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Serologic assays for influenza surveillance, diagnosis and vaccine evaluation

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Serological techniques play a critical role in various aspects of influenza surveillance, vaccine development and evaluation, and sometimes in diagnosis, particularly for novel influenza virus infections of humans. Because individuals are repeatedly exposed to antigenically and genetically diverse influenza viruses over a lifetime, the gold standard for detection of a recent influenza virus infection or response to current vaccination is the demonstration of a seroconversion, a fourfold or greater rise in antibody titer relative to a baseline sample, to a circulating influenza strain or vaccine component. The hemagglutination-inhibition assay remains the most widely used assay to detect strain-specific serum antibodies to influenza. The hemagglutination-inhibition assay is also used to monitor antigenic changes among influenza viruses which are constantly evolving; such antigenic data is essential for consideration of changes in influenza vaccine composition. The use of the hemagglutinin-specific microneutralization assay has increased, in part, owing to its sensitivity for detection of human antibodies to novel influenza viruses of animal origin. Neutralization assays using replication-incompetent pseudotyped particles may be advantageous in some laboratory settings for detection of antibodies to influenza viruses with heightened biocontainment requirements. The use of standardized protocols and antibody standards are important steps to improve reproducibility and interlaboratory comparability of results of serologic assays for influenza viruses.

Keywords: antigenic characterization • hemagglutination inhibition • influenza virus • vaccine responses • virus neutralization

Influenza viruses represent an ever changing target of global public health concern. In 2009–2010, the world experienced the first influenza pandemic in over 40 years. The pandemic strain emerged from swine and was first detected in humans in North America, contrary to widely held beliefs that the next pandemic virus would arise from avian sources in Asia. Ongoing epizootics of highly pathogenic avian influenza (HPAI) H5N1 viruses continue to result in occasional human infections with high fatality rates. These events highlight the unpredictable and diverse nature of influenza and underscore the need for global viral surveillance among humans and animals. Serological techniques play a critical role in various aspects of influenza surveillance, vaccine development and evaluation, and occasionally in diagnosis, particularly for novel influenza virus infections of humans. Because individuals are repeatedly exposed to antigenically and genetically diverse series of influenza viruses over a lifetime, assays that are strain-specific are essential for optimal characterization of viruses and detection of immunity to recent infection or vaccination. Here we review serological methods to measure strain-specific influenza antibody responses and their applications to understand human infection, and develop and evaluate influenza vaccines. We will focus on the use of the serologic assays that measure antibody responses to the major influenza virus surface antigen, the hemagglutinin (HA), in particular, the hemagglutination inhibition (HI) and virus neutralization (VN) assays, and other HA-based serologic methods where appropriate.

Influenza viruses are single-stranded, negative-sense, enveloped RNA viruses, of the family *Orthomyxoviridae*. Three influenza types, A, B and C, exist, which are classified based on serological relatedness of internal viral proteins. Although all types cause human disease, only influenza A and B viruses will be considered in this article, since they are primarily responsible

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for global outbreaks of illness among humans and the targets of licensed seasonal influenza vaccines. Influenza A viruses also infect a wide variety of avian and mammalian species [1]. By contrast, influenza B viruses are primarily human pathogens, and are only rarely isolated from other species [2]. Influenza A and B viruses possess two major surface glycoproteins, the HA, which mediates attachment to host cell receptors and virus entry, and the neuraminidase (NA), which facilitates progeny virus release from the infected cell [3,4]. Antibodies directed against the HA block virus–receptor binding or otherwise inhibit virus entry or uncoating, and are considered the primary mediator of host defense against the virus [5–7]. Antibodies to the NA, while unable to prevent infection, reduce the amount of virus shed and can ameliorate disease [8,9]. The HA and NA form the basis of serologically distinct virus subtypes of influenza A viruses. There are currently 16 HA (H1–H16) and nine NA (N1–N9) subtypes identified, all of which exist in wild water birds, the natural reservoir for all influenza viruses [1,10,11]. Influenza B viruses are not classified into distinct subtypes, but are comprised of two antigenically distinct lineages, B/Victoria and B/Yamagata, that circulate in various proportions at different times and geographic locations [12,13].

Influenza viruses have a remarkable capacity to undergo variation in their surface glycoproteins in two fundamentally different ways. The first is the process known as 'antigenic drift' which results from a gradual accumulation of point mutations in the genes encoding the HA and NA, resulting in the emergence of variant strains that are no longer sufficiently inhibited by antibody to older strains, allowing the variants to predominate and become the next epidemic strain. Occasionally, viruses emerge with a novel HA or HA and NA combination which has not circulated recently in humans and for which little serologic immunity exists in the population. The second process is termed 'antigenic shift' and may occur either due to a reassortment event whereby human influenza viruses acquire a novel HA and one or more viral genes from an animal influenza virus, or by the introduction of a wholly animal virus into humans, followed by acquisition of point mutations to facilitate adaptation to humans. If such a virus has the capacity to spread efficiently and cause disease among humans, a pandemic can result. Only H1, H2 and H3 subtypes have caused widespread and sustained human disease outbreaks. Their introduction into humans resulted in four pandemics in the previous 100 years: the pandemics of 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) and 2009 (H1N1), the latter three pandemics being caused by the emergence of animal–human reassortant viruses. Despite occasional interspecies transmission of avian H5, H7 and H9 viruses to humans, primarily through contact with infected domestic poultry, these viruses have failed to acquire the ability to spread efficiently from person-to-person [14]. Nevertheless, avian H5N1 influenza viruses have caused over 500 laboratory-confirmed human cases since 2003, with a fatality rate of >50%, highlighting the need for public health vigilance, and continued global surveillance to detect new instances of human disease, as well as to monitor their molecular evolution and pandemic potential.

Rates of seasonal influenza infection are highest among children, whereas rates of serious illness and death are highest among adults aged ≥65 years, children ≤2 years of age, and persons of any age with underlying medical conditions [15–18]. During the 2009 H1N1 pandemic, the risk for influenza hospitalization was greater for adults aged 19–64 years, compared with the risk typically seen for seasonal influenza [19,20]. Vaccination is the most effective means of reducing influenza morbidity and mortality in the community. Seasonal trivalent influenza vaccines containing influenza A/H1N1, A/H3N2 and a type B virus must be updated annually to reflect the genetic and antigenic characteristics of circulating viruses. In addition, vaccines targeting avian H5, H7 and H9 viruses have been produced for pandemic preparedness purposes. Two different types of seasonal influenza vaccines are licensed, one that contains inactivated viruses, and one that contains live-attenuated viruses. Because the main target of inactivated vaccines is the induction of strain-specific anti-HA antibodies, vaccines are standardized only by quantification of the HA protein content. Live-attenuated influenza vaccines (LAIVs) are based on cold-adapted, temperature-sensitive donor strains that limit the growth of the live virus vaccine following intranasal introduction to the upper respiratory tract. The efficacies of either type of vaccine depend on several factors, including the antigenic match between the vaccine strain and the circulating virus, and the age and health status of the vaccine recipients [21–23].

Serologic assays for detection of anti-HA antibodies

Hemagglutination inhibition

Hemagglutination inhibition is the most widely used assay for detection of antibodies to influenza viruses. The test was first developed by Hirst who fortuitously discovered the ability of influenza viruses to agglutinate chicken red blood cells (RBCs) [24]. The demonstration that immune sera could inhibit agglutination has led to the development of the HI test with later modifications by Salk [24–26]. Influenza virus HA binds to terminal sialic acids of glycoproteins and glycolipids that serve as receptors on cell membranes. Human influenza viruses, preferentially bind to α2,6-linked sialic acids while avian viruses bind predominantly to α2,3-linked sialic acids, with the linkage typically to a galactose residue [27,28]. Different avian and mammalian RBCs express different proportions of these receptors on their surface [29]. Antibodies to HA that bind or block the receptor binding site inhibit hemagglutination. HI measures the inhibition of hemagglutination at a fixed virus and RBC concentration. Within laboratories, HI titers correlate well with those detected by more complicated VN assays [30].

The source, species and quality of RBCs can greatly affect results. In general, turkey, guinea pig or human type O RBCs are preferred for HI assays with contemporary human viruses, while horse RBCs are preferred for HI assays with H5N1 viruses and other avian subtypes [31–37], but other species, including goose RBCs may be useful in regions where sources of other RBC species are limited [38,39]. In the standard HI assay for influenza, twofold serial dilutions of serum are combined with an equal volume of live or killed virus containing 4 hemagglutination units (HAUs; one HAU is defined as the amount of virus needed to agglutinate an equal volume of a standardized RBC suspension [201]). Following an incubation to allow for the binding of antibodies to the virus, a standard quantity of RBCs is added and the HI end point titer is read as the reciprocal of the highest dilution

of serum that completely inhibits hemagglutination. The assay is complicated by the fact that all sera tested must be pretreated with receptor-destroying enzyme (RDE) overnight to remove the various sialic acid-containing glycans in sera which may bind to the virus HA and mimic the binding of influenza-specific anti-HA antibodies resulting in a false positive. RDE treatment is followed by heat inactivation of the RDE and serum complement. In addition, some sera must also be adsorbed with RBCs to remove nonspecific agglutinins of RBCs to prevent a false-negative result [40,201]. Influenza B viruses often require ether treatment prior to use in an HI assay to increase sensitivity for the detection of human serologic responses. However, ether treatment is not recommended for antigenic characterization of influenza B viruses because it may reduce assay specificity and therefore compromise detection of antigenic differences among viruses.

Virus neutralization

Virus neutralization is a highly sensitive and specific method for detecting strain-specific antibodies that inhibit virus entry or otherwise block virus replication, including HA-mediated fusion of the viral envelop and the endosomal membrane [3,31,41]. Neutralization is performed in three steps: a virus–antibody reaction step, in which the virus is mixed with dilutions of serum and allowed to react; an inoculation step, in which the mixture is inoculated into the appropriate host system; and a procedure to detect virus or viral antigen as a read-out step. The absence of infectivity constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in the serum sample. Classical neutralization methods measured reduction of virus plaque formation or inhibition of virus growth in primary cells or embryonated chicken eggs. Nowadays, microneutralization (MN) assays using cultured Madin Darby canine kidney (MDCK) cells, a continuous cell line widely used for propagation of influenza viruses, in microtiter plates are the preferred method for higher throughput detection of neutralizing antibodies. As long as the amount of virus utilized in the assay is standardized and low-passage MDCK cells, free from microbial contamination, are used while in log-phase growth, the main variable between assays is the method of detecting virus infectivity postneutralization. Virus or viral antigen is often detected through hemagglutination activity in culture supernatants and/or cytopathic effect (CPE) of cell monolayers on days 3–7 postneutralization. The use of colorimetric detection of cell viability has recently been reported [42]. More commonly, ELISA has been used in MN assays to detect viral antigen, typically the nucleoprotein (NP), expressed in infected cells following only overnight culture [43]. The presence of influenza virus is detected in fixed cells by a virus-specific mouse monoclonal antibody followed by the addition of an enzyme-labeled antimouse immunoglobulin antibody and a colorimetric substrate which can be either insoluble for detection of foci of infected cells [30] or soluble for spectrophotometric absorbance quantification of viral replication [43,202]. This method allows for calculation of an end point titer, the reciprocal of the serum dilution exhibiting at least 50% neutralization, by automated data analysis. Neutralization assays using ELISA to detect viral-infected cells tend to be less variable than neutralization evaluated using CPE [44,45]. An alternative to ELISA detection is the use of immunofluorescence coupled with an enzyme-linked immunosorbent spot-type reader.

The MN assay primarily detects antibodies to the virus HA and identifies functional, predominantly strain-specific antibodies in animal and human sera, although it can be modified to detect antibodies to either HA or NA [31,46,47]. Owing to the strain specificity of the MN assay, as well as the HI assay, it is critical that the virus used for testing is the same or very similar to the virus that caused the infection or elicited an antibody response through vaccination. Numerous studies have demonstrated the correlation between HI and MN results and the higher sensitivity of VN or MN assays, particularly for detection of antibodies to influenza B viruses [48–50].

Pseudotype neutralization

As an alternative to using live virus in neutralization assays, retroviral vectors pseudotyped with influenza HA have been developed [51–54]. Pseudotyped virus particles can attach to cells expressing terminal sialic acid glycoproteins that function as receptors, undergo the steps of viral entry, and following reverse transcription and integration express a reporter gene such as luciferase. Pseudotyped particles cannot produce replication-competent virions [51,54] and therefore their use is most appealing for measuring neutralizing antibody to viruses that require enhanced biocontainment for use, including highly pathogenic avian influenza viruses and noncontemporary pandemic strains [55]. Unlike HI and MN, pseudotype neutralization (PN) assays do not require live virus since the HA gene can be synthesized for cloning into the expression plasmid. However, the assay requires 3 days to complete owing to the 48 h required for expression of the reporter gene and can only detect neutralizing antibodies directed against the HA.

Hemagglutinin-pseudotyped particles are produced using retroviral vectors in a two- or three-plasmid system. Human embryonic kidney (HEK)293T cells are transfected, exogenous NA added at 18–24 h postinfection to induce the release of the HA pseudotyped particles, and the pseudotype harvested 48 h postinfection. Quantification of pseudotypes is done by measuring the reporter gene expression. For neutralization assays, twofold serial dilutions of serum are mixed with a predetermined amount of pseudotype, incubated at 37°C for 1 h, and then added to microtiter plates containing MDCK or 293T cells. Infectivity is measured 48 h later, typically using a luciferase reporter gene system, with output in relative light units (RLUs). A 90% reduction of RLUs is the inhibitory concentration (IC) and titer end point. Other IC end points may be used [51–54]. PN titers are often higher than the MN titers for the same samples [54,56,57], but this alone does not indicate increased sensitivity. However, one study has shown increased sensitivity of the PN assay compared with the MN and HI assays for the detection of very low levels of neutralizing antibody following vaccination [57]. Within laboratories, results from PN and MN [54], and between PN, MN, HI and single radial haemolysis (SRH) assays correlate well [53,57,58].

Single radial hemolysis

Single radial hemolysis, based on immunodiffusion of antibodies in agarose gel containing influenza virus bound to RBC and complement, is used to detect antibodies to HA. The assay was developed in 1975 as a modified single radial diffusion technique and has an advantage over HI since it is not necessary to RDE treat serum samples prior to testing [59,60]. Undiluted serum is directly added to wells in the prepared immunodiffusion plate and allowed to diffuse overnight at 4°C. Incubation at 37°C the next day results in complement-mediated lysis of the RBCs when antibodies are present. The diameter of the lysis zone is measured and typically the zone annulus, calculated from the area of zone minus the area of well, is determined. Studies have shown a good correlation between HI titer and the area of the hemolyzed zone in the SRH assay, although the SRH may be somewhat less strain specific [61–63]. Some studies show a slightly higher sensitivity for the SRH assay as compared with HI when evaluating responses to vaccination in clinical trials [64,65]. Higher sensitivity has been especially noted with influenza B viruses [62–64,66]. A drawback of the SRH assay is the lack of automation in both performance and reading of the assay, making large studies challenging.

ELISA

Unlike the aforementioned assays, which all measure functional antibodies, the ELISA measures antibodies that bind to influenza virions or, more recently, to purified recombinant HA. Although there are many variations, typically virus or recombinant HA is adsorbed to the wells of a microtiter plate followed by the sequential addition of serum sample, an enzyme-labeled antibody to detect immunoglobulin bound to the virus or HA, and substrate for colorimetric detection of binding. Early studies using virus as antigen demonstrated that antibodies specific to influenza A and B could be discriminated [67–69], but that antibodies to influenza A subtypes could not be distinguished [70]. This lack of subtype specificity, also reported for H5 subtype antibodies, is a shortcoming of traditional ELISA-based methods [71,72]. The value of ELISA is that it can measure serum and mucosal IgM, IgA and IgG subclass responses to influenza infection or vaccination [73–77]. Modifying the ELISA using low concentrations of a denaturing agent in the wash buffer allows for evaluation of serum antibody avidity, the strength of interaction between antibody and a multivalent antigen [78]. In addition, unlike the other assays mentioned, an ELISA can be completed within a few hours and can be fully automated for high-throughput testing. Recently, the use of the recombinant HA1 domain rather than the full-length HA as antigen has been reported as an approach to improve assay specificity [79] . In addition, an epitope-blocking ELISA, using a monoclonal antibody to a highly conserved epitope in the HA1 region of H5 HA has been reported to be highly sensitive and 100% specific for detecting antibodies to the H5 subtype [80].

Use of serologic methods for identification of human infections

Owing to the time taken to develop an antibody response to infection, serology is not typically used for the diagnosis of acute infection [31,46]. Instead, methods that directly detect viral nucleic acids or viral proteins in a respiratory tract specimen are commonly used. Although classical techniques such as growth of specimen in cell culture, detection via immunoflurorescence with an anti-NP antibody, and detection via commercial antigen detection kits [46,81–83] are still widely used, recently, real-time (RT)-PCR is more often used for detection of viral infection because of its high sensitivity. While not playing a role in patient management, influenza serology is essential for epidemiological studies of virus transmission, for determining the seroprevalence of potentially protective antibodies, and for evaluating the effectiveness of vaccines. Serology is useful for detecting asymptomatic infections in outbreak investigations. The constant antigenic drift influenza viruses undergo and their widespread circulation means that people may be repeatedly infected or vaccinated throughout their lifetime and have an antibody repertoire that reflects their history. Antibodies to closely related, drifted viruses may be cross-reactive, even in HI and VN assays [78,84,85], making it impossible to determine, based on a single convalescent serum sample, if antibody detected is due to a recent influenza virus infection or to prior infection or vaccination.

The gold standard for detection of a recent influenza virus infection is the demonstration of a seroconversion, a fourfold or greater rise in antibody titer relative to a baseline sample, to the circulating influenza strain suspected of causing the infection. Ideally, an acute serum sample is collected within 7 days of symptom onset and a convalescent sample is collected 2–3 weeks later [31,46]. The kinetics of the serum antibody responses to infection depends on age and prior exposure history. In general, antibodies may be detected by 10–14 days postsymptom onset and peak by 21–28 days post symptom onset, the optimal timing for detection of seroconversion. Studies by Miller *et al.* and Veguilla *et al.* provide examples of the kinetics of HI and neutralizing antibody responses in individuals infected with the 2009 H1N1 pandemic strain [86,87]. Although other viral infections are diagnosed by the detection of virus-specific IgM [88], repeated exposure to influenza antigens means that detection of IgM, a marker of a primary infection, is not generally suitable for influenza serodiagnosis [31,89,90]. It should be noted that a small proportion of individuals may not mount any serum antibody response following influenza infection [86,91].

Although seroconversion remains the gold standard for influenza serodiagnosis, in the case of a novel HA subtype infecting humans, it is possible to establish serologic criteria for diagnosis on the basis of a single convalescent serum sample when seroprevalence of cross-reactive antibody is low in unexposed populations. The establishment of serodiagnostic criteria is based on the analysis of serum samples, typically in HI and/or MN assays, collected prior to the emergence of the novel HA subtype to determine the background levels of antibody reactivity. The criteria are also based on the analysis of serum samples from virologically confirmed cases of the novel virus. These data are used to determine assay sensitivity and specificity and establish a minimum antibody level considered to be seropositive. Serodiagnostic criteria based on a convalescent serum sample have been established for human infections with avian H5N1 viruses and have been applied to confirm H5N1 virus-infected cases, in situations where virologic confirmation of infection was not possible [71,92,203]. Such criteria

have also been applied for the detection of mild or asymptomatic infection with avian H5 and H7 viruses in persons exposed to infected birds, people or environments [93–96]. Similarly, following the introduction of the novel 2009 H1N1 virus [97], we established criteria that detected infection with the novel virus among participants of seroepidemiologic investigations conducted during the early stages of the pandemic [87].

As the recent pandemic demonstrated, there is a need to be better prepared to evaluate human cross-reactive and strain-specific serum antibody responses to newly emerging influenza viruses. Ideally, the use of convalescent serum from virologically confirmed cases to understand the basis of authentic responses, and age-matched sera collected before the virus emerged to understand the extent of serologic cross-reactivity that is pre-existing in populations, can be used to establish threshold titers which can guide early seroepidemiologic investigations that seek to characterize the scope of and risks associated with human infection. Using such an approach during the 2009 H1N1 pandemic, we established minimum MN and HI titers for seropositive convalescent serum samples with a sensitivity of 90% and a specificity of 96% [87]. The MN assay was more sensitive for the detection of seroconversion, detecting seroconversion in 91% of 55 infected individuals compared with 84% by the HI assay [87].

Serologic methods for virus characterization & vaccine strain selection

The ability of influenza viruses to circumvent immunity acquired through infection or vaccination by progressive antigenic drift necessitates regular updating of the composition of influenza vaccines to reflect contemporary viruses [98,99]. This requires continuous global monitoring for the emergence of influenza viruses that are distinct from current vaccine strains. Vaccine strain selection is a process carried out by the WHO Global Influenza Surveillance Network (GISN) twice annually to coincide with Northern Hemisphere and Southern Hemisphere influenza seasons and allowing for the time involved in the development, production and validation of influenza vaccines [204]. New vaccine strain recommendations are based on an assessment of the future impact of circulating viruses and, in particular, of any emerging antigenic variants. The scientific evidence that forms the basis for vaccine recommendation includes the antigenic and genetic characteristics of viruses, their prevalence, geographic distribution and rate of spread; the availability of suitable vaccine strains is also taken into consideration [100,204].

The degree to which immunity induced by one strain is effective against another depends on the antigenic difference between strains; thus, the analysis of antigenic differences is critical for surveillance and vaccine strain selection. The HI test is the test of choice for monitoring antigenic relationships among viruses and is essential for consideration of changes in vaccine composition recommended by the WHO [100]. Three major components of the HI assays for antigenic characterization are: viruses with sufficient hemagglutination titers; strain-specific antisera and RBCs from avian or mammalian species such as chicken, turkey, guinea pig or type O human. Ferrets which are naturally susceptible to influenza viruses are used to prepare exquisitely strain-specific antisera. Seronegative animals are infected intranasally and undergo a primary influenza infection. Sera collected 2–4 weeks later can discriminate between the infecting virus and variant strains [101].

To determine antigenic relationships between two viruses, ferret antisera to both viruses must be used in a so-called 'two-way' HI test. If the homologous and heterologous HI titers of the two viruses are equal to or within fourfold of each other, the viruses are considered antigenically related (like) [100]. Presently, antigenic characterization for large numbers of circulating influenza viruses are carried out largely in 'one-way' HI tests (i.e., field viruses are tested against a panel of reference antisera along with their homologous reference viruses including the current vaccine strain). Generally, a virus is considered 'vaccine or reference virus-like' if its HI titer is equal to or within fourfold of the homologous HI titer against the vaccine or reference strains [100]. Antisera used in the tests are newly prepared throughout the year, representing viruses that are circulating at the time. The HI assay can be performed on a comparatively large scale (up to 100 test viruses) yet can be completed within a work day. Table 1 demonstrates the use of the HI test to discriminate antigenic differences of influenza A H3N2 viruses circulating between 1968 and 2009.

Disadvantages of the HI test include that it is labor intensive, it requires removal of nonspecific inhibitors of hemagglutination that occur naturally in sera, reference and test antigens must be standardized each time a test is performed, specialized expertise is required in reading the results of the test (currently, a plate reader for reading HI results is not available) and that the assay has considerable test-to-test variation (the average variation of HI tests results between individual tests is approximately twofold). Additional challenges that complicate performance of the assay and interpretation of HI data include changes in the ability of influenza viruses to agglutinate different RBCs. In the late 1990s, H3N2 viruses dramatically reduced or even lost their capacity to agglutinate chicken RBCs that were widely used in HI tests. Since then, other RBC species, especially turkey RBCs, have become the popular choice for hemagglutination and HI assays [32,100,102]. Amino acid changes in the receptor binding site of the HA contribute to the inability of some viruses to agglutinate chicken erythrocytes [32,102]. Recently, many H3N2 viruses have exhibited reduced or no agglutination of turkey RBCs, necessitating the use of mammalian RBCs (guinea pig or human) in antigenic HI assays [100,103]. Furthermore, viruses derived from different culture systems may present different HI reaction patterns owing to the difference of receptor binding specificity [104]. Egg-derived and mammalian (MDCK) cell-derived viruses show different levels of reactivity to postinfection ferret antisera as determined by the HI assay [105,106], with lower HI titers being seen for viruses isolated in mammalian cells than viruses that have been adapted to replication in hen eggs [100]. In recent years, modified MDCK cells, such as MDCK SIAT-1 [107], have been used by some laboratories because they offer advantages for virus isolation and propagation, particularly for H3N2 viruses [108,109]. However, viruses derived from MDCK SIAT-1 cell lines often exhibited reduced HI titers compared with viruses derived from traditionally used MDCK cells in HI tests using turkey RBCs, thus complicating

Table 1. Guinea pig red blood cell hemagglutination-inhibition reactions of influenza A H3N2 viruses circulating between 1968 and 2009.

Strain designation	Vaccine strain (years)	Ferret antisera against[†]													
		HK/68	*ENG/72*	*TX/77*	*BAN/79*	*PHI/82*	*SHI/87*	*BE/89*	*BE/92*	*JHB/94*	*NAN/95*	*SYD/97*	*FJ/02*	*BRIS/07*	*PER/09*
A/Hong Kong/08/1968	1968–1973	160	640	20	40	10	5	10	10	5	5	5	5	5	5
A/England/42/1972	1973–1974	40	1280	40	40	10	5	20	10	5	5	5	5	5	5
A/Texas/01/1977	1978–1980	5	80	2560	320	640	10	20	40	5	5	5	5	5	5
A/Bangkok/01/1979	1980–1983	5	10	640	640	640	10	40	40	5	5	5	5	5	5
A/Philippines/2/1982	1984–1986	5	10	20	40	320	10	10	10	5	5	5	5	5	5
A/Shanghai/11/1987	1989–1990	5	20	40	40	40	1280	160	80	10	5	5	5	5	5
A/Beijing/353/1989	1991–1993	20	20	40	40	40	320	320	80	20	5	5	5	5	5
A/Beijing/32/1992	1993–1994	5	10	40	40	20	10	40	640	80	10	5	5	5	5
A/Johannesburg/33/1994	1995–1996	20	20	40	20	20	5	20	80	1280	40	5	5	5	5
A/Nanchang/933/1995	1996–1998	5	10	20	20	20	5	10	80	80	1280	80	10	5	5
A/Sydney/05/1997	1998–2000	20	10	10	10	20	5	20	40	10	80	5120	320	5	5
A/Fujian/411/2002	2004–2005	5	5	5	5	5	5	5	5	5	5	80	1280	40	5
A/Brisbane/10/2007	2008–2010	5	5	5	5	5	5	5	5	5	5	20	80	1280	5
A/Perth/16/2009	2010–2011	5	5	5	5	5	5	5	5	5	5	5	5	10	320

The hemagglutination-inhibition titer is the reciprocal of the highest dilution of antiserum that completely inhibits hemagglutination.
[†]Underlined values indicate reference ferret antiserum titer to homologous virus.

the interpretation of HI results. This effect is somewhat reduced but not eliminated when mammalian RBCs are used [100]. Interference by NA in hemagglutination and HI assays to influenza A (H3N2) viruses has been reported recently [103], suggesting that precautions need to be taken when performing and interpretating HI tests for recent H3N2 viruses. Further studies, for example, evaluation of receptor binding ability of NA in the presence or absence of HA are needed to better understand this phenomenon. The use of reference antisera prepared in different animals, for example chicken, rabbit or sheep, is another factor that can cause difficulties in interpretation of the HI data. Antisera prepared by intravenous or intramuscular inoculation, often involving a booster inoculation, may induce antibodies that cross-react with distantly related viruses compared with a single infection protocol [100]. However, influenza B viruses usually generate ferret antisera with lower homologous HI titers compared with influenza A viruses [100], requiring additional boosting of animals.

Because of these factors that complicate the HI assay, alternative techniques such as the VN assay are now also used to characterize antigenic relationships between circulating viruses and reference or vaccine viruses [30,50,110]. In particular, Matrosovich *et al.* described a neutralization-plaque reduction assay which is currently used as a confirmatory assay of HI for assessment of virus antigenic relationships [100,111]. VN and MN are currently used as supplementary assays to HI for virus antigenic characterization but each test needs 3–4 days to complete and only a handful of viruses can be characterized in one test. Influenza antigenic cartography is a new computational method that allows a graphic visualization of antigenic evolution that has been used as an additional tool for the WHO vaccine strain selection process for the past several years [100,112–114]. Based on results of HI tests, antigenic cartography projects influenza antigens into a 2D or 3D map through which antigenic distances between influenza viruses are determined, just as geographic distances between cities are visualized by geographic cartography.

Genetic or sequence analyses of influenza viruses for vaccine strain selection have became increasingly important during the past two decades [100,115–117]. Currently, approximately 10% of the viruses analyzed antigenically are also analyzed genetically by sequencing the HA and/or other genes [114]. Sequence analysis provides a molecular basis of virus antigenic evolutions by identifying the location and type of changes relative to HA or NA structures in known antigenic sites or virus receptor binding sites, as well as recognizing particular amino acid changes that may have resulted from host cell selection [32,102]. Phylogenetic analysis reveals evolutionary relationships among the circulating viruses and also leads to the discovery of newly emerging genetic groups (e.g., lineages and clades), and helps to monitor the spread or evolution of a particular

genetic group [115–117]. Another essential type of data gathered to guide vaccine strain selection decisions is the extent of cross-reactivity in human postvaccination sera to antigenically drifted variants compared with the current vaccine strains. This process is described in the next section.

Serologic methods for vaccine evaluation & licensure

The ability to elicit HA strain-specific antibodies in the serum of immunized persons is the major indicator of influenza vaccine immunogenicity and a marker of protective efficacy. While HI, SRH and VN assays are all used to measure levels of strain-specific anti-HA antibodies, the HI is the most widely because of its relative simplicity and because HI titers are associated with protection. Currently, human serology is an important component of the vaccine strain selection process: pre- and post-vaccination sera are collected from subjects who received the most recent vaccine and are tested, mainly by HI assay, against current vaccine strains and representative circulating viruses. These sera panels are selected from different age groups and different geographical areas and distributed among WHO Influenza Collaborating Centers [100]. Postvaccination geometric mean HI titers (GMTs) of the serum panels to current vaccine strains are compared with those of circulating viruses; a significant reduction (>50%) of post-GMTs of a newly emerged virus compared with homologous GMTs of the vaccine strain usually suggests that the antibodies induced by the vaccine may not fully protect the individuals from infection with the new virus. An update of the current vaccine, therefore, may be required.

The HI assay has been used in clinical studies to establish a serological correlate of protection. Meiklejohn *et al.* demonstrated protection from natural H1N1 virus infection in vaccinated subjects with detectable HI antibodies [118]. The seminal study of Hobson *et al.* found that HI titers of 18–36 were associated with protection of 50% of individuals against experimental infection with an H2N2 virus [119]. These findings have been translated to a generally acceptable convention in influenza vaccine studies of using an HI titer of 40 as a measure of a 50% reduction in the risk of infection in susceptible populations or a 50% protective titer. More recently, meta-analyses of data collected in multiple studies, primarily in healthy adults less than 65 years of age, have confirmed that an HI titer of 40 is an acceptable approximation of a 50% protective titer, regardless of the virus type, subtype or strain [120,121]. However, although an HI titer of 40 provides a threshold value of protection in a population, there is no absolute HI titer that guarantees protection from infection. Furthermore, higher HI titers are associated with higher rates of protection [119,120]. Although high rates of serum HI antibody responses to LAIV may be seen in children, only a modest proportion of adults receiving LAIV develop serum HI antibody titers of ≥32 or 40 [122].

Additionally, it is not clear that the seroprotective HI titer 40 applies to protection in older adults, other groups at high risk from complications of influenza infection and in cases of infections with highly virulent H5N1 viruses, since data in such populations are lacking. Indeed, some studies suggest that cellular, rather than humoral, immune responses correlate with protection in older adult populations [123,124]. To detect responses to vaccination, paired serum samples collected at baseline and typically 14–28 days after vaccination, at the peak of the response in healthy, previously primed persons, are analyzed [125–127]. Antibody levels may wane by up to 50% within 6 months of immunization with inactivated vaccines [128]. Elderly individuals generally respond less well to influenza vaccines; responses may be delayed and fewer seroconversions detected compared with younger adults [129].

The extent of prior exposure to influenza virus through infection or vaccination, which becomes more complex with increasing age, influences timing, postvaccination titer achievement and quality of the antibody response. In addition, health status, and likely also host genetic factors, contribute to the robustness of a serum antibody response to vaccination. In populations that are seronegative at baseline, the postvaccination antibody GMT can provide a meaningful estimation of the robustness of a vaccine response. However, in seropositive populations, parameters including the mean fold increase in titer (postvaccination divided by prevaccination titer), seroprotective rate (proportion of individuals achieving an HI titer of ≥40) and seroresponse rate (proportion of individuals that have a fourfold or greater rise in titer from pre- to post-vaccination titer, where seronegative individuals achieve a titer of ≥40) are conventionally used. As shown in Table 2, these criteria are also applied by regulatory authorities within the EU and USA, requiring age-specific minimum criteria for licensure of influenza vaccines [130–132]. In the EU, the SRH assay is accepted as an alternate serologic assay for the regulatory evaluation of influenza vaccines. For vaccine evaluation, a 50% increase in SRH is accepted as a significant response and achievement of an SRH area of 25 mm^2 or greater is considered a 50% protective titer [62,132,133]. While serum HI antibody is a widely accepted immune correlate of protection for inactivated influenza vaccines, serum antibody and nasal wash IgA are independent correlates of protection for live-attenuated vaccines [9,134].

The greater sensitivity of VN assays have made them a useful adjunct for detection of serum antibody responses to prepandemic vaccines targeting avian subtypes, particularly H5 and H7, which generally demonstrate poor immunogenicity in naive human populations [135,136]. However, because there is no known protective titer for neutralizing serum antibodies, regulatory authorities generally rely on the HI assay and the criteria established for interpandemic vaccines to evaluate and license vaccines against avian subtypes. To improve sensitivity for the detection of antibodies to H5 and H7 subtypes, the traditional HI assay has been modified by substituting horse RBCs for those of avian species [35]. EU guidelines recommend that neutralizing antibody also should be measured when evaluating avian subtype vaccines.

ELISA is a useful method for detecting immunoglobulin subclasses induced by vaccination, which can provide further information as to the quality of the response [75]. The sensitivity of the ELISA may be particularly beneficial for detection of nasal wash immunoglobulin stimulated in response to LAIV. In previously seronegative recipients of LAIV, resistance to experimental influenza A virus challenge has been correlated with detection of nasal wash IgA by a HA-specific indirect ELISA [9,73]. However,

Table 2. Recommended immunogenicity criteria to support licensure of inactivated influenza vaccines[†].

Regulatory agency	Age group (years)	Seroconversion rate (%)[‡]	Seroprotection rate (%)[§]	Seroconversion factor[¶]
EMA Committee for Human Medicinal Products	18–60	>40	>70	>2.5
	>60	>30	>60	>2
US FDA Center for Biologics Evaluation and Research	18–64	≥40[#]	≥70[#]	Not stated
	≥65	≥30[#]	≥60[#]	Not stated

[†]For annual relicensure of influenza vaccines in the EU or new licensure of an influenza vaccine in the USA [130,131].
[‡]The seroconversion rate is the proportion of subjects with a ≥fourfold rise from a hemagglutination inhibition (HI) titer of <10 to ≥40 or a ≥fourfold rise in postvaccination HI titer in subjects with a prevaccination titer of ≥10; in the EU, the proportion of subjects with a prevaccination single radial haemolysis (SRH) titer of 0 and postvaccination SRH titer of ≥25mm^2 or ≥50% increase in SRH zone size may be used.
[§]The seroprotection rate is the proportion of subjects with a HI titer of ≥40; in EU SRH titer of ≥25mm^2 may be used.
[¶]The seroconversion factor is the ratio of postvaccination HI geometric mean titers (GMTs) to prevaccination HI GMT; in the EU SRH titers may be used.
[#]Lower limit of a two-sided 95% CI.

there is no standardized method or reagents for the detection of local IgA antibodies. Sample collection method (e.g., nasal wash versus nasal wick or swab), as well as assay procedure, can influence the sensitivity and reproducibility of antibody detection. In particular, it is important to standardize serial samples collected from the same or different individuals, based on total protein or total IgA content. Recently, Taalat *et al.* reported that detection of rises in serum IgA by indirect ELISA was a more sensitive measure of response to an avian H7N3 LAIV than either serum horse erythrocyte HI or VN assays [137]. Similarly, serum IgA responses were detected more frequently than serum HI or VN responses in volunteers given avian H5N1 LAIV [138]. However, the biological significance of serum IgA detection and its relevance to protection remains unknown.

Recently there has been renewed interest in the role of anti-NA antibodies in influenza virus infection and the potential role that vaccines strategically incorporating NA may have in eliciting immunity [139,140]. While clearly not the primary humoral immune response, antibodies to NA have been shown to inhibit the release of virus from infected cells, thereby reducing virus replication and preventing disease [8,141–144]. Anti-NA antibody response is measured by a NA inhibition assay using fetuin as the substrate. One method for detecting NA activity, based on the classical NA assay [145], detects released sialic acid by the periodate-thiobarbituric acid reaction [146]. Another method is based on the detection of galactose, which is exposed when the sialic acid is removed [147]. Currently, licensed, inactivated vaccines are all likely to contain NA, but the quantity and quality of NA is not defined. Optimizing the antibody response to NA would require the standardization of the NA content of inactivated vaccines [148].

Standardization of assays

Interlaboratory variability in serological techniques and determination of assay end points poses a considerable challenge for comparing the immunogenicity of candidate influenza vaccines in clinical trials, which may complicate vaccine licensure. Recent global interest in performing seroprevalence studies to better understand the extent of human infection with the pandemic 2009 H1N1 virus has also highlighted the need for improved standardization of assays and study design. Although standard protocols are used by experienced laboratories, and assay variability within laboratories is usually low, several international collaborative studies have confirmed that assay reproducibility between laboratories remains a concern. One study compared the reproducibility of the HI performed with avian erythrocytes compared with the SRH assay using a standard panel of sera and viruses. In this study, interlaboratory variability for the HI was considerably higher than for the SRH assay [63]. The species and method of standardization of RBCs as well as the efficiency with which sera are treated by receptor-destroying enzymes to remove nonspecific inhibitors may contribute to variability in HI results between laboratories. More recently Stephenson *et al.* reported that both inter- and intra-laboratory variation among VN assay results were significantly poorer than for the HI assay detecting human antibodies to H3N2 viruses, although the latter still showed variation between laboratories consistent with the earlier findings [44,63]. Operator experience, lack of common assay protocols, quality of cells used and, in particular, the amount and standardization of virus used, may contribute to poor reproducibility and interlaboratory VN assay variability. Nevertheless, in both studies, the use of a standard serum to normalize results within a laboratory substantially reduced variation between laboratories. Although the use of bioassay standards to improve interlaboratory agreement is well recognized for other infectious agents, the antigenic diversity and continuing evolution of influenza viruses, coupled with the time taken to develop and validate such reagents complicate their use for interpandemic serologic analyses [149,205]. However, the urgency for developing immunogenic and effective vaccines against HPAI H5N1 viruses and more recently the 2009 H1N1 virus has stimulated efforts to prepare, assess and validate international serology standards for the global evaluation of prepandemic H5N1 and pandemic H1N1 vaccines. The use of a candidate international standard (07/150), prepared from pooled plasma from persons immunized with a clade 1 H5N1 virus, was shown to reduce interlaboratory variability in horse erythrocyte HI and VN assays by at least 50% [45]. However, this effect was clade-specific as the same standard did not reduce variability of assays using clade 2 H5N1 viruses. The WHO Expert Committee on Biologic Standards assigned a unitage of 1000 IU to 07/150 as an antibody standard for clade 1 H5N1 HA. Using a similar approach in 2009,

a pandemic H1N1 candidate international standard was prepared and validated in just a few short months [150]. These reagents are the first two human antibody international standards for use in influenza serologic assays, and therefore represent a new approach to improve interlaboratory agreement for serologic assessment of influenza vaccines and seroprevalence studies. The use of standard operating procedures to qualify serologic assays should also improve intra- and inter-laboratory variability [151].

Expert commentary & five-year view

At present, HI and VN assays remain the most reliable and specific serologic methods for the antigenic characterization of influenza viruses, detection of human infections and assessment of influenza vaccine responses. Because they use live or inactivated unpurified virus as an antigen source, which can be prepared within days of identification of a novel virus, they also provide the most rapid approach to address questions of antigenic identity and cross-reactivity of a newly emerging virus, as was recently demonstrated for the 2009 H1N1 virus [152]. However, both methods are limited by the complexity of assays that employ cell-based systems to measure interactions between polyclonal serum and a complex, multi-epitope antigen. The need for manual detection of end point titers for the HI and some VN assays also limits full automation of assays which is a priority for many influenza laboratories. The development of a cell-free system, such as the use of glycan receptor-coated beads or other matrices, is one approach to improve reproducibility and throughput of serologic methods that detect strain-specific functional antibodies that inhibit receptors. However, more detailed knowledge of the structures of glycans recognized by influenza viruses on natural receptors on respiratory epithelium, as well as the multiple molecular determinants on the HA that influence receptor binding are needed to facilitate the optimal development of synthetic glycan systems for serologic methods. Simplification of VN assay procedures should also improve reproducibility and sample throughput. Recently, Hossain *et al.* described the development of a MDCK cell luciferase reporter system that could provide a simplified, yet sensitive approach to detect virus replication in neutralization assays [153]. Baculovirus expression of recombinant proteins is now relatively routine including the preparation of native trimeric HA [154,155]. This should allow for the development of immunoassays with greater specificity for detection of at least subtype-specific, and optimally strain-specific, responses. One such application is the use of label-free biolayer interferometry technology to measure binding between serum antibodies and recombinant trimeric HA immobilized on a fiberoptic biosensor [156]. Initial studies have shown a good correlation for the reactivity of ferret antisera in this assay compared with the HI assay. However, at present, such assays do not discriminate total antibody binding to HA from those with receptor-binding inhibition or VN properties. Future use of this or similar technology may offer micro-array binding formats and the ability to detect antibody inhibition of a synthetic glycan receptor(s) to improve specificity and detection of functional antibodies and use of standardized, cell-free reagents that reduce interlaboratory variability [156]. Improved sensitivity and specificity of antibody-binding assays that detect influenza virus-specific antibodies at mucosal surfaces including optimal and unified methods for sample collection [157] are urgently needed to better understand correlates of protection for live-attenuated vaccines and other mucosally delivered next-generation influenza vaccines under development [150]. Since new influenza vaccine targets include antibodies directed against the stem region of the HA and non-HA targets such as influenza A virus NA and M2 proteins, development and validation of standardized serologic assays to assess such antibodies are also needed [158–159].

The 2009 H1N1 pandemic stimulated global interest in performing serosurvey studies to better understand the frequency of and risk factors associated with human infection in different settings and populations. This effort highlighted the difficulties often associated with rapidly obtaining and storing serum samples due to the need for trained clinical and laboratory staff and facilities, lack of participant compliance with venous blood collection, or ethical considerations, particularly for pediatric cohorts. For these reasons, simpler sampling methods, including collection of filter paper-dried blood spots warrant future investigation [160]. In conclusion, regardless of the sampling method, the target antigen, or assay used to detect antibody to influenza, it is increasingly apparent that laboratories conducting influenza surveillance and vaccine evaluation should agree on the use of standardized protocols and antibody standards to assess comparability of results.

Key issues

- Humans are repeatedly exposed to an antigenically and genetically diverse series of influenza viruses over a lifetime. Therefore, strain-specific assays are essential for optimal characterization of viruses and detection of immunity to recent infection or vaccination.
- Hemagglutination inhibition (HI) and virus neutralization assays are reliable and specific methods for serologic confirmation of human infection with influenza viruses, monitoring virus antigenic variation and evaluation of responses to influenza vaccines.
- The gold standard for detection of a recent influenza virus infection or response to vaccination is the demonstration of a seroconversion, a fourfold or greater rise in antibody titer relative to a baseline sample, to the circulating influenza strain or vaccine strain.
- An HI titer of 40 or more has become established as a correlate of a 50% reduction in the risk of influenza infection in susceptible populations and is one criteria used to evaluate the immunogenicity of influenza vaccines for licensure.
- Progressive antigenic drift and circulation of multiple types and subtypes of influenza necessitates regular updating of the composition of influenza vaccines to reflect contemporary viruses. The HI assay is used to monitor antigenic relationships among viruses which is essential for consideration of changes in influenza vaccine composition.
- The use of qualified serologic assays and international antibody standards will improve intra- and inter-laboratory variability, respectively.

The development of the first international antibody standards for human influenza serologic investigations is an important step in this direction.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC or the US Department of Health and Human Services.

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