simple, reproducible and was successfully adopted by most of the manufactures in the field.
- Clinical trials of Vi conjugates made with various clinical relevant toxoids consistently reaffirmed the safety and demonstrated superiority over the licensed Vi polysaccharide vaccine.
- Key physicochemical and serological markers are compiled based on existing clinical studies. It can be helpful to establish product profile for new Vi conjugates and allows new product to surrogate with existing clinical data for potency evaluation [101].
- Giving the success of the investigational vaccine Vi-rEPA, in order for Vi conjugate to advance into production level, there are still many questions unanswered, such as: the ideal dosage, formulation, stability, cold chain requirement or improvement, optimum number of injections and immunization schedule, especially for children under 2 years old.
- Currently, there exists an estimated protective level of anti-Vi IgG. This value appears to be too conservative. A closer study to derive a more realistic protective level is incumbent.
- Consideration should be given to include Vi conjugate in combination form with other vaccines. An ideal vaccine to control enteric fever would be a combination vaccine composed of Vi conjugate and non-typhoidal conjugates that are still under development.
- During the past decade, global non-profit organizations including the Bill Melinda Gates Foundation, Sabin Vaccine Institute and International Vaccine Institute, joint forces with local public health organizations build up comprehensive strategies for typhoid control, including integrated vaccination program along with supply of clean water and sanitation improvement. The participation of the new generation Vi conjugate vaccine will accelerate the process of typhoid elimination [102–104].

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