

REVIEW

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The application of pseudotypes to influenza pandemic preparedness

Francesca Ferrara¹, Eleonora Molesti¹ & Nigel Temperton^{*1}

ABSTRACT Human and animal populations are constantly exposed to multiple influenza strains due to zoonotic spillover and rapid viral evolution driven by intrinsic error-prone replication and immunological pressure. In this context, antibody responses directed against the hemagglutinin protein on the surface of the virus are of importance since they have been shown to correlate with protective immunity. Serological techniques, detecting these responses, play a critical role in influenza pandemic preparedness in particular with regard to the measurement of vaccine immunogenicity. As the recent human pandemics (H1N1) and avian influenza outbreaks (H5 and H7) have demonstrated, there is an urgent need to be better prepared to assess the contribution of the antibody response to protection against newly emerged viruses and to evaluate the extent of pre-existing heterosubtypic immunity in populations. This review compares pseudotype-based assays with wild-type and virus-like particle virus assays and discusses their place in the pandemic preparedness against the influenza virus. It additionally addresses the state-of-the-art developments of pseudotype-based assays (chimeric hemagglutinins, multiplex and post-attachment) including the development and future deployment of assay kits and approaches toward standardization to both preclinical and clinical endpoints. Progress toward the development of an influenza pseudotype library for the purposes of pandemic preparedness is also outlined and discussed.

Influenza: virus

Influenza viruses belong to the *Orthomyxoviridae* family and are negative sense segmented RNA viruses that can infect different avian and mammalian species, including humans. They are enveloped pleomorphic particles with a 120 nm average diameter, and length that can reach and exceed 300 nm (filamentous particles) [1]. On the basis of internal protein antigenic and phylogenetic characteristics, three influenza types, A, B and C, can be distinguished [2]. However, viruses with homology to influenza C have been described and provisionally classified as influenza D [3]. In their viral envelope (a lipid bilayer of cellular origin) influenza viruses harbor three different proteins: two glycoproteins – hemagglutinin (HA) [4] and neuraminidase (NA) [5] – and the transmembrane ion-channel M2 [6]. Influenza A viruses are then further classified into subtypes using antigenic characteristics (historical classification [2]) and phylogenetic characteristics (modern classification [7]) of the envelope glycoproteins HA and NA. Currently, 18 different HA subtypes are recognized and are divided into two lineages (Figure 1): group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, H18) and group 2 (H3, H4, H7, H10, H14, H15) [7–10]. The NAs are classified into 11 subtypes divided into two groups (group I: N1, N4, N5, N8; group II: N2, N3, N6, N7, N9; N10 and N11 are not included in the classification) [11]. However, there is debate about considering

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- neutralization assays
- pandemic preparedness
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¹Viral Pseudotype Unit, School of Pharmacy, University of Kent, Chatham Maritime, Kent, ME4 4TB, UK

^{*}Author for correspondence: Tel.: +44 1634 202957; n.temperton@kent.ac.uk

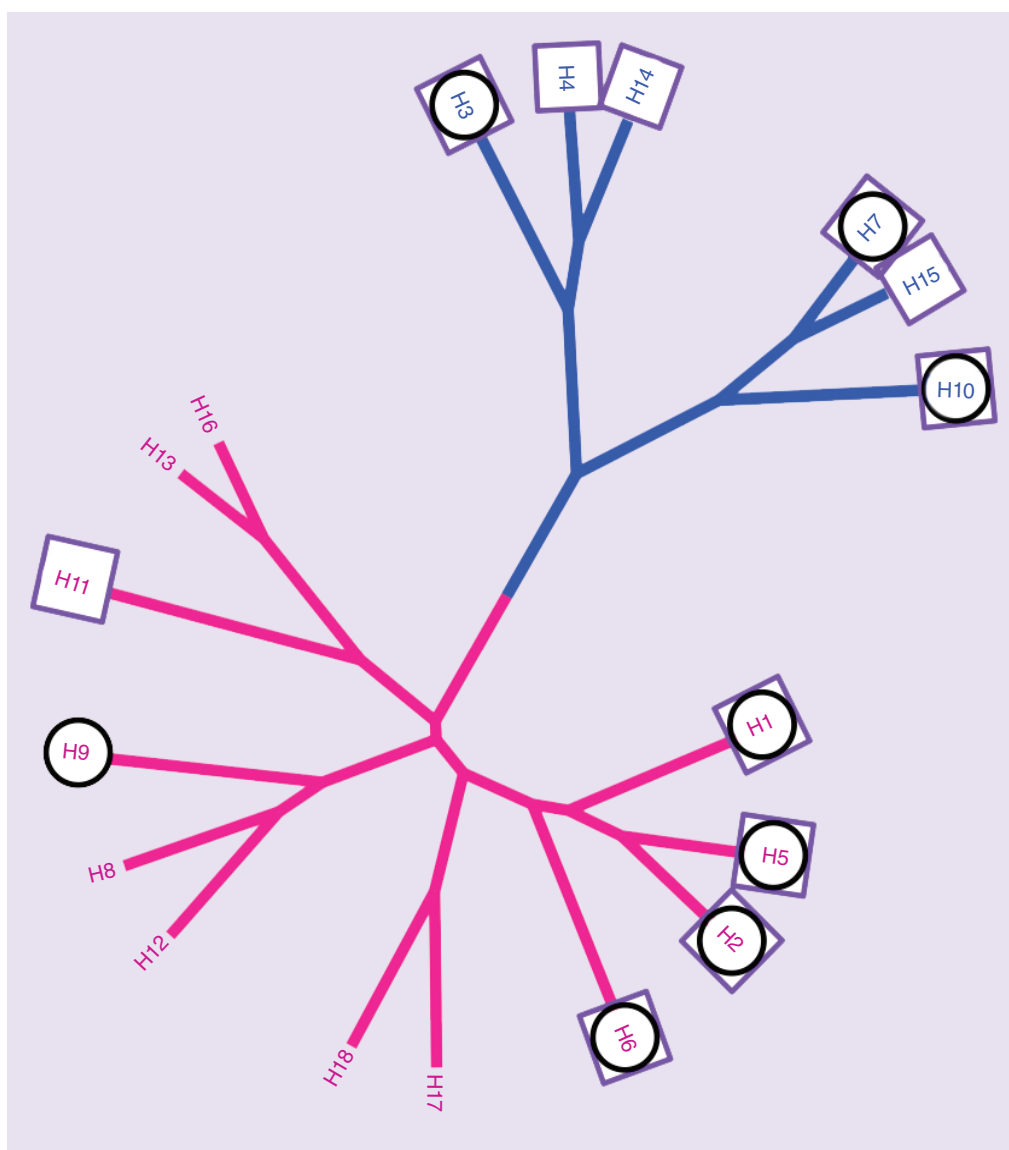


Figure 1. A phylogenetic tree of the influenza hemagglutinins H1 to H18. The HAs are divided into two groups (group 1 HA in pink and group 2 HA in blue). Highlighted with a circle are those subtypes that currently pose a pandemic risk. Highlighted with a box are those subtypes that have been pseudotyped. The tree was constructed using BEAST and FigTree.

HA: Hemagglutinin.

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N10 and N11 subtypes in the classical NA classification, as these proteins do not show sialidase activity [10,12–14].

The HA is a rod-shaped homotrimer with a stalk region and a globular head [15] that is necessary for viral attachment to the cellular receptor, sialic acid [16]. Usually the HA of avian origin viruses has higher affinity for α -2,3 sialic acid, while human viruses preferentially bind α -2,6 sialic acid [17]. Furthermore, the HA also mediates envelope–endosome fusion, since a pH-mediated

structural change [18] exposes a fusion peptide present in the stalk region (reviewed in [19]). The NA [5] is a sialidase that removes sialic acid from influenza-infected cells to facilitate viral progeny release [20,21]; *in vivo* its activity is also necessary to facilitate virus penetration of the mucus surface thus allowing access to the lung epithelium level [22,23]. In influenza C virus, the two glycoprotein functions are substituted by one, the hemagglutinin–esterase–fusion (HEF), that possesses both HA and NA activity [24,25].

For influenza A, the ion channel M2 has numerous functions during the influenza replication cycle: viral uncoating, stabilization of HA, viral particle assembly and membrane scission. For influenza B and C, the ion channel is named B/M2 and C/M2, respectively.

Present in the internal core of the influenza virus is the matrix protein M1 [26], the viral ribonucleoprotein complex within which the negative sense RNA genome is associated with the nucleoprotein and the polymerase complex (PA, PB1 and PB2) [27,28]. The viral core additionally contains the nonstructural protein 2 (NS2; nuclear export protein) but not the nonstructural protein 1, which is expressed during the cell cycle and has IFN-inhibiting activity.

Influenza: epidemiology & pandemic preparedness

Influenza viruses cause respiratory disease with variable severity of clinical symptoms. The infection can be asymptomatic, self-limiting to the respiratory tract or can be associated with extremely severe complications (e.g., pneumonia, myocarditis, influenza-associated myositis, rhabdomyolysis, encephalitis, Reye syndrome and Guillain-Barré syndrome). Classic influenza symptoms include fever, cough, sore throat, runny or stuffy nose, muscle or body aches, headache and fatigue [29]. The sudden onset and presence of fever, myalgia, headache and fatigue allows differential clinical diagnosis and permits the distinguishing of influenza from the common cold (rhinoviruses) and respiratory disease caused by other pathogens [30]. The clinical course depends on the viral virulence and the infected individual: age (child or elderly), pre-existing immunity, the presence of prior morbidity affecting heart (cardiopathic disease and/or hypertension) or lungs (e.g., asthma), diabetes, pregnancy, the presence of immunosuppression are all risk factors for influenza complication [31,32]. Some genetic determinants can also play a role in the development of severe disease [33,34].

The three influenza virus types infect different mammalian species. Influenza C infects exclusively humans and, even though its spread is limited in the human population, it can cause severe disease (e.g., pneumonia [35]). Influenza B infects humans causing seasonal epidemics [36] and is included in human seasonal influenza vaccines [37]. Influenza B has also been reported to infect other mammalian species, such as seals [38]. Influenza A viruses are disease-causing

agents in mammals (humans, pigs and horses primarily) and birds, and the viruses harboring H1–H16 have a reservoir in wild birds, especially *Charadriiformes* (gulls) and *Anseriformes* (ducks) [39,40]. To date, viruses harboring H17 and H18 have only been isolated in bats [9,10]. In humans only influenza A viruses that harbor H1 or H3 HA in their envelope circulate currently during winter seasons, causing annual epidemics [31,41], and for this reason are included in the human seasonal vaccine [42]. H2 strains circulated in humans between 1957 and 1968 and were associated with significant morbidity and mortality.

Human H1 and H3 influenza A viruses and influenza B viruses undergo antigenic changes of the surface glycoproteins (antigenic drift) leading to the appearance of new virus variants. Every year the WHO changes the seasonal vaccine composition on the basis of surveillance data in order to match the predicted circulating strains [42]. This choice is made twice each year, once for the northern hemisphere (in February for the next winter), the other for the southern hemisphere in October. The choice is made in advance since for vaccine production, clinical trials and stockpiling, at least 6 months are necessary [42,43].

In addition to viruses with H1, H2 and H3 HA, viruses of avian origin from other HA subtypes (e.g., H5, H7, H9 and more recently H6 and H10) have been shown to directly infect humans [44–50] and if these viruses were to acquire efficient human-to-human transmission they could potentially cause pandemics.

Furthermore, influenza viruses possess a segmented genome and reassortment between human, avian and/or swine viruses can occur (antigenic shift) if they infect the same host. It is thought that pigs can act as a mixing vessel for this reassortment. This was observed with the emergence of past influenza pandemics. In 1918, an avian-like human adapted H1N1 virus started to circulate in the human population causing the first pandemic of the 20th century [51,52]. Subsequently the 1918 virus underwent antigenic drift mutations and it has circulated as a seasonal strain until 1957 at which time a reassortment with an avian virus occurred [53]. This new virus had acquired the avian origin envelope proteins of the H2 and N2 subtypes. H2N2 viruses circulated for 10 years until, in 1968, they waned from the human population after a further reassortment event occurred, and the

H3N2 pandemic strain emerged [53]. After the pandemic, H3N2 strains have continued circulating even after the reappearance of an H1N1 virus in 1976 [54]. This virus has subsequently become a seasonal strain [55].

At the start of the 21st century it was believed that the next pandemic could emerge either via a reassortment between human viruses and viruses belonging to the highly pathogenic avian influenza (HPAI) H5 or H7 subtypes (predominantly circulating in poultry), or from an adaptation of these HPAI H5 or H7 viruses to the human host. For this reason active surveillance, collaborative networks, vaccines and pandemic plans were developed to monitor and block the circulation of these subtypes in poultry populations around the world. This in part may have led to the surveillance of other virus subtypes being neglected: for example, the emergence of new avian or swine-origin H1 or H3 viruses was believed not to be of concern, because previously, pandemics have always originated from viruses with HA subtypes different from the subtype already circulating in the human population [56,57]. Ultimately a quadruple H1N1 reassortant virus emerged causing the first pandemic of the 21st century after reassortment between two swine viruses: one was a Eurasian avian-like swine virus, the other originated previously through reassortment of North American avian, classical swine and human (H3N2) viruses [58,59].

In China, in 2013, a recombinant low pathogenic avian influenza (LPAI) H7N9 virus emerged [47]. This virus can easily infect humans causing severe respiratory syndrome but does not have the capacity for efficient human-to-human transmission [60]. Studies have shown that this virus and similar viruses have been circulating undetected in the avian reservoir and probably in poultry, and that this was ongoing for at least a year before the emergence of the human strains [61,62].

All these instances have shown that, to carry out effective pandemic preparedness of zoonotic viruses such as influenza, it is necessary to implement continuous animal surveillance. For example, HPAI H7 and H5 viruses are symptomatic and require notification to the authorities, and when outbreaks are confirmed, culling of farm animals is rapidly implemented to block the spread of the disease. This has permitted the monitoring of the spread of these viruses [57]. However LPAI influenza and swine influenza are often asymptomatic and they require the

monitoring of both sick and healthy animals (especially livestock) since these viruses can infect and lie undetected for extended periods of time. For this reason it is necessary to monitor the viruses circulating in all animal reservoirs through virus isolation and identification (with classical and molecular biology methods) [63,64] and via seroepidemiology studies in animals and humans to predict possible emerging viruses [65]. Another important monitoring tool is the collection of viral gene sequences [66–68], as using bioinformatic analysis it is now possible to identify the presence of molecular markers that can help to recognize viruses that pose a pandemic risk [69]. This ‘One Health’ approach (‘To improve health and well-being through the prevention of risks and the mitigation of effects of crises that originate at the interface between humans, animals and their various environments’ [One Health Global Network]) is now widely accepted by the international scientific community and applied with common effort by the Food and Agricultural Organization of the United Nations (FAO), World Organization for Animal Health (OIE), WHO, and US Centers for Disease Control and Prevention (CDC).

Influenza: antibody response – from anti-HA head to anti-HA stalk antibodies

The quantification of antibody responses induced by natural infection and vaccination, directed against influenza HAs, is an important facet of pandemic preparedness, since it permits evaluation of whether a population is subclinically infected and additionally prediction of the actual geographical spread of circulating viruses (assuming no assay cross-reactivity between subtypes). Furthermore, understanding how the immune response against influenza functions is of paramount importance for the design of vaccines with higher efficacy [70–73].

The mechanisms by which antibodies can neutralize influenza virus are now well characterized and have been explained in detail in a review by Han and Marasco [74]. From the initial studies on the influenza antibody response, it was clear that antibodies directed against HA were able to prevent viruses binding to the cellular receptor, blocking viral infectivity [75,76]. Initially, considering the characteristic antigenic drift of influenza virus and hemagglutination inhibition (HI) assay results, it was thought that minimal mutation in the HA could block the immunoglobulin (Ig) binding and that antibody

cross-reactivity between closely related strains was absent [77]. However, in the 1990s it became clear that antibodies able to neutralize different HA strains and subtypes could be generated *in vivo* and *in vitro* (Hoag presents an interesting historical perspective of this discovery [78]).

Recently, using different approaches, monoclonal antibodies showing pan- and heterosubtypic neutralization activity conferred by their ability to bind conserved regions on the HA stalk have been isolated in humans (F10, CR6261, CR8020, FI6v3, CR9114, CR8043) [79–84]. This has opened up the possibility not only for the use of these as therapeutic agents in antiviral therapy [85–87] but also has increased interest in stimulating such neutralizing antibodies using novel ‘universal’ vaccine approaches (reviewed in Krammer and Palese) [88].

Influenza: serology & HA antibody testing

The development of new ‘universal’ vaccines is however problematic: first, it is necessary to evaluate whether antibodies that can bind to the HA-conserved region are already present in the human population and, if present, at what level; subsequently, it is necessary to assess whether these new vaccines will be able to induce or boost a heterosubtypic stalk-directed neutralizing antibody-mediated response. Classical serological assays, such as single radial hemolysis (SRH), HI and microneutralization (MN) assays, have demonstrated low sensitivity for the detection of cross-neutralizing antibodies, especially those directed against epitopes in the HA stalk region [79–82,89]. Using ELISA or other binding assays (immunofluorescence and western blotting), it is possible to detect HA specific antibodies present in sera and other relevant samples (e.g., nasal wash, throat wash) [90] but it cannot differentiate whether these antibodies can neutralize influenza virus infection, since this assay evaluates the binding ability, and not the antibody function [91]. For this reason, over the past decade many laboratories have tried to develop new and more sensitive assays that possess all the advantages and characteristics of classical neutralization assays but without the risk associated with using wild-type viruses (essential for HPAI strains). For this purpose, surrogate recombinant viruses (pseudotypes) can be used.

Pseudotype particles

Pseudotype particles or pseudotypes are replication defective viruses that harbor on their

envelope a protein of one virus, but their core is constituted of the internal proteins of another virus, and in their genome a reporter gene is encoded [92]. Different viral cores are commonly used: vesicular stomatitis virus [93], gammaretroviruses (e.g., murine leukemia virus [MLV]) [94] and lentivirus (HIV) [95,96]. Lentiviral cores and lentiviral vectors are particularly useful since lentiviruses can integrate into the cellular genome of dividing and nondividing cells and this has led to their use in gene therapy and the development of many lentiviral vectors (reviewed in Sakuma *et al.*) [97].

Lentiviral pseudotypes are produced utilizing highly transfectable cell lines (usually human embryonic kidney HEK293 cells or derivative cell lines) and introducing plasmid constructs to produce the core proteins, the lentiviral genome encoding a reporter gene, and one encoding the envelope protein of the virus of interest. The pseudotyped viruses generated acquire the cellular tropism of the virus that donated the envelope protein, and the lentiviral capacity to integrate their genome into the target cell line (Figure 2) [98].

The reporter gene permits measurement of virus transduction of target cells and quantification of the viral contents (titer). Since they harbor in their envelope foreign proteins, pseudotypes are a useful tool to study both viral entry and antibody response. Another major advantage associated with the use of pseudotypes is that since they are replication defective viruses they can be used in low biosafety level laboratories [96]. The use of pseudotypes in serology for emerging and re-emerging viruses was recently reviewed by Mather *et al.* [99]. For influenza specifically, Garcia and Lai and Carnell *et al.* have reviewed their use in detail [100,101]. Here we will focus on the use of influenza lentiviral pseudotypes in neutralization assays, especially to detect stalk-directed neutralizing antibody responses as this has a greater relevance to pandemic preparedness against multiple HA subtype viruses originating from different species.

New & established serological assays for influenza

• Established assays

HI assay

The HI assay [102] is based on the principle that antibody binding to the HA globular head can inhibit the HA ability to agglutinate red blood cells (RBCs). Since the agglutination process

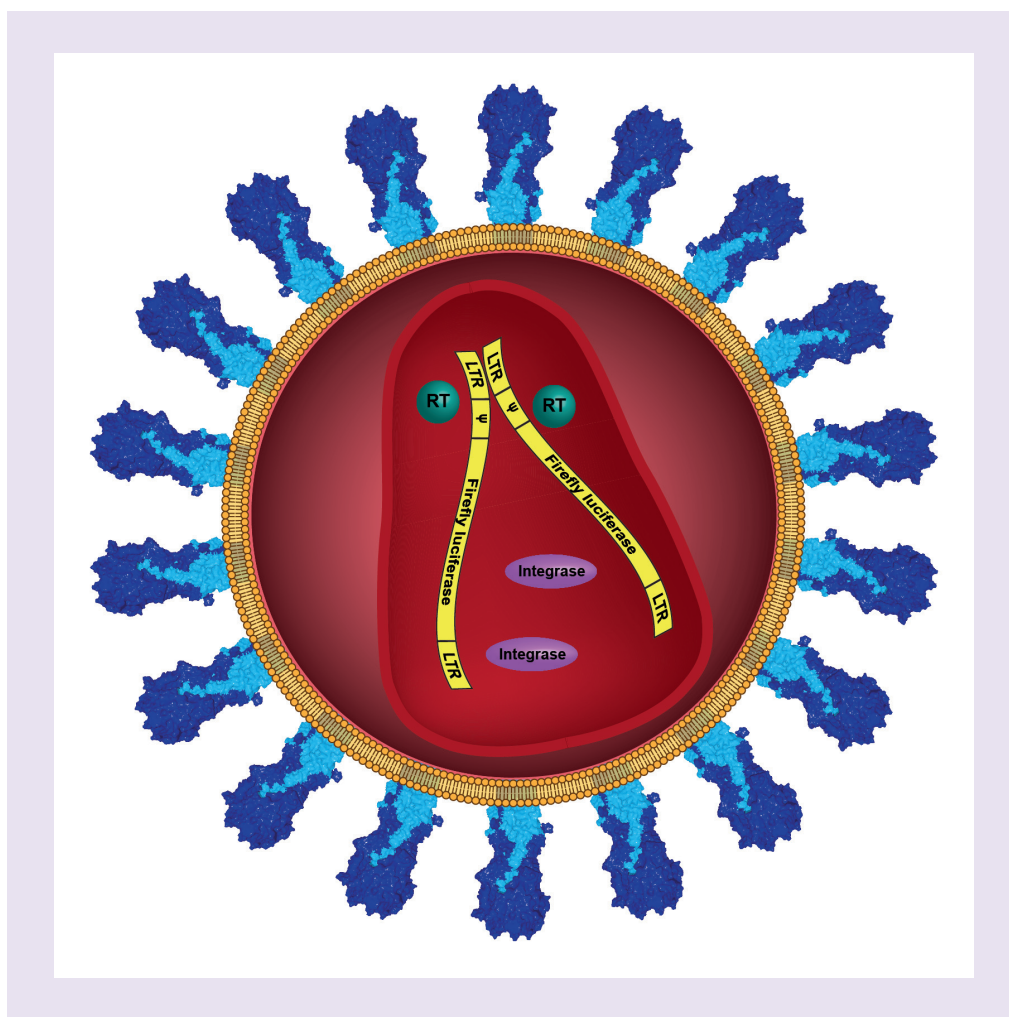


Figure 2. A schematic representation of an influenza hemagglutinin-pseudotyped lentiviral particle that carries a luciferase reporter. Hemagglutinin structure in light and dark blue representing HA2 and HA1 regions, respectively. LTR: Long terminal repeat; RT: Reverse transcriptase.

is mediated through the attachment of the HA receptor binding sites to the erythrocytes' sialic acid residues creating a virus-RBC lattice, the ability of antibodies to block this binding is an indirect measure of the ability to block viral attachment to target cells and in this way block viral infectivity. In the HI assay, two-fold serial dilutions of sera are prepared and a fixed amount of recombinant or viral origin HA antigen (previously calculated via HA assay) is added. After a 15 min to 1 h incubation, RBCs (0.5% chicken and turkey, 0.75% guinea pig and human type O or 1.0% horse RBCs) are added. Erythrocyte agglutination indicates the absence of virus specific antibodies (negative) and RBC precipitation shows the presence of antibodies (positive). Assay consistency and

correct interpretation of results is ensured by the addition of antibody positive/negative reference antisera controls, and RBC in absence/presence of HA antigens/viruses [103,104].

The major advantage of the HI assay is that correlation with protection against influenza has been described. Different studies have shown that HI titers >40 are associated with at least 50% protection from infection in adult humans [105–108]. Studies performed on children have shown that higher HI titers are necessary to attain 50% protection [109,110]. Even if the complexity of influenza antibody response in humans is difficult to summarize using cutoff values, the correlates are widely accepted and recognized, and this permits the use of HI as the preferred assay in vaccine evaluation studies [111].

However, as shown in different monoclonal antibody studies, a major disadvantage is that the HI assay cannot detect immunoglobulins that do not bind near the sialic-acid receptor site, such as the antibodies binding to the HA stalk region [79–82,89] and, for this reason, it cannot be effectively used in the evaluation of vaccines that aim to elicit such antibodies, the so termed ‘next-generation’ or ‘universal’ vaccines. Another disadvantage is the fact that sera can contain nonspecific inhibitors of hemagglutination that need to be inactivated prior to the testing procedure [112]. Furthermore, the standard HI assay is less sensitive than MN in detecting antibodies against avian influenza viruses [113]. This becomes a problem when the assay is applied to the evaluation of H5 prepandemic vaccines. For this reason, a modified HI assay that uses horse instead of turkey erythrocytes was developed [114]. Horse RBCs are richer in α -2,3 sialic acid and thus are bound with greater efficiency by avian influenza viruses. This modified version has shown good correlation with the MN assay for a wide range of avian influenza viruses [115]. Despite HI being widely used and easy to perform, problems with standardization and inter-laboratory reproducibility/agreement still exist. In different collaborative studies [116–118], it was observed that intra-laboratory assay variability is relatively low, but inter-laboratory variability is significant. Nevertheless, the same studies show that the use of an internal reference standard can improve the inter-laboratory results [116–118]. An interesting new adaptation of the HI assay is the use of an influenza pseudotype as the virus antigen in lieu of wild-type virus, which warrants further study [119]. This would additionally permit easy manipulation of key amino acids in the synthetic pseudotype HA to study their effect on HI titer.

SRH assay

The SRH assay [120,121] was developed as an antigen sparing modification of the single radial immunodiffusion test to offer an alternative to the more commonly used HI. In this assay, inactivated virus-coated RBCs are mixed with agarose and guinea-pig complement to form gel immunoplates in which, after cutting out circular wells, it is possible to add the de-complemented sera to be tested, usually undiluted. During the overnight incubation step if anti-HA antibodies are present, they can bind to the virus and, in the presence of complement, erythrocyte

lysis occurs. In this way the presence of antibodies appears as a zone of hemolysis around the well, the diameter of which is positively correlated to the quantity of antibody present.

The SRH assay is robust and shows little intra-laboratory variation [121]; also in inter-laboratory studies it exhibits less variation when results are expressed in the same format (e.g., surface area) [117]. Furthermore, it was observed that a $>25 \text{ mm}^2$ SRH area usually correlates with protection [107]. Together with the fact that it does not require wild-type viruses, this makes the SRH one of the assays of choice for vaccine evaluation studies, especially for influenza B where the HI assay is relatively insensitive compared with MN [111].

Disadvantages of SRH application in detecting antibodies exist: the assay detects only immunoglobulin subclasses (IgG1, IgG3 and IgM) that bind the complement complex (precisely the C1q complement protein, the first protein in the complement cascade) and, for example, since immunoglobulins of class A do not bind C1q, application of this assay to the study of the mucosal IgA-mediated response is not possible [121]. In addition, when performed against H5 viruses to test human sera, the samples require preadsorption against H1 and H3 viruses to remove possible cross-reactivity against viral internal proteins [111,122].

Hemagglutinin ELISA

ELISAs are regularly used to detect antibodies against proteins of interest. In influenza serology, they are applied to detect antibody responses in sera against HA and other influenza proteins. The focus here is on ELISA for HA antibody detection.

In the most widely used approach (indirect ELISA), the HA is coated onto the wells of a 96-well plate. After a blocking step, dilutions of the sera to be tested are added and the plate is incubated. Then a labeled secondary antibody that can recognize the test antibodies is added. After a further incubation, the assay is developed using an appropriate substrate and measured using a plate reader. An advantage of this assay is that by using specific secondary antibodies it is possible to differentiate antibody subclasses and test other sample types, such as nasal washes [123]. This is particularly useful for the detection of mucosal antibodies. The assay has high sensitivity but specificity needs to be improved. For example, to distinguish

between anti-head or anti-stalk responses, other approaches such as competitive ELISA [82,124–125] need to be used. Furthermore, ELISA detects only antibody binding (seroreactivity), it cannot give information about the neutralizing activity of the antibodies [91].

Plaque-reduction neutralization & MN assays

Plaque-reduction neutralization and MN assays are the only assays that directly evaluate the neutralizing antibody activity against wild-type virus [103]. In the plaque-reduction neutralization assay, sera are diluted and incubated with virus. After incubation to permit antibody attachment, the antibody–virus mixture is added to a target cell monolayer. After an hour incubation in which the unbound virus can enter the target cells, cells are covered with agarose to block the spreading of the viral progeny that will be produced after infection. In this way, instead of a generalized cytopathic effect, clear lysis plaques will appear after removing the agarose and staining with crystal violet. The neutralization is then evaluated as the reduction of the number of plaques in comparison to the virus infection control (in the absence of antibodies). The MN assay is performed in 96-well plates [126]. Since the plate format does not permit the counting of viral plaques, agarose is not used and the general viral cytopathic effect is evaluated. The neutralization activity is measured as the ability of sera to reduce the cytopathic effect due to inhibition of viral entry and subsequent replication. A more sensitive and quantitative approach is represented by the ELISA-MN. In this assay the readout is quantitative: an ELISA to measure influenza nucleoprotein is performed, and this correlates directly with viral infection. Another approach is the use of recombinant viruses in the neutralization assay [127]. This recombinant virus presents in its genome a green fluorescent protein (GFP) gene instead of the HA gene and is produced by exploiting the reverse genetic system (in this case an HA protein expressing plasmid is also necessary). Using this method it is not necessary to use an extra detection step since the readout is the GFP signal that is produced after viral infection. Furthermore, the system is also safe since lacking a proper HA genome segment, the recombinant virus cannot replicate in cells that do not express HA [127]. Neutralization assays can detect antibodies that are able to neutralize viral attachment and viral entry and for this reason they are able to detect also antibodies

binding to the HA stalk region [89]. For this, MN should be the assay of choice to evaluate influenza antibody response. However, it is not widely used since it requires wild-type virus and correlation of disease protection is not established despite the WHO routinely indicating a 1:80 titer as a cutoff and correlating this with protection [128].

Other binding assays

Western blotting and immunofluorescence represent two classical methods to detect antibody response [129]. As with ELISA, these assays can only detect the presence of binding antibodies and they do not give information about neutralization activity [91]. Western blotting can also be used in the characterization of epitopes recognized by the antibodies: during electrophoresis in the presence of reducing agents, the two HA subunits (HA1 and HA2) can be separated and antibodies against HA2 and HA1 can be distinguished, while antibodies that recognize stalk conformational epitopes can detect only the full HA protein [82,89,125].

Cell–cell fusion inhibition assay

Infection with viruses harboring proteins with fusion activity (such as *Paramyxoviridae* viruses) is usually evaluated by observing syncytia formation [130]. In its native state, influenza virus HA does not possess fusion activity but, when exposed to acid pH, it undergoes conformational changes and acquires fusion activity [19]. Since this function is mediated by the rearrangement of the stalk region, it is possible to evaluate antibodies that can bind to the HA stalk. Furthermore, all the broadly neutralizing monoclonal antibodies have been shown to inhibit this step, thus this assay (or a modification thereof) represents a direct approach to assess stalk antibody function activity [79–83,85,89].

The assay [89,131] is not easy to perform. Cells need to be transfected with an HA-expressing plasmid and after incubation to permit HA expression, cells are treated with TPCK-trypsin to enable the HA cleavage necessary to trigger the conformational change and exposure of the fusion peptide. Cells are then incubated in the presence of monoclonal antibodies or sera. After incubation, media is removed and low pH media added. Subsequently, formation of polykaryons (cells that possess more nuclei) is observed by microscopy; sometimes usage of tissue/cell staining can facilitate their visualization [79,132]. The

presence of polykaryons indicates the absence of antibodies that block the cell–cell fusion. Furthermore, another assay complication is the fact that some HA mutations have been shown to change the HA fusion pH [133,134], and between avian and human viruses fusion pH variations also exist [135]. Different groups [136] have tried to simplify the fusion assay using dyes or reporter gene systems, however, the use of this complex assay is still not widespread.

• New assays

Pseudotype virus neutralization assay

The adaptability of pseudotypes has been exploited not only for influenza basic research but also as an alternative/adjunct tool for serological surveillance and (pre) clinical applications, in particular for highly pathogenic strains such as H5N1 and H7N1. It has been demonstrated that influenza pseudotypes are highly sensitive when used in *in vitro* assays, and they can be used to rapidly, safely and accurately evaluate the neutralizing antibody responses elicited by vaccine candidates and/or by natural infections. The use of pseudotypes as alternatives to wild-type influenza viruses, including HPAI strains and pandemic strains, has been implemented in neutralization assays, also referred to as pseudotype-based neutralization (pp-NT) assays. Previous studies have reported that results from HI, MN and SRH assays correlate well with those obtained by pp-NT assay [128,137–139]. For the pp-NT assay, twofold serial dilutions of sera are incubated with a predetermined amount (quantification is done via the measurement of reporter gene expression) of pseudotypes and incubated for 1 h at 37°C in a 96-well plate. Subsequently, a fixed amount of target cells is added to the wells of the 96-well plate and transduction by the reporter is measured routinely after 48 h. As is the case for traditional virus neutralization assays, the titer of antibody can be expressed as the highest dilution of serum sample that inhibits transduction by usually 50 or 90% [140].

Different laboratories have shown good correlation between the pseudotype neutralization assays and other serological assays using sera of different origin [95–96,119,128,141–142]. Furthermore, Alberini *et al.* have used the correlation between an H5 pseudotype neutralization assay and MN to infer a possible cutoff titer associated with protection in the pseudotype assay, which is 1:357 [128]. Similar studies are yet to be

performed using other influenza subtype pseudotype strains. As observed in different studies the pseudotype neutralization assay shows higher sensitivity in detecting HA stalk-directed antibodies probably due to the lower HA density on the pseudotype surface in comparison with wild-type influenza virus [82,143].

Other functional antibody assays

To study HA-directed antibody responses, the application of the enzymatic virus-like particle (VLP) assay is a valid approach [144]. This assay is used to evaluate viral entry and is performed using VLP (particles harboring the original envelope viral proteins and original viral core proteins but without genomic material) in which the M1 protein is modified to a fusion protein with the β -lactamase enzyme. In this way when these particles enter into cells, a signal can be detected after adding the appropriate substrate. Since the incubation time is only required to permit viral entry, and it is not necessary to wait for the expression of reporter genes, this assay is very rapid. Furthermore, Tscherne *et al.* show that VLPs can be used as surrogate antigen in HI assays also [144].

Post-attachment pseudotype neutralization assay

Using pseudotypes as surrogate viruses, Oh *et al.* have modified the post-attachment neutralization assay for influenza developed by Edwards *et al.* [145,146]. In this assay, pseudotypes are incubated at 4°C with cells to permit the synchronization of the virus binding to the cell surface and to block viral endocytosis. Diluted sera are then added, and following another 4°C incubation, plates are transferred to 37°C to permit transduction [145]. In the post-attachment pseudotype neutralization assay, neutralization is observed only when the antibody is blocking the endocytosis step and subsequently HA conformational changes necessary for virus-endosome fusion. Antibodies that have neutralizing activity through impeding viral attachment will produce negative results in this assay. For this reason the assay is useful for evaluation of stalk-directed antibodies that do not inhibit viral attachment [145]. Post-attachment neutralization assays have the ability to differentiate stalk-directed antibodies from head-directed antibodies and can be used to verify the presence of stalk antibodies. Another possible approach could be to use pseudotypes harboring mutated

HAs mimicking the HAs of broadly neutralizing mAb escape-mutant viruses. This last approach may also give precise information about the recognized epitopes: if the antibodies present in the sera recognize the same epitopes of broadly neutralizing mAbs, sera will not be able to neutralize the mAb escape-mutant HA pseudotypes.

Chimeric HA pseudotype particle neutralization assay

To study the antibody response directed against the HA stalk, the Palese group has developed pseudotype particles and influenza viruses exhibiting on their envelope a chimeric HA. This chimeric HA has a stalk region and a globular head originating from two different HA subtypes or strains [147]. To create this chimeric construct, they have exploited the presence in the HA of two cysteines, Cys52 and Cys277, that are engaged in a disulphide bond and that separate the head and stalk regions. In fact, the HA gene portion between the Cys52 and Cys277 codons encodes for the amino acids that constitute the HA globular head and thus it was possible using DNA recombinant technology to exchange the HA head of one virus with the HA head of another influenza strain. Additionally, the same cysteines have been used to construct a headless HA to be used as a vaccine to induce stalk-directed antibodies [148]. The concept behind the idea of using these modified HAs to detect exclusively HA stalk-directed antibodies is that since the antibody response is principally directed against the HA head, testing sera against an HA that has a different head will permit easy detection of antibodies against the more conserved stalk region instead of the antibodies generated against the original HA globular head since they will not recognize an antigenically unrelated head. Furthermore, these chimeric HAs can also be used as a vaccine to elicit stalk-directed antibodies [149,150]. In different studies [147,151], the chimeric HA, even if not utilized in the pseudotype platform, but in ELISA, has been demonstrated to be a useful approach to study and detect exclusively cross-reactive antibody responses directed against the stalk.

Influenza: pseudotype resource/library

One facet of pandemic preparedness that pseudotypes particularly lend themselves to is the establishment (prepandemic) of a pseudotype resource library consisting of influenza HA and

NA envelope glycoprotein expression vectors and their cognate retroviral pseudotypes. In order to prioritize the strains that will constitute a high-utility library, the following procedures (among others) can be performed: use of WHO/OiE/ProMed news feeds, conference abstracts, queries instigated by potential library end users via email, website, social networks, published literature (human/animal outbreaks/surveillance data/vaccine studies), phylogenetic analysis and antigenic cartography [152]. This information can then inform custom gene synthesis of surface influenza glycoproteins (HA and NA) from candidate viruses and subcloning into expression vectors using an array of different promoters (CMV, β -actin) to effect high-level expression in producer cells. Once nucleic acid sequence and expression has been confirmed, HIV or MLV vector systems can be used for pseudotyping as detailed previously, using a GFP reporter as it allows visualization of individually transduced cells. In parallel with the collection of library strains, the use and further development of retroviral vectors carrying different reporter genes (GFP/RFP/YFP, luciferase, B-gal, secreted alkaline phosphatase) for use by end users in a range of sectors (pharma/high-throughput, low resource/field, multiplex, etc.) should be prioritized. The resource would additionally lend itself to optimization of assay protocols and standardization of virus stocks depending on end-user assay requirements and laboratory facilities and to the training of external users. In a manner analogous to the establishment of vaccine seed stocks in readiness for a pandemic caused by a new influenza strain [153], a pseudotype library would enable neutralization assays to be rolled out very soon after strain identification. While there may be some mismatch at the amino acid level, a comprehensive HA library would permit a surrogate cross-reactive strain to be employed as a preliminary measure until the outbreak strain had been sequenced and deposited in the NCBI [68] or other database [66] to allow for synthetic gene synthesis. Starting from a synthetic gene, production can be achieved in 2–3 weeks as methodologies have been largely optimized and streamlined. In fact, influenza HA pseudotypes have now been constructed by many groups based on multiple HA subtypes originating from different species, human, avian, swine and equine. In one such study, the full complement of Group 2 HA pseudotypes was constructed via the cotransfection of

the TMPRSS2 protease in order to enable the cleavage of HA necessary for infectivity [154].

Influenza: multiplexing pseudotype neutralization assays

A further refinement of the pseudotype neutralization assay is the ability to multiplex, which is infrequently described in the context of functional serological assays. This is particularly relevant for avian influenza. Outbreaks of H5 and H7 lineage LPAI and HPAI viruses in poultry have been increasing over many years with concomitant implications for both public and animal health [65]. Evolution of these subtypes (sometimes under vaccine pressure) and the emergence of new antigenic variants can make it difficult to undertake effective surveillance and to implement pandemic preparedness strategies aimed at stemming avian influenza outbreaks. The development of a multiplex assay for the study of neutralizing antibodies that are directed against both influenza H5 and H7 HA has been described recently [155]. In this study, multiplexing was achieved using lentiviral pseudotype viruses carrying two different luciferase reporters, firefly and renilla (Figure 3), although in theory two different fluorescent reporters could also be used (GFP and RFP for example). Given appropriate test

sera, this novel assay permits the quantification of neutralizing antibody responses against two genetically and antigenically distinct HAs (H5 belongs to group 1, H7 to group 2; see Figure 1) in the same serum/plasma sample, thus increasing the amount and quality of serological data that can be acquired from an often-limited volume of sera. This assay can therefore be described as serum sparing compared with traditional serological assays. Sera obtained from chickens vaccinated with a monovalent H5N2 vaccine, chickens vaccinated with a bivalent H7N1/H5N9 vaccine or turkeys naturally infected with an H7N3 virus were evaluated by multiplex assay and the results were found to correlate significantly with data obtained by HI assay, the predominant assay used for avian influenza surveillance [155]. Additionally, with relevance to both the standardization of pseudotype-based assays and also the development of kits for easy worldwide deployment, the study additionally showed that influenza pseudotypes are highly stable under basic cold-chain storage conditions and following multiple rounds of freeze thaw.

Influenza: pseudotype neutralization kits

Molesti *et al.* showed that influenza pseudotypes are highly stable under basic cold-chain storage

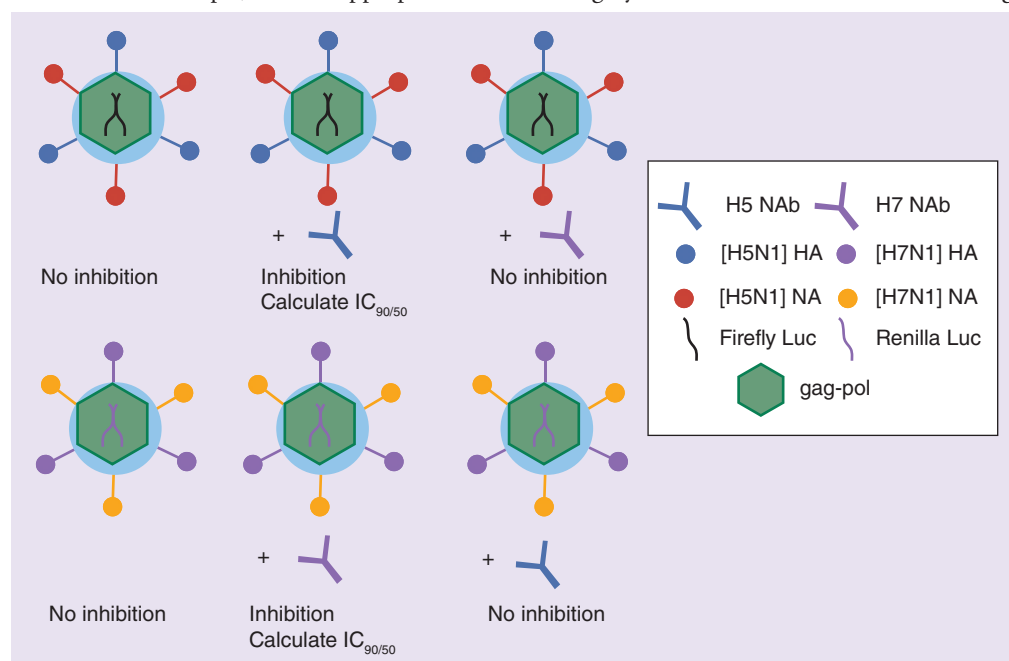


Figure 3. A schematic showing the multiplex neutralization assay for highly pathogenic avian influenza H5N1 and H7N1 by using retroviral vectors carrying firefly (for H5N1) and renilla (for H7N1) luciferase.

NAb: Neutralizing antibody.

conditions and following multiple rounds of freeze-thaw. This may thus have practical utility for in-field serosurveillance and vaccine studies in resource-limited regions worldwide [155]. However, one logistical issue with developing comprehensive collections of influenza HA pseudotype stocks for pandemic preparedness is that they necessitate -80°C long-term storage and cold-chain maintenance during transport. This limits the scope of dissemination and application throughout resource-limited laboratories. A recent study has investigated the effects of lyophilization on influenza pseudotype stability, with a view to developing a pseudotype virus neutralization assay based kit suitable for affordable global distribution [156]. Infectivity of the influenza pseudotype was calculated after lyophilization and immediate reconstitution, as well as subsequent to incubation of freeze-dried pellets at varying temperatures, humidities and time-points. The integrity of HA glycoprotein structure following lyophilization treatment was additionally assessed by employing lyophilized pseudotype in downstream neutralization assays. In the presence of 0.5 M sucrose-PBS cryoprotectant, the freeze-dried influenza pseudotype was stably stored for 4 weeks at up to 37°C and could be neutralized to the same potency as unlyophilized pseudotype when employed in a neutralization assay. These results confirm the viability of a freeze-dried pseudotype-based kit, which could significantly facilitate low-cost serology for a wide portfolio of influenza subtypes.

Influenza: pseudotype standardization

The R&D and ultimate licensing of a broadly neutralizing universal influenza vaccine will likely require the use of more sensitive assays than those currently used for seasonal vaccines [111]. Further development and validation of influenza pseudotype-based assays as described above is highly complementary to studies on understanding the breadth of universal vaccine-induced immune responses in preclinical animal models and ultimately in humans. To be able to demonstrate the reliability of a new serological assay, such as the pseudotype-based neutralization assay, a validation process is required. A validation process for the pseudotype assay is required to be carried out according to International Guidelines (specifically the ICH Harmonized Tripartite Guideline Q2). As part of the validation protocol the criteria of specificity, accuracy,

linearity, precision and robustness of the method need to be studied and evaluated. The specific reagents and protocols for influenza pseudotype production and for the pseudotype neutralization assay will need to be studied and standardized as factors during the validation process. With respect to robustness criteria, the impact of changes linked to these (operative steps of pseudotype assay, and reagent set changes) require evaluation. Examples of changes linked to the protocol are influenza pseudotype concentration, incubation time (with sera) and incubation temperature. Example of changes linked to the use of a different reagent is the use of an alternative production lot or different cell culture medium.

Input of pseudotype virus is of paramount importance in standardization of this assay. To this end, the standardization of the pp-NT assay could be performed by normalizing on the basis of the transduction activity (relative luminescence units, RLUs) of each separate pseudotype. This approach is optimal since it permits direct comparison with MN assays (in a neutralization assay 50% Tissue Culture Infectious Dose, TCID_{50} , is used as indicator of viral infectivity). However, some laboratories have argued for the need to normalize on the basis of other variables, such as the HA content, or by other methods to permit comparison between results [100].

Different laboratories exploiting pseudotypes determine the viral input (pseudotype titer) to be used in neutralization assays by evaluating the number of viral particles [119,157–158]. These quantification methods usually consist either in quantification of the HIV viral core protein p24 content (directly correlating with number of particles) of pseudotype preparation, or by quantification of pseudotype genomes through real-time reverse transcriptase (RT)-PCR or, in theory, evaluating pseudotype RT activity. Evaluation of p24 protein content and of pseudotype RT activity can be readily achieved using commercial kits that are commonly employed in HIV testing. Evaluating pseudotype titer using particle number does not necessarily correlate with viral entry since particles without envelope glycoprotein cannot enter target cells (an issue for p24 measurement or quantitative RT-PCR, since these methods quantify the particle number and not the ability of the particle to enter into cells, the factor that is evaluated in a neutralization assay). Technical problems can be encountered also

when normalizing viral input on the basis of HA content. In fact, the HA content could be interpreted as the total HA protein amount, the amount of only the active (cleaved) HA or the HA units. Evaluation of total HA quantity can be easily achieved using ELISAs that are already commercially available or that can be developed in-house; however the price of these commercial assays is still high and it is necessary to use a specific kit for each and every HA subtype or strain. Quantification of HA units can be easily performed with a hemagglutination assay; however, it has been shown that normalizing the virus input using HA units instead of reporter gene expression can result in lower reporter signal levels for some pseudotypes since a low number of particles are used [147]. This is probably due either to differential HA expression on the viral surface or to a decrease of HA affinity for sialic acid and consequentially a reduction in viral entry.

To quantify cleaved and uncleaved HA, quantitative western blotting using densitometry needs to be performed. This assay requires a complex development and standardization: internal loading controls need to be used, membrane transfer needs to be consistent between different experiments, quantity standards need to be prepared and validated. Furthermore this assay requires the use of specific antibodies against the protein of interest, and for this reason it will be necessary to isolate at least two antibodies (or polyclonal preparation) for each HA subtype that needs to be tested: one that recognizes HA1 and one that recognizes HA2; a third antibody that recognizes HA0 could also be useful.

For standardization and validation of the pseudotype neutralization assay, a fine characterization of pseudotype input using HA quantity measures, preferentially the amount of cleaved and uncleaved HA simultaneously, will be necessary to support statistical analysis and will require further study (e.g., quantification method development and standardization). Correlation between HA content (however this is measured) and transduction units (RLU or GFP) or particle numbers (p24 or electron microscopy) needs to be established during any validation process. The difficulty to quantify cleaved and uncleaved HA content in pseudotype preparations could make direct comparison between IC_{50} obtained using different HA subtypes problematic, since IC_{50} is related to

antibody affinity that is dependent additionally on the antigen content (e.g., some pseudotypes could be intrinsically easier to neutralize than others because they harbor a smaller number of HA molecules in their surface). However, it should be noted that in classic neutralization and MN assays the HA content of the viral input is not usually evaluated and the same problems also apply. As the state of the art, it is the authors' opinion that the use of RLU for normalization is the most effective method.

The added value of pseudotype neutralization assay development consists of the ability to perform a reliable neutralization assay in a laboratory with a low biosafety level, and additionally the take up of this assay as an analytical service to Big Pharma, WHO, CDC and OIE. The pseudotype neutralization assay will allow analysis of large numbers of samples in a limited time and consequently with a low cost/benefit ratio.

Conclusion

In conclusion, it can be seen that lentiviral and retroviral vectors, originally developed for gene therapy purposes, have rapidly gained an impressive foothold in the pandemic preparedness canon via the pseudotyping of influenza HA onto their surface in order to produce surrogate influenza viruses. These influenza pseudotypes have been developed into serological assays that are being increasingly used by many sectors for serosurveillance, vaccine immunogenicity and monoclonal antibody testing. They are inherently safe to use with the full array of unmodified HAs and have shown increased sensitivity for the screening of stalk-based antibodies, thus placing them as the assay of choice for universal vaccine research, a major cornerstone of pandemic preparedness.

Future perspective (5–10 years)

It is envisaged that protocols will be available for the efficient production of influenza pseudotypes based on all 16 HA subtypes circulating in wild birds. These protocols will incorporate optimized HA cleavage conditions (using cotransfected proteases) and where applicable the cotransfection of other influenza proteins (NA or M2). The standardization of the pseudotype neutralization assay to clinical endpoint will have ideally been performed and recommended protocols will be made available by the WHO and OIE to all interested end users. Also,

antibody standards will have been prepared by public health and regulatory bodies and distributed to key reference laboratories to enable extensive inter- and intra-laboratory variability assays. There will be widespread usage of influenza HA pseudotypes on high-throughput liquid-handling assay systems for use in serosurveillance, vaccine immunogenicity testing and for the screening of antivirals and therapeutic monoclonals.

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EXECUTIVE SUMMARY

Influenza: serological assays for pandemic preparedness

- Antibody responses directed against hemagglutinin (HA) on the surface of the influenza virus are important as they have been shown to correlate with protective immunity.
- Serological assays play a critical role in pandemic preparedness as they permit evaluation of pre-existing immunity within populations and also the evaluation of vaccine immunogenicity.
- The most widely used serological assays for the detection of antibodies against HA are hemagglutination inhibition (HI), single radial hemolysis (SRH), ELISA, and microneutralization (MN).

Influenza: new serological assay developments

- Classical serological assays, such as single radial hemolysis, hemagglutination inhibition and microneutralization assays, have demonstrated low sensitivity for the detection of cross-neutralizing antibodies, especially those directed against epitopes in the HA stalk region.
- Influenza pseudotypes having a lower density of HA on their surface than their wild-type counterparts are highly sensitive for the measurement of antibody responses against both the HA globular head and the stalk.
- Post-attachment pseudotype neutralization assays have the ability to differentiate stalk-directed antibodies from head-directed antibodies and can be used to verify the presence of stalk antibodies.
- Chimeric HA pseudotypes have been produced that have a stalk region and a globular head from two different HA subtypes. These can be exploited in neutralization assays to delineate antibody responses against either the head or the stalk.

Influenza: pseudotype resource/library

- A pseudotype resource library consisting of influenza HA and neuraminidase envelope glycoprotein expression vectors and their cognate retroviral pseudotypes can contribute significantly to pandemic preparedness.
- An influenza HA pseudotype library would enable neutralization assays to be rolled out very soon after strain identification as was done for the recent H7N9 strain.
- The use and further development of retroviral vectors carrying different reporter genes (GFP/RFP/YFP, luciferase, B-gal, secreted alkaline phosphatase) for use by end users in a range of sectors (pharma/high-throughput, low resource/field, multiplex etc.) is being carried out.

Influenza: multiplexing pseudotype neutralization assays

- A multiplex assay for the study of neutralizing antibodies that are directed against both influenza H5 and H7 HA using pseudotype viruses carrying two different luciferase reporters, firefly and Renilla, has been developed. This assay should be readily adaptable to other pairs of subtypes, for example, H1 and B or H3 and B (for seasonal vaccine testing).
- The multiplex neutralization assay permits the measurement of responses against two antigenically distinct HAs in the same serum/plasma sample thus increasing the amount and quality of serological data that can be acquired from valuable sera.

EXECUTIVE SUMMARY (CONT.)

- A robust multiplex assay may have practical utility for in-field serosurveillance and vaccine studies in resource-limited regions worldwide.

Influenza: pseudotype neutralization kits

- Lentiviral pseudotypes displaying influenza virus HA can be lyophilized and retain high levels of infectivity after reconstitution.
- Freeze-dried pseudotype pellets can withstand storage for 4 weeks at temperatures up to 37°C and remain employable in downstream neutralization assays.
- Lyophilized influenza pseudotypes can be neutralized by antibody-positive sera with the same potency as their unlyophilized counterparts.
- HA glycoprotein integrity remains intact following lyophilization – pseudotype infectivity and neutralization are not significantly hindered.
- Reconstitution of lyophilized pseudotypes in either supplemented DMEM or nuclease free H₂O has a negligible effect on titer retention.
- A lyophilized, pseudotype-based neutralizing antibody kit, suitable for global distribution and application is a viable proposition.

Influenza pseudotype standardization

- For them to take a prominent role in pandemic preparedness, pseudotypes and pseudotype-based neutralization assays require standardization and validation to international standards.
- The main methods of pseudotype input standardization include relative luminescence units, HA content and particle number (p24).
- Normalizing on the basis of the transduction activity (relative luminescence units) of influenza pseudotypes is optimal since it permits direct comparison with microneutralization assays (in a neutralization assay 50% tissue culture infectious dose is used as indicator of viral infectivity).

References

- Harris A, Cardone G, Winkler DC *et al.* Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proc. Natl Acad. Sci. USA* 103(50), 19123–19127 (2006).
- Tumova B, Schild GC. Antigenic relationships between type A influenza viruses of human, porcine, equine, and avian origin. *Bull. World Health Organ.* 47(4), 453–460 (1972).
- Hause BM, Collin EA, Liu R *et al.* Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. *MBio*. 5(2), e00031–e00014 (2014).
- Hirst GK. Adsorption of influenza hemagglutinins and virus by red blood cells. *J. Exp. Med.* 76(2), 195–209 (1942).
- Gottschalk A. Neuraminidase: the specific enzyme of influenza virus and *Vibrio cholerae*. *Biochim. Biophys. Acta* 23(3), 645–646 (1957).
- Lamb RA, Zebedee SL, Richardson CD. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell* 40(3), 627–633 (1985).
- Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. *Virology* 217(2), 508–516 (1996).
- Fouchier RA, Munster V, Wallensten A *et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* 79(5), 2814–2822 (2005).
- Tong S, Li Y, Rivaller P *et al.* A distinct lineage of influenza A virus from bats. *Proc. Natl Acad. Sci. USA* 109(11), 4269–4274 (2012).
- Tong S, Zhu X, Li Y *et al.* New world bats harbor diverse influenza A viruses. *PLoS Pathog.* 9(10), e1003657 (2013).
- Xu J, Davis CT, Christman MC *et al.* Evolutionary history and phylodynamics of influenza A and B neuraminidase (NA) genes inferred from large-scale sequence analyses. *PLoS ONE* 7(7), e38665 (2012).
- Li Q, Sun X, Li Z *et al.* Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. *Proc. Natl Acad. Sci. USA* 109(46), 18897–18902 (2012).
- Garcia-Sastre A. The neuraminidase of bat influenza viruses is not a neuraminidase. *Proc. Natl Acad. Sci. USA* 109(46), 18635–18636 (2012).
- Zhu X, Yang H, Guo Z *et al.* Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. *Proc. Natl Acad. Sci. USA* 109(46), 18903–18908 (2012).
- Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289(5796), 366–373 (1981).
- Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* 333(6172), 426–431 (1988).
- Matrosovich M, Stech J, Klenk HD. Influenza receptors, polymerase and host range. *Rev. Sci. Tech.* 28(1), 203–217 (2009).

- 18 Skehel JJ, Bayley PM, Brown EB *et al.* Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl Acad. Sci. USA* 79(4), 968–972 (1982).
- 19 Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* 69, 531–569 (2000).
- 20 Palese P, Tobita K, Ueda M, Compans RW. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 61(2), 397–410 (1974).
- 21 Lentz MR, Air GM. Loss of enzyme activity in a site-directed mutant of influenza neuraminidase compared with expressed wild-type protein. *Virology* 148(1), 74–83 (1986).
- 22 Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J. Virol.* 78(22), 12665–12667 (2004).
- 23 Cohen M, Zhang XQ, Senaati HP *et al.* Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol. J.* 10, 321 (2013).
- 24 Nakada S, Creager RS, Krystal M, Aaronson RP, Palese P. Influenza C virus hemagglutinin: comparison with influenza A and B virus hemagglutinins. *J. Virol.* 50(1), 118–124 (1984).
- 25 Rosenthal PB, Zhang X, Formanowski F *et al.* Structure of the haemagglutinin-esterase-fusion glycoprotein of influenza C virus. *Nature* 396(6706), 92–96 (1998).
- 26 Baudin F, Petit I, Weissenhorn W, Ruigrok RW. *In vitro* dissection of the membrane and RNP binding activities of influenza virus M1 protein. *Virology* 281(1), 102–108 (2001).
- 27 Compans RW, Content J, Duesberg PH. Structure of the ribonucleoprotein of influenza virus. *J. Virol.* 10(4), 795–800 (1972).
- 28 Area E, Martin-Benito J, Gastaminza P *et al.* 3D structure of the influenza virus polymerase complex: localization of subunit domains. *Proc. Natl Acad. Sci. USA* 101(1), 308–313 (2004).
- 29 Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical signs and symptoms predicting influenza infection. *Arch. Intern. Med.* 160(21), 3243–3247 (2000).
- 30 Eccles R. Understanding the symptoms of the common cold and influenza. *Lancet Infect. Dis.* 5(11), 718–725 (2005).
- 31 Monto AS. Epidemiology of influenza. *Vaccine* 26(Suppl. 4), D45–D48 (2008).
- 32 Junge C. Morbidity: a personal response. *Nature* 480(7376), S14–S15 (2011).
- 33 Horby P, Nguyen NY, Dunstan SJ, Baillie JK. The role of host genetics in susceptibility to influenza: a systematic review. *PLoS ONE* 7(3), e33180 (2012).
- 34 Lin TY, Brass AL. Host genetic determinants of influenza pathogenicity. *Curr. Opin. Virol.* 3(5), 531–536 (2013).
- 35 Principi N, Scala A, Daleno C, Esposito S. Influenza C virus-associated community-acquired pneumonia in children. *Influenza Other Respir. Viruses* 7(6), 999–1003 (2013).
- 36 Chen R, Holmes EC. The evolutionary dynamics of human influenza B virus. *J. Mol. Evol.* 66(6), 655–663 (2008).
- 37 Dolin R. The quadrivalent approach to influenza vaccination. *J. Infect. Dis.* 208(4), 539–540 (2013).
- 38 Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA. Influenza B virus in seals. *Science* 288(5468), 1051–1053 (2000).
- 39 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* 56(1), 152–179 (1992).
- 40 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. *Science* 312(5772), 384–388 (2006).
- 41 Graham-Rowe D. Epidemiology: Racing against the flu. *Nature* 480(7376), S2–S3 (2011).
- 42 Gerdil C. The annual production cycle for influenza vaccine. *Vaccine* 21(16), 1776–1779 (2003).
- 43 Wood JM, Levandowski RA. The influenza vaccine licensing process. *Vaccine* 21(16), 1786–1788 (2003).
- 44 Claas EC, De Jong JC, Van Beek R, Rimmelzwaan GF, Osterhaus AD. Human influenza virus A/HongKong/156/97 (H5N1) infection. *Vaccine* 16(9–10), 977–978 (1998).
- 45 Apisarnthanarak A, Kitphati R, Thongphubeth K *et al.* Atypical avian influenza (H5N1). *Emerg. Infect. Dis.* 10(7), 1321–1324 (2004).
- 46 Koopmans M, Wilbrink B, Conyn M *et al.* Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* 363(9409), 587–593 (2004).
- 47 Gao R, Cao B, Hu Y *et al.* Human infection with a novel avian-origin influenza A (H7N9) virus. *N. Engl. J. Med.* 368(20), 1888–1897 (2013).
- 48 Yuan J, Zhang L, Kan X *et al.* Origin and molecular characteristics of a novel 2013 avian influenza A (H6N1) virus causing human infection in Taiwan. *Clin. Infect. Dis.* 57(9), 1367–1368 (2013).
- 49 Chen H, Yuan H, Gao R *et al.* Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. *Lancet* 383(9918), 714–721 (2014).
- 50 Jia N, De Vlas SJ, Liu YX *et al.* Serological reports of human infections of H7 and H9 avian influenza viruses in northern China. *J. Clin. Virol.* 44(3), 225–229 (2009).
- 51 Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. *Emerg. Infect. Dis.* 12(1), 15–22 (2006).
- 52 Reid AH, Taubenberger JK, Fanning TG. Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. *Nat. Rev. Microbiol.* 2(11), 909–914 (2004).
- 53 Smith GJ, Bahl J, Vijaykrishna D *et al.* Dating the emergence of pandemic influenza viruses. *Proc. Natl Acad. Sci. USA* 106(28), 11709–11712 (2009).
- 54 Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87(1), 13–20 (1978).
- 55 Both GW, Sleight MJ, Cox NJ, Kendal AP. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *J. Virol.* 48(1), 52–60 (1983).
- 56 Capua I, Kajaste-Rudnitski A, Bertoli E, Vicenzi E. Pandemic vaccine preparedness – have we left something behind? *PLoS Pathog.* 5(6), e1000482 (2009).
- 57 Peiris JS, Poon LL, Guan Y. Public health. Surveillance of animal influenza for pandemic preparedness. *Science* 335(6073), 1173–1174 (2012).
- 58 Dawood FS, Jain S, Finelli L *et al.* Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med.* 360(25), 2605–2615 (2009).
- 59 Zimmer SM, Burke DS. Historical perspective – emergence of influenza A (H1N1) viruses. *N. Engl. J. Med.* 361(3), 279–285 (2009).
- 60 Liu D, Shi W, Shi Y *et al.* Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. *Lancet* 381(9881), 1926–1932 (2013).
- 61 Van Ranst M, Lemey P. Genesis of avian-origin H7N9 influenza A viruses. *Lancet* 381(9881), 1883–1885 (2013).

- 62 Kageyama T, Fujisaki S, Takashita E *et al.* Genetic analysis of novel avian A (H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro. Surveill.* 18(15), 20453 (2013).
- 63 Cattoli G, Terregino C. New perspectives in avian influenza diagnosis. *Zoonoses Public Health* 55(1), 24–28 (2008).
- 64 Belak S, Kiss I, Viljoen GJ. New developments in the diagnosis of avian influenza. *Rev. Sci. Tech.* 28(1), 233–243 (2009).
- 65 Capua I, Cattoli G. One flu for one health. *Emerg. Infect. Dis.* 16(4), 719 (2010).
- 66 Squires RB, Noronha J, Hunt V *et al.* Influenza research database: an integrated bioinformatics resource for influenza research and surveillance. *Influenza Other Respir. Viruses* 6(6), 404–416 (2012).
- 67 Liu Q, Ma J, Liu H *et al.* Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States. *Arch. Virol.* 157(3), 555–562 (2012).
- 68 Bao Y, Bolotov P, Dernovoy D *et al.* The influenza virus resource at the national center for biotechnology information. *J. Virol.* 82(2), 596–601 (2008).
- 69 Smith DJ. Applications of bioinformatics and computational biology to influenza surveillance and vaccine strain selection. *Vaccine* 21(16), 1758–1761 (2003).
- 70 Ben-Yedidia T, Arnon R. Epitope-based vaccine against influenza. *Expert Rev. Vaccines* 6(6), 939–948 (2007).
- 71 Li OT, Poon LL. One step closer to universal influenza epitopes. *Expert Rev. Anti. Infect. Ther.* 7(6), 687–690 (2009).
- 72 Chen GL, Subbarao K. Attacking the flu: neutralizing antibodies may lead to 'universal' vaccine. *Nat. Med.* 15(11), 1251–1252 (2009).
- 73 Rose MA, Zielen S, Baumann U. Mucosal immunity and nasal influenza vaccination. *Expert Rev. Vaccines* 11(5), 595–607 (2012).
- 74 Han T, Marasco WA. Structural basis of influenza virus neutralization. *Ann. NY Acad. Sci.* 1217, 178–190 (2011).
- 75 Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 289(5796), 373–378 (1981).
- 76 Jackson DC, Murray JM, White DO, Gerhard WU. Enumeration of antigenic sites of influenza virus hemagglutinin. *Infect. Immun.* 37(3), 912–918 (1982).
- 77 Archetti I, Horsfall FL Jr. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. *J. Exp. Med.* 92(5), 441–462 (1950).
- 78 Hoag H. A universal problem. *Nat. Med.* 19(1), 12–14 (2013).
- 79 Sui J, Hwang WC, Perez S *et al.* Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 16(3), 265–273 (2009).
- 80 Ekiert DC, Bhabha G, Elsliger MA *et al.* Antibody recognition of a highly conserved influenza virus epitope. *Science* 324(5924), 246–251 (2009).
- 81 Ekiert DC, Friesen RH, Bhabha G *et al.* A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 333(6044), 843–850 (2011).
- 82 Corti D, Voss J, Gamblin SJ *et al.* A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 333(6044), 850–856 (2011).
- 83 Dreyfus C, Laursen NS, Kwaks T *et al.* Highly conserved protective epitopes on influenza B viruses. *Science* 337(6100), 1343–1348 (2012).
- 84 Friesen RH, Lee PS, Stoop EJ *et al.* A common solution to group 2 influenza virus neutralization. *Proc. Natl Acad. Sci. USA* 111(1), 445–450 (2014).
- 85 Friesen RH, Koudstaal W, Koldijk MH *et al.* New class of monoclonal antibodies against severe influenza: prophylactic and therapeutic efficacy in ferrets. *PLoS ONE* 5(2), e9106 (2010).
- 86 Mancini N, Solfrosi L, Clementi N, De Marco D, Clementi M, Burioni R. A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza. *Antiviral Res.* 92(1), 15–26 (2011).
- 87 Marasco WA, Sui J. The growth and potential of human antiviral monoclonal antibody therapeutics. *Nat. Biotechnol.* 25(12), 1421–1434 (2007).
- 88 Krammer F, Palese P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr. Opin. Virol.* 3(5), 521–530 (2013).
- 89 Okuno Y, Isegawa Y, Sasao F, Ueda S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* 67(5), 2552–2558 (1993).
- 90 Reber A, Katz J. Immunological assessment of influenza vaccines and immune correlates of protection. *Expert Rev. Vaccines* 12(5), 519–536 (2013).
- 91 Plotkin SA. Vaccines: correlates of vaccine-induced immunity. *Clin. Infect. Dis.* 47(3), 401–409 (2008).
- 92 Temperton NJ, Wright E. Retroviral pseudotypes. In: *eLS*. Wiley, Chichester, UK (2009).
- 93 Cheresiz SV, Kononova AA, Razumova YV *et al.* A vesicular stomatitis pseudovirus expressing the surface glycoproteins of influenza A virus. *Arch. Virol.* 159(10), 2651–2658 (2014).
- 94 Hatzioannou T, Valsesia-Wittmann S, Russell SJ, Cosset FL. Incorporation of fowl plague virus hemagglutinin into murine leukemia virus particles and analysis of the infectivity of the pseudotyped retroviruses. *J. Virol.* 72(6), 5313–5317 (1998).
- 95 Temperton NJ, Hoschler K, Major D *et al.* A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies. *Influenza Other Respir. Viruses* 1(3), 105–112 (2007).
- 96 Nefkens I, Garcia JM, Ling CS *et al.* Hemagglutinin pseudotyped lentiviral particles: characterization of a new method for avian H5N1 influenza sero-diagnosis. *J. Clin. Virol.* 39(1), 27–33 (2007).
- 97 Sakuma T, Barry MA, Ikeda Y. Lentiviral vectors: basic to translational. *Biochem. J.* 443(3), 603–618 (2012).
- 98 Cronin J, Zhang XY, Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. *Curr. Gene Ther.* 5(4), 387–398 (2005).
- 99 Mather S, Scott S, Temperton N, Wright E, King B, Daly J. Current progress with serological assays for exotic emerging/re-emerging viruses. *Future Virol.* 8(8), 745–755 (2013).
- 100 Garcia JM, Lai JC. Production of influenza pseudotyped lentiviral particles and their use in influenza research and diagnosis: an update. *Expert Rev. Anti. Infect. Ther.* 9(4), 443–455 (2011).
- 101 Carnell GW, Ferrara F, Grehan K, Thompson CP, Temperton NJ. Pseudotype-based neutralization assays for influenza: a systematic analysis. *Front. Immunol.* 6, 161 (2015).
- 102 Hirst GK. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science* 94(2427), 22–23 (1941).
- 103 Klimov A, Balish A, Veguilla V *et al.* Influenza virus titration, antigenic characterization, and serological methods for antibody detection. *Methods Mol. Biol.* 865, 25–51 (2012).

- 104 CONWISE. Consensus protocols for laboratory assay of antibody to influenza A (H1N1)pdm09 (2014). <https://conwise.tghn.org/articles/consensus-protocols-laboratory-assay-antibody-influenza-h1n1pdm09/>
- 105 Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J. Hyg. (Lond)*. 70(4), 767–777 (1972).
- 106 Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. *Br. Med. Bull.* 35(1), 69–75 (1979).
- 107 Al-Khayatt R, Jennings R, Potter CW. Interpretation of responses and protective levels of antibody against attenuated influenza A viruses using single radial haemolysis. *J. Hyg. (Lond)*. 93(2), 301–312 (1984).
- 108 Cox RJ. Correlates of protection to influenza virus, where do we go from here? *Hum. Vaccin. Immunother.* 9(2), 405–408 (2013).
- 109 Davies JR, Grilli EA. Natural or vaccine-induced antibody as a predictor of immunity in the face of natural challenge with influenza viruses. *Epidemiol. Infect.* 102(2), 325–333 (1989).
- 110 Black S, Nicolay U, Vesikari T *et al.* Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. *Pediatr. Infect. Dis. J.* 30(12), 1081–1085 (2011).
- 111 Trombetta C, Perini D, Mather S, Temperton N, Montomoli E. Overview of serological techniques for influenza vaccine evaluation: past, present and future. *Vaccines* 2(4), 707–734 (2014).
- 112 Subbarao EK, Kawaoka Y, Ryan-Poirier K, Clements ML, Murphy BR. Comparison of different approaches to measuring influenza A virus-specific hemagglutination inhibition antibodies in the presence of serum inhibitors. *J. Clin. Microbiol.* 30(4), 996–999 (1992).
- 113 Rowe T, Abernathy RA, Hu-Primmer J *et al.* Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J. Clin. Microbiol.* 37(4), 937–943 (1999).
- 114 Stephenson I, Wood JM, Nicholson KG, Zambon MC. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J. Med. Virol.* 70(3), 391–398 (2003).
- 115 Kayali G, Setterquist SF, Capuano AW, Myers KP, Gill JS, Gray GC. Testing human sera for antibodies against avian influenza viruses: horse RBC hemagglutination inhibition vs. microneutralization assays. *J. Clin. Virol.* 43(1), 73–78 (2008).
- 116 Stephenson I, Das RG, Wood JM, Katz JM. Comparison of neutralising antibody assays for detection of antibody to influenza A/H3N2 viruses: an international collaborative study. *Vaccine* 25(20), 4056–4063 (2007).
- 117 Wood JM, Gaines-Das RE, Taylor J, Chakraverty P. Comparison of influenza serological techniques by international collaborative study. *Vaccine* 12(2), 167–174 (1994).
- 118 Wood JM, Major D, Heath A *et al.* Reproducibility of serology assays for pandemic influenza H1N1: collaborative study to evaluate a candidate WHO International Standard. *Vaccine* 30(2), 210–217 (2012).
- 119 Wang W, Xie H, Ye Z, Vassell R, Weiss CD. Characterization of lentiviral pseudotypes with influenza H5N1 hemagglutinin and their performance in neutralization assays. *J. Virol. Methods* 165(2), 305–310 (2010).
- 120 Schild GC, Pereira MS, Chakraverty P. Single-radial-hemolysis: a new method for the assay of antibody to influenza haemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza. *Bull. World Health Organ.* 52(1), 43–50 (1975).
- 121 Russell SM, Mccahon D, Beare AS. A single radial haemolysis technique for the measurement of influenza antibody. *J. Gen. Virol.* 27(1), 1–10 (1975).
- 122 Molesti E, Ferrara F, Lapini G, Montomoli E, Temperton N. Discordant correlation between serological assays observed when measuring heterosubtypic responses against avian influenza H5 and H7 viruses in unexposed individuals. *Biomed. Res. Int.* 2014, 231365 (2014).
- 123 El-Madhun AS, Cox RJ, Haaheim LR. The effect of age and natural priming on the IgG and IgA subclass responses after parenteral influenza vaccination. *J. Infect. Dis.* 180(4), 1356–1360 (1999).
- 124 Postel A, Ziller M, Rudolf M *et al.* Broad spectrum reactivity versus subtype specificity-trade-offs in serodiagnosis of influenza A virus infections by competitive ELISA. *J. Virol. Methods* 173(1), 49–59 (2011).
- 125 Throsby M, Van Den Brink E, Jongeneelen M *et al.* Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS ONE* 3(12), e3942 (2008).
- 126 Okuno Y, Tanaka K, Baba K, Maeda A, Kunita N, Ueda S. Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *J. Clin. Microbiol.* 28(6), 1308–1313 (1990).
- 127 Martinez-Sobrido L, Cadagan R, Steel J *et al.* Hemagglutinin-pseudotyped green fluorescent protein-expressing influenza viruses for the detection of influenza virus neutralizing antibodies. *J. Virol.* 84(4), 2157–2163 (2010).
- 128 Alberini I, Del Tordello E, Fasolo A *et al.* Pseudoparticle neutralization is a reliable assay to measure immunity and cross-reactivity to H5N1 influenza viruses. *Vaccine* 27(43), 5998–6003 (2009).
- 129 Qiu D, Tannock GA, Barry RD, Jackson DC. Western blot analysis of antibody responses to influenza virion proteins. *Immunol. Cell. Biol.* 70(Pt 3), 181–191 (1992).
- 130 Horvath CM, Paterson RG, Shaughnessy MA, Wood R, Lamb RA. Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. *J. Virol.* 66(7), 4564–4569 (1992).
- 131 White J, Helenius A, Gething MJ. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. *Nature* 300(5893), 658–659 (1982).
- 132 Vanderlinden E, Goktas F, Cesur Z *et al.* Novel inhibitors of influenza virus fusion: structure–activity relationship and interaction with the viral hemagglutinin. *J. Virol.* 84(9), 4277–4288 (2010).
- 133 Reed ML, Yen HL, Dubois RM *et al.* Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein. *J. Virol.* 83(8), 3568–3580 (2009).
- 134 Cotter CR, Jin H, Chen Z. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. *PLoS Pathog.* 10(1), e1003831 (2014).
- 135 Shelton H, Roberts KL, Molesti E, Temperton N, Barclay WS. Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. *J. Gen. Virol.* 94(Pt 6), 1220–1229 (2013).
- 136 Paterson RG, Russell CJ, Lamb RA. Fusion protein of the paramyxovirus SV5: destabilizing and stabilizing mutants of fusion activation. *Virology* 270(1), 17–30 (2000).
- 137 Yang J, Li W, Long Y *et al.* Reliability of pseudotyped influenza viral particles in

- neutralizing antibody detection. *PLoS ONE* 9(12), e113629 (2014).
- 138 Wallerstrom S, Lagerqvist N, Temperton NJ *et al.* Detection of antibodies against H5 and H7 strains in birds: evaluation of influenza pseudovirus particle neutralization tests. *Infect. Ecol. Epidemiol.* doi:10.3402/iee.v4.23011 (2014).
 - 139 Scott S, Molesti E, Temperton N, Ferrara F, Bottcher-Friebertshauser E, Daly J. The use of equine influenza pseudotypes for serological screening. *J. Mol. Genet. Med.* 6, 304–308 (2012).
 - 140 Molesti E, Milani A, Terregino C, Cattoli G, Temperton NJ. Comparative serological assays for the study of h5 and h7 avian influenza viruses. *Influenza Res. Treat.* 2013, 286158 (2013).
 - 141 Garcia J, Sovero M, Torres AL *et al.* Antiviral resistance in influenza viruses circulating in Central and South America based on the detection of established genetic markers. *Influenza Other Respir. Viruses* 3(2), 69–74 (2009).
 - 142 Garcia JM, Lagarde N, Ma ES, De Jong MD, Peiris JS. Optimization and evaluation of an influenza A (H5) pseudotyped lentiviral particle-based serological assay. *J. Clin. Virol.* 47(1), 29–33 (2010).
 - 143 Corti D, Suguitan AL, Jr., Pinna D *et al.* Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J. Clin. Invest.* 120(5), 1663–1673 (2010).
 - 144 Tscherne DM, Manicassamy B, Garcia-Sastre A. An enzymatic virus-like particle assay for sensitive detection of virus entry. *J. Virol. Methods* 163(2), 336–343 (2010).
 - 145 Oh HL, Akerstrom S, Shen S *et al.* An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. *J. Virol.* 84(16), 8275–8286 (2010).
 - 146 Edwards MJ, Dimmock NJ. Hemagglutinin 1-specific immunoglobulin G and Fab molecules mediate postattachment neutralization of influenza A virus by inhibition of an early fusion event. *J. Virol.* 75(21), 10208–10218 (2001).
 - 147 Hai R, Krammer F, Tan GS *et al.* Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. *J. Virol.* 86(10), 5774–5781 (2012).
 - 148 Steel J, Lowen AC, Wang TT *et al.* Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio.* 1(1), 1–9 (2010).
 - 149 Krammer F, Pica N, Hai R, Margine I, Palese P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J. Virol.* 87(12), 6542–6550 (2013).
 - 150 Margine I, Krammer F, Hai R *et al.* Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. *J. Virol.* 87(19), 10435–10446 (2013).
 - 151 Miller MS, Gardner TJ, Krammer F *et al.* Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. *Sci. Transl. Med.* 5(198), 198ra107 (2013).
 - 152 Fouchier RA, Smith DJ. Use of antigenic cartography in vaccine seed strain selection. *Avian Dis.* 54(1 Suppl.), 220–223 (2010).
 - 153 Wood JS, Robertson JS. Reference viruses for seasonal and pandemic influenza vaccine preparation. *Influenza Other Respir. Viruses* 1(1), 5–9 (2007).
 - 154 Ferrara F, Molesti E, Bottcher-Friebertshauser E *et al.* The human transmembrane protease serine 2 is necessary for the production of group 2 influenza A virus pseudotypes. *J. Mol. Genet. Med.* 7, 309–314 (2012).
 - 155 Molesti E, Wright E, Terregino C, Rahman R, Cattoli G, Temperton NJ. Multiplex evaluation of influenza neutralizing antibodies with potential applicability to in-field serological studies. *J. Immunol. Res.* 2014, 457932 (2014).
 - 156 Mather ST, Wright E, Scott SD, Temperton NJ. Lyophilisation of influenza, rabies and Marburg lentiviral pseudotype viruses for the development and distribution of a neutralisation-assay-based diagnostic kit. *J. Virol. Methods* 210, 51–58 (2014).
 - 157 Wang SY, Su CY, Lin M *et al.* HA-pseudotyped retroviral vectors for influenza antagonist screening. *J. Biomol. Screen* 14(3), 294–302 (2009).
 - 158 Wang W, Butler EN, Veguilla V *et al.* Establishment of retroviral pseudotypes with influenza hemagglutinins from H1, H3, and H5 subtypes for sensitive and specific detection of neutralizing antibodies. *J. Virol. Methods* 153(2), 111–119 (2008).