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Steps toward a Universal Influenza Vaccine: Research Models and Comparison of Current Approaches

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Abstract

The ability of influenza virus to adapt to various species and evade natural immunity makes the ubiquitous pathogen particularly difficult to eradicate. Annual reformulation of influenza vaccines is costly and time-consuming and has varying efficacy against influenza virus strains. Therefore, worldwide efforts aim to develop a universal influenza vaccine to prevent potential healthcare emergencies such as pandemic influenza threats, such as the 1918 Spanish Flu and pandemic Swine Flu of 2009. Efficacy of a universal influenza vaccine must overcome current challenges with subtype diversity, antigenic drift, and adequately protect against emerging reassortants from both environmental and agricultural sources. Furthermore, the manufacturing and production of vaccines largely influence the effectiveness of a vaccine and technological advancements may soon rival current vaccine strategies. This review discusses the evolution and diversity of influenza viruses, how viral glycoprotein hemagglutinin plays a dominant role in influenza surveillance and assessment of protection and compares the methodologies of current and upcoming vaccine options. While the obstacles remain daunting, growing knowledge of influenza evolution and immunity may lead to more viable candidates that protect against broader varieties of influenza viruses and help prevent future international health crises.

Keywords: influenza, vaccine development, universal flu, flu strategies, immunology

1. Introduction

The World Health Organization (WHO) estimates 3–5 million cases of severe influenza worldwide will result between 250,000 and 500,000 deaths annually [1]. Within the United States, 24.7 million cases of seasonal influenza of various severities are predicted and an estimated

3,300–49,000 deaths occurred per year between 1979 and 2001 [2]. From 2013 to 2014, the hospitalization rate in the United States was 24.6 per 100,000 persons [3], although rates as high as 309 per 100,000 have been reported from 1993 to 2008, among elderly >65 years of age [4]. Including direct medical costs and loss of productivity and earnings associated with work absenteeism, seasonal influenza contributes an \$87 billion burden on the US economy [5]. Furthermore, the spread of pandemic swine influenza virus from 2009 to 2010 attributed to 42–89 million cases of pandemic H1N1 (pdm H1N1) and an estimated 8870–18,300 deaths. Consequently, there is still a need to enhance our understanding of influenza virus evolution and manage the spread of pandemic influenza viruses.

Global influenza virus monitoring and surveillance systems track significant trends or real-time changes to influenza epidemiology and identify predominant viral subtypes during a particular season. Clinical symptoms include acute onset of cough, fever, and myalgia [6–9], and viral presence is confirmed by WHO-mandated laboratory standard operating procedures, such as immunostaining, cell culture growth, real-time polymerase chain reaction (RT-PCR), and detection of hemagglutination-inhibiting or viral neutralizing antibodies [10]. While viral culture in mammalian cells remains vital for characterization, RT-PCR remains the most rapid and sensitive diagnostic test available, with detection rates enhanced twofold over cell culture [8, 11, 12]. Alternatively, increases in hemagglutination-inhibiting antibody titers more than fourfold are counted as positive seroconversion and following viral infection or vaccination, which assists with monitoring immunogenicity. Collectively, data are shared through the WHO's Global Influenza Surveillance and Response System (GISRS), which includes 143 institutions in 113 WHO Member States [13], to help alert the emergence of antigenic variants or the beginning of a pandemic.

In April 2009, the Centers of Disease Control and Prevention (CDC) cited the first incidence of human-to-human transmission of pdmH1N1, also referred to as swine influenza A, which was antigenically distinct from other circulating human H1N1 [14]. As the first influenza pandemic of the twenty-first century, pandemic (pdm) H1N1 was not included in the annual trivalent vaccine regimen, leaving a large majority of the population unprotected from the newly emerging pathogen. In response to the increasing number of illnesses associated with pdm H1N1, satellite surveillance sites throughout the world rapidly collected clinical samples, surveyed seroconversion rates, and monitored the spread of the pandemic. Under the recommendation of WHO and CDC, the standard trivalent vaccine regimen of 2009–2010 [15] was reformulated to accommodate for the pdm H1N1 influenza strain (A/California/07/2009-like) [16, 17], and a median seroconversion rate of 59% was observed among healthy individuals ranging from 18 to 65 years of age [18]. During the 2010–2011 season, predicted efficacy in Sweden among individuals between 6 months and 64 years was between 63% and 80%, when 60% of the Swedish population received a monovalent AS03-adjuvanted vaccine against pdm H1N1 [19]; importantly, laboratory diagnosis of pdm09 H1N1 was utilized in this study. As of 2016, A/California/07/2009-like pdm H1N1 remains the predominant H1N1 strain circulating in the human population and remains a component in the annual trivalent or quadrivalent vaccine schedule. The remaining components of the annual vaccine, including prototype strains of Group B influenza strains and subtype H3N2, are frequently updated due to frequent

antigenic changes to the hemagglutinins (HA). Ultimately, the WHO and CDC provide recommendations for vaccine formulation and identify what particular health concerns need to be addressed by the public, healthcare workers, and pharmaceutical/biomedical research industry.

2. Influenza diversity, tropism, and evolution

Currently, there are three types of influenza (A, B, and C), however, only types A and B infect humans. Influenza belongs to the family of Orthomyxoviridae and contains a negative sense, single segmented RNA genome. The influenza virion is pleomorphic or filamentous in shape, budding to approximately 80–120 nm in diameter, and attaches to sialic acid receptors found on numerous tissues in a diverse variety of hosts, including birds and mammals, such as swine, equine, canine, ferrets, and primates [20–23]. Upon budding from host cells, a lipid bilayer envelopes the viral ribonucleoprotein complex and presents surface viral glycoproteins, which are various combinations of hemagglutinins (HA, H1-H18) and neuraminidases (NA, N1-N11); the arrangement of HA and NA help distinguish the large diversity of influenza subtypes [24].

Avian influenza strains are ubiquitous in the wild; circulating in predominantly the wild ducks Anatidae bird populations [25–27] through an indirect fecal-oral transmission route and often results in little to no pathology [25, 28]. Replication of avian influenza virus (AIV) occurs in the intestinal tract of waterfowl and is shed in feces, such that contaminated waters infect other hosts; certain strains of AIV can persist in aquatic environments for 9 to 200 days, depending on salinity, pH, and temperature [29–31]. Consequently, poor water quality control and cohabitation of wild species increase the likelihood of AIV transmission into domestic birds markets [27, 32, 33] and livestock. Surveillance of live poultry markets in Asia indicate high prevalence of low pathogenic avian influenza strains, such as H3N2, H5N2, and H9N2 [32, 34–37]; isolates collected from poultry populations in Korea were used to experimentally infect mice and demonstrate zoonotic adaptation for mammalian hosts [38].

The human influenza virus threats are derived from avian [21, 39–42] or swine [14, 43] influenza predecessors, but these viruses have adapted to transmit and to replicate in the human host. Highly pathogenic avian influenza H5N1 (HPAIV H5N1) outbreaks during the end of the twentieth century led to lethal disease in domestic poultry and waterfowl, and eventually human intervention was needed to quarantine and cull millions of contaminated flocks worldwide [41, 44, 45]. Although most avian influenza strains typically do not cause disease in humans, HPAIV H5N1 was spread to humans in Hong Kong in 1997. From 2003 to January 2014, the WHO reported 386 human deaths out of the 650 confirmed H5N1 cases [46]. In February 2013, a novel avian influenza virus subtype, H7N9, was also detected in humans [47] and was thought to have arisen from nonpathogenic H7 strains.

In humans, influenza spreads through aerosolized respiratory droplet transmission and the virus attaches to primarily columnar epithelial cells lining the respiratory tract. Infection of influenza spreads to airway epithelial cells [48–50] and alveolar macrophages [51], although

dendritic cells [52], natural killer cells [53], and mast cells are semi-permissive for influenza infection *in vitro* and *in vivo*. Typical incubation period for influenza virus is 1–4 days, in which an afflicted individual begins shedding virus and continues to shed up to 10 or more days after the onset of symptoms [9, 54]. Unlike human seasonal influenza, most AIV transmits directly from animal-to-human (zoonotic), but infrequently transmits between human hosts; however, since 1918, four influenza pandemics occurred after antigenic shift enabled human adaption and no prior immunity existed in the population. Since influenza hemagglutinin binds to sialic acid receptors that are on respiratory epithelial as well as erythrocytes, a rapid assay for sialic acid receptor binding is the red blood cell agglutination and hemagglutination inhibition (HAI) assays. Developed in the 1940s and based on the ability of influenza hemagglutinin to bind receptors of target cells and agglutinate red blood cells (RBCs), the HAI assay has become the standard serological assay for screening influenza hemagglutinin-reactive antibodies in sera [10]. Serial dilutions of heat-inactivated, receptor-destroying enzyme (RDE)-treated sera are incubated with a fixed amount of virus titrated in hemagglutination units and then RBCs are added to the virus-sera mixture to permit agglutination. The presence of influenza-specific, receptor-binding antibodies prevents RBC agglutination and potentially reduces infectivity. By standard, an antibody titer of >32, or most commonly 40, is correlated with up to 50% protection from influenza disease [14], and seroconversion is assumed when greater than fourfold increase is observed when comparing of pre- and post-vaccination HAI titers [13]. However, as important as the standard agglutination and HAI assays are for vaccine immunogenicity surveillance, new challenges arise when viruses have reduced species-specific RBC agglutination [55, 56] or when HAI titers do not correlate with protection [15]. Consequently, additional verification approaches are needed and applied to assess efficacy beyond immunogenicity of a vaccine candidate; the greatest obstacle, however, is overcoming the antigenic diversity in influenza strains and eliciting vaccine-induced protection prior to antigenic drifts or shifts.

Influenza glycoprotein hemagglutinin (HA) amino acid sequence variation fluctuates rapidly amidst cross-species infections, leading to shifts within HA subtypes. For example, the influenza viruses that spread internationally in 2009 were antigenically unique to seasonal H1N1 strains, but resembled the pandemic H1N1 strains isolated in 1918. The emergence of H1N1 into the human population in 1918 caused the first influenza pandemic of the twentieth century and caused ~50 million deaths worldwide [57]. Cross-reactive antibodies to the pdm H1N1 strains of 2009 were identified in the sera of older people [58] and therefore this implied that people infected with the 1918 H1N1 viruses elicited long-lasting antibodies that cross-reacted with the pdm H1N1 infecting people 90 year later. H3N2 influenza strains emerged in 1968 as a human pathogen after reassortment of HA & PB1 genes with avian source (1918 H1N1 (avian/human)→1957 H2N2 (avian)→1968 H3N2 (avian/human)) [43] leading to the loss of immune recognition among human hosts. The timeline in **Figure 1** illustrates the reassortment events that transitioned between avian influenza viruses into human pathogens and the emergence of pdm H1N1 or swine flu remained circulating in the swine population since early twentieth century and was reintroduced in the early 2000s.

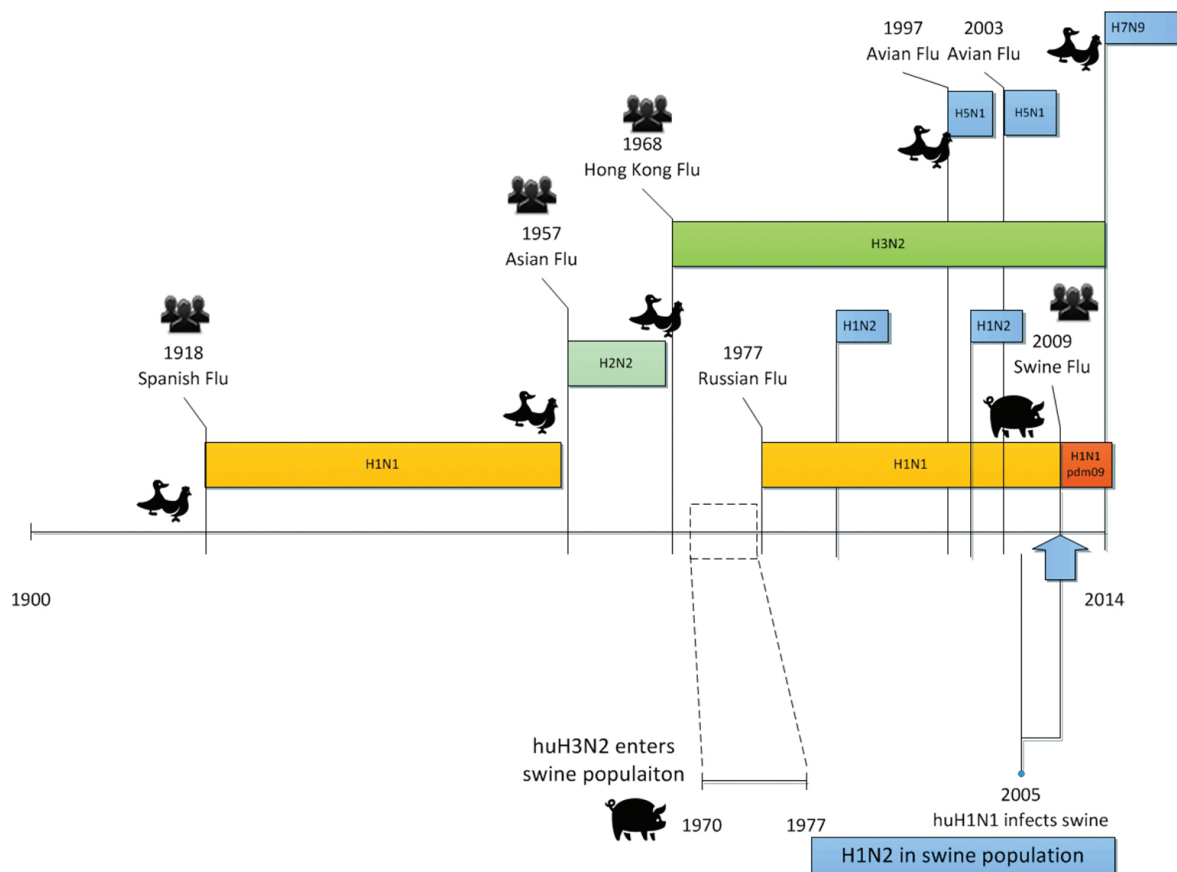


Figure 1. Timeline of influenza viruses and circulation among avian, swine and human populations.

The emergence of the 1918 Spanish Flu into the human population resulted from divergent reassortment of avian-like influenza and caused substantial morbidity and mortality worldwide. Further reassortment of the HA subsequently led to H2N2 and H3N2, which currently circulates abundantly in the human population. Introduction of H3N2 into the swine population during the 1970s and circulation of H1N2 permitted additional reassortment in the form of novel Swine Flu or pdm H1N1. The recent incidence of avian strains H5N1 and H7N1 is further evidence that zoonotic exchange of influenza viruses increases the likelihood of introduction to human populations.

Influenza HA mediates the binding to host cell surface receptors, dominantly sialic acid, which tends to be specific to species and tissue. HA proteins expressed by avian influenza strains preferentially bind to the sialic acids bound by an alpha 2-3 glycosidic bond, whereas an alpha 2-6 linkage is preferred by human adapted strains. Importantly, the upper human respiratory tract is lined with epithelial cells expressing sialic acids with alpha 2-6, whereas the lower respiratory tract contains sialic acids containing the alpha 2-3[41] potentially increasing susceptibility to lower respiratory tract infections by avian influenza viruses [42]; however, direct droplet transmission between humans is inefficient and remains limited.

In addition to receptor-binding specificity, the influenza HA sequence contains a critical cleavage site that is necessary for initiating pH-dependent fusion into the host cell. Influenza

virus HA is synthesized as a precursor HA0, which is composed of a globular surface “head” HA1 and the stalk-like HA2. The HA2 portion contains a transmembrane domain spanning the viral membrane [23, 59, 60]. Within the HA1 subunit is the receptor-binding site (RBS) and a vestigial esterase domain that alters pH stability and ultimately, confers high or moderate pathogenicity [61, 62]. The exposure of the fusion peptide sequence is essential for conformational changes in the coiled-coil trimeric HA structure during a prefusion event, leading to extension of the HA, fold-back, hemifusion and eventual fusion of viral membrane to host membrane [63]. **Figure 2** is reprinted from a study [63] that analyzed the fusion event between influenza hemagglutinin and cell membrane of the infected cell, which utilizes a similar coiled-coil hemifusion state as other viral pathogens, such as HIV, respiratory syncytial virus, and Ebola virus [64].

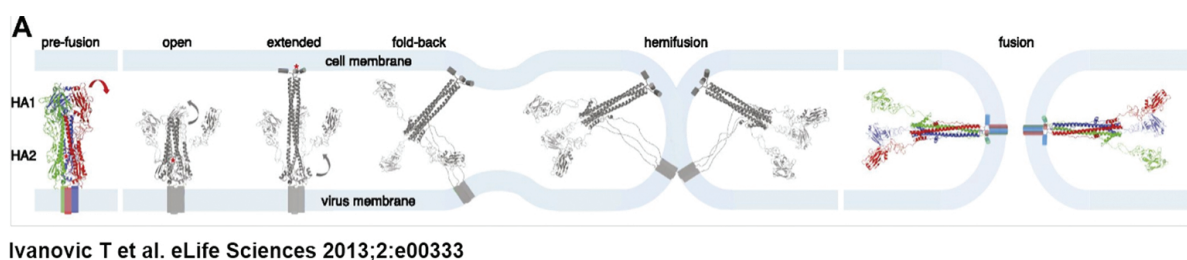


Figure 2. Fusion event of influenza hemagglutinin on host cell. Adapted from Ivanovic et al. [63].

Utilizing the coiled-coil motif to extend into a host cell, the hemagglutinin fusion peptide serves as an anchoring “hook” to attach to the cell membrane; through pH-mediated hairpin folding, the hemifusion event occurs, followed by complete fusion of the two membranes and release of viral components into the host cytoplasm.

The majority of low pathogenic influenza strains have HA0 containing a monobasic arginine residue that is cleaved by extracellular enzymes; within the human respiratory tract, the transmembrane protease, serine 2 (TMPRSS2) and human airway trypsin-like protease (HAT) are capable of mediating mono-basic cleavage to yield disulfide-bonded HA1 and HA2 subunits [65]. Several highly pathogenic avian influenza strains (H1N1v, H5 and H7) contain multi-arginine motifs that are cleaved by subtilisin-like cellular proteases and are often present in the intestinal and respiratory tracts of birds and mammals; cleavage by host proteinases, such as furin and PC6, occurs intracellularly upon exit of the endoplasmic reticulum, and proteolysis is blocked by inhibitors of serine proteases, such as aprotinin [50, 66]. Interestingly, within a human large intestine carcinoma cell line Caco-1, aprotinin treatment and disruption of the late Golgi transport mechanisms by brefeldin A inhibit cleavage of HA0, regardless whether the influenza strain contains mono- or multi-basic proteolytic sites [66]. **Figure 3** illustrates the domains HA1 and HA2 in a homotrimer of HA from A/Fujian/411/2002-H3N2 influenza predicted using SWISS-MODEL service [67–70], with antigenic sites highlighted. Sites in H3 HA have been best characterized, with antigenic epitopes defined as A, B, C, D, and E [71–73]. Alternatively, H1 contains five antigenic sites, Cb, Sa, Sb, Ca1, and Ca2, that were originally modeled from H3 structure [74].

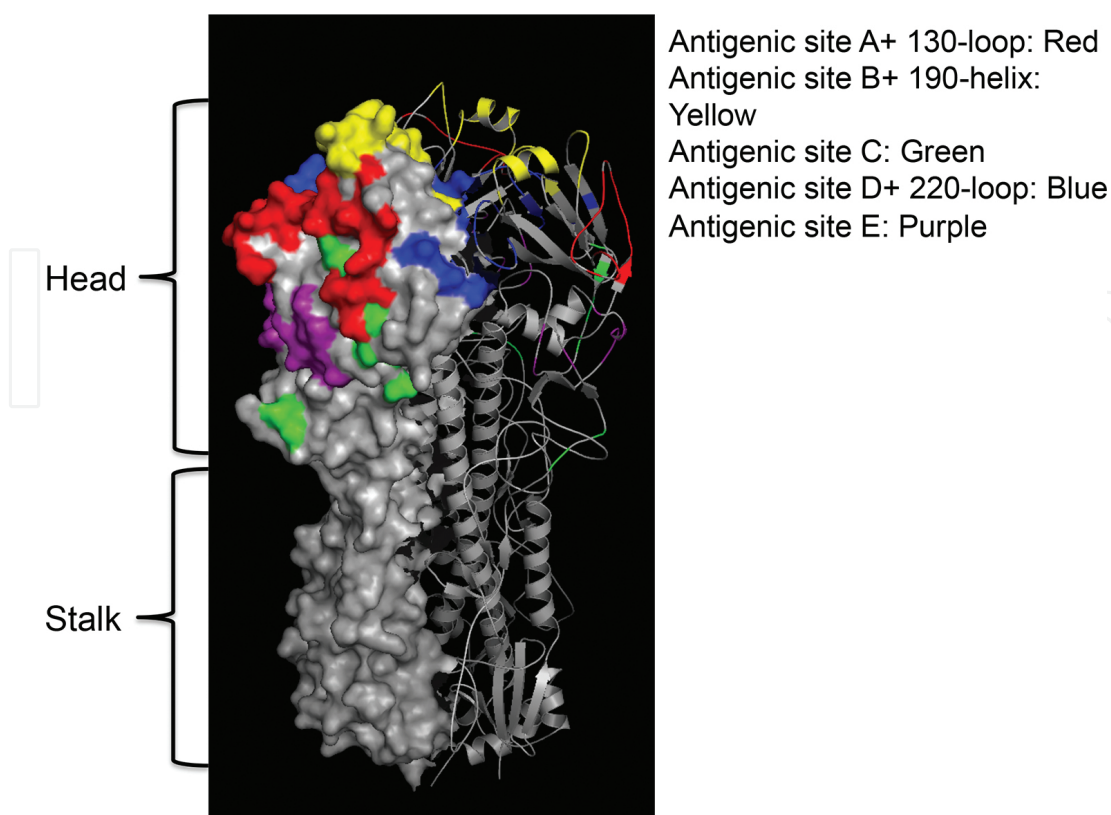


Figure 3. Structure of influenza hemagglutinin with antigenic sites and domains highlighted.

Influenza hemagglutinin is a homotrimer of a globular head and stalk domains. The HA of A/Fujian/411/2002-H3N2 influenza was modeled to PDB 2yp2.1.a using structure homology software SWISS-MODEL and further manipulated with PyMol. One monomer is shown with surface, whereas the remaining two monomers illustrated in ribbon-cartoon form. Highlighted antigenic sites A (red), B (yellow), C (green), D (blue), and E (purple) are present in H3 HA (shown), whereas Cb, Sa, Sb, Ca1, and Ca2 are the known antigenic epitopes in H1 influenza subtypes. Sites used were derived from 73.

Incomplete cleavage of HA0 yields lower viral titers [50, 65, 75] and virions incapable of HA0 cleavage are less infectious upon subsequent replication cycles, with more than 4 log-units lower than cleaved HA [50, 76]. Current cell-based approaches to influenza propagation using Madin-Darby canine kidney (MDCK) cells require artificial addition of trypsin or serine protease treatment to yield appreciable viral titers, but native enzymes in egg-based culture sufficiently cleave HA; therefore, no addition treatment is required [77, 78]; consequently, post-translational HA processing is substantially different between the two standard methods [79]. Recent human isolates, particularly H3N2 subtype, are difficult to propagate in conventional egg or MDCK cell lines; advancements in stable-overexpression of $\alpha 2-6$ sialic acids on the MDCK cell line (MDCK-SIAT1) have improved virus isolation rates [56] and typically retains HA sequence identity better than the aforementioned egg or MDCK approaches. These characteristic of HA and sialic acid binding affinity determine species- and tissue-specificity, and further complicate the likelihood of zoonotic diseases and transmission among humans.

Fears of an avian HA and NA reassortment with a human seasonal influenza virus are well justified given the emergence of avian-to-human and human-to-human transmissible HPAI H5N1 [45, 80] and subsequent fatality rates averaging 56% [81]. Human infection with H7N9 [47, 82–85] and H10N8 [86, 87] is high risks to individuals working or residing near live poultry markets, and mammalian receptor adaptation remains a significant concern for health officials. While H10N8 does not immediately appear to pose an imminent threat to global health, there are at least 450 confirmed cases of H7N9 by June 2014 since its initial emergence in China in March 2013. Historically associated with a highly pathogenic avian influenza strain, the H7 subtype strains, such as H7N2, H7N3, and H7N7 [47, 88, 89], have the preferential binding to α 2–6-linked sialic acids, which increase their affinity to human upper respiratory epithelial cells [88, 90]; however, incidence of H7 avian and rare human infections remained isolated [91]. Experimental aerosol transmission studies in 2012 [92, 93] and 2013 [94, 95] using viruses in subtypes H5 and H7 showed that, similar HPAI H5N1, the H7N9 viruses potentially poses greater threat to humans since it has a limited capacity to transmit through aerosol droplets (REFS) and therefore further investigations are needed to elucidate H7 pathogenesis and HA evolution.

3. Challenges of generating a universal influenza vaccine

The diversity of influenza virus strains and subtypes exacerbates the challenge for generating a universal vaccine against influenza. The WHO makes recommendations for influenza vaccine composition for each flu season based on international surveillance systems and compares the ability of monovalent vaccine prototypes to elicit cross-reactive antibodies against prevalent circulating strains [16, 96, 97]. Unfortunately, delays in surveillance and the generation and evaluation of serology data hinder the selection of the optimal candidates and seasonal efficacy depends on prompt production by vaccine manufacturers and subsequent distribution. Moreover, the emergence of antigenic drift variants after selection of vaccine candidates may result in poor efficacy and wasted production time and resources [98]. Consequently, there is a demand for a universal, or more broadly reactive, vaccine against influenza virus. Utilizing various manufacturing platforms, adjuvants, and targets, researchers worldwide are currently seeking ways to improve current influenza vaccination strategies [99]. The following traits are desired in an ideal universal vaccine candidate: [1] recognition of antigenic drift variants; [2] elicitation of long-lasting memory responses; and [3] minimal manufacturing lag time between vaccine formulations. Additional desirable features may include cost-efficient production methods, predictive measures to protect against future antigenic variants, and efficacy among immunocompromised target populations, infants and elderly over the age of 65. Despite nearly a century of research, human influenza vaccine production is largely based on the traditional technologies. The rapid emergence of antigenically unique subtypes continues to challenge the current pace of diagnosis, prototype selection, and egg-based vaccine distribution. Furthermore, the importance of pre-existing immunity determines the level of protection from novel strains and depends on the presence of circulating cross-reactive antibodies. For example, individuals born prior to 1957 reportedly had fewer complications associated with

pdm H1N1 than the younger, naïve population potentially because the elderly population encountered similar historical H1N1 strains during their lifetime and therefore had pre-existing neutralizing antibodies to antigenically similar strains [100].

Common name	Distributor	Inactivation	Detergent	Platform	Derived from:	Ref
Inactivated influenza, Trivalent (IIV3) or Quadrivalent (IIV4), intramuscular						
Afluria®	CSL Limited	beta-propiolactone	sodium taurodeoxycholate	Split virus	Embroynated chicken eggs	[110]
Fluarix®	GlaxoSmithKline	formaldehyde	sodium deoxycholate	Split virus	Embroynated chicken eggs	[111]
FluLaval®	ID Biomedical Corporation of Quebec (GSK)	UV, formaldehyde	sodium deoxycholate	Split virus	Embroynated chicken eggs	[112]
Fluvirin®	CSL Limited (Formerly Novartis Vaccines)	beta-propiolactone	nonylphenol ethoxylate	Subunit	Embroynated chicken eggs	[114]
Fluzone®	Sanofi Pasteur	formaldehyde	Triton X-100	Split virus	Embroynated chicken eggs	
Flucelvax®	CSL Limited (Formerly Novartis Vaccines)	beta-propiolactone	Cetyltrimethylammonium bromide	Subunit	MDCK mammalian cells	[115]
Inactivated influenza, Trivalent (IIV3) or Quadrivalent (IIV4), intradermal						
Fluzone®	Sanofi Pasteur	formaldehyde	Triton X-100	Split virus	Embroynated chicken eggs	[116]
Live, Trivalent (LAIV3) or Quadrivalent (LAIV4), intranasal						
FluMist®	MedImmune	N/A-cold-adapted, temperaturesensitive, or attenuated		Live reassortant	Embroynated chicken eggs	[117]
Recombinant Influenza Vaccine, Trivalent (RIV3)						
FluBlok®	Protein Sciences	none-purified HA	Triton X-100	Recombinant HA	expresSF+ insect cells	[118]
Trivalent, inactivated with adjuvant						
FLUAD™	Seqirus™ (CSL Limited)	formaldehyde	Cetyltrimethylammonium bromide	Split virus, MF59C.1	Embroynated chicken eggs	

Table 1. A list of FDA-approved influenza vaccines in US market as of March 2016.

In contrast, recent evidence suggests that sequential exposure to seasonal strains confers greater protection against novel antigenic drift variants; consequently, the question remains whether the existence of protective neutralizing antibodies in the elderly is due to immunological recall, or rather, enhanced broadly reactive response due to immunological boosts of heterologous strains over a lifetime. This has since been modeled in the ferret animal models and, without prior exposure to the pdm H1N1, ferrets sequentially challenged with seasonal H1N1 [101, 102] or immunized with inactivated seasonal vaccine [103] strains mount protective responses to pdm H1N1. In summary, seroprotection against influenza may also depend on the individual's pre-existing immunity, as well as the platform and strategy implemented.

Current 2016–2017 influenza vaccines recommended by the WHO are available as an inactivated trivalent or quadrivalent formulation delivered intramuscularly or as live attenuated mixture of influenza strains (LAIV) administered through intranasal route [104]. The various doses and formulations may or may not contain ovalbumin or mercury, and eligibility for some formulations is age restricted. Vaccine manufacturers have proprietary methods for virus propagation and inactivation, which often alters the antigenic properties of the vaccine candidate. Vaccine antigens may be prepared as whole virus, detergent or solvent disrupted split virions [105, 106], or subunit vaccines [107–109]. **Table 1** displays the 2016–2017 FDA-approved influenza vaccines used in the United States and exhibits formulation differences as described in the package inserts of each product [110–118]. Essentially, all current formulations aim to elicit protective antibody responses, predominantly against HA, and therefore protect against antigenically similar viral strains to the vaccine strain. Since the vaccine is often generated as a reassortant (6:2) vaccine strain from a high yield genetic backbone A/Puerto Rico/8/34 (H1N1), the HA and NA selected as a seasonal vaccine recommendation must be reformulated each season and validated for cross-reactivity to antigenically similar strains using HAI assay [98].

Adverse reactogenicity remains a safety concern for the young, immunocompromised, and elderly, therefore split or subunit vaccines are preferred over live or whole inactivated virus, despite enhanced humoral responses elicited by whole virus preparations [119]. Efforts to reduce the risks associated with LAIV are particularly important since humoral and cross-reactive T-cell responses are superior to that of inactivated vaccines in experimental swine and ferret studies [120–122]; among children, LAIV, but not inactivated vaccine, led to enhanced CD4+, CD8+, and $\gamma\delta$ T-cell responses [123]. In contrast to inactivated vaccines that require reformulation each year, there is evidence that LAIV induces cross-reactivity with heterologous strains. This cross-reactivity phenomenon makes LAIV a promising candidate for the development of a universal vaccine platform. The route of administration, absence of active viral replication, and process of inactivation are different between influenza vaccine formulations. Further studies are needed to understand the mechanisms that promote influenza-specific T-cell responses upon LAIV immunization.

The WHO recommends propagating influenza viruses in embryonic chicken eggs, and this methodology is predominant source of high yield vaccine strains [124]. A list of FDA-approved

influenza vaccines, majority of which are derived from embryonated chicken eggs, is provided in **Table 1**.

Table 1 shows differences in inactivation, detergents, and platforms used in the current vaccine market.

Despite the frequent use of the relatively inexpensive but highly productive virus production egg-based platform, some challenges are arising. Spontaneous mutations due to egg adaptation were recognized as early as 1951 [125] and, as a recent vaccine efficacy (VE) study showed, may present unanticipated challenges to vaccine development. In the 2012–2013 influenza season, MDCK-grown prototypes were selected for vaccine production and classical reassortant methodology was performed; in brief, co-culture of high-yield A/Puerto Rico/8/34 (H1N1) reassortant (6:2) with the HA and NA gene products from A/Victoria/361/2011 (H3N2) in the presence of antiserum to A/Puerto Rico/8/34 yielded reassortant A/Victoria/361/2011(H3N2)-IVR-165 [126]. During vaccine production, the high growth strain acquired several substitutions within the antigenic site B, which reduced reactogenicity against the original prototype A/Victoria/361/2011 and antigenically similar circulating field strains, resulting in a disappointing 39% (95% CI, 29%–47%) rate in humans vaccinated with the 2012–2013 egg-based preparations [127]. Consequently, the low VE for the 2012–2013 H3N2 season is attributed to changes in the HA due to vaccine manufacturing, not antigenic drift. In contrast, growth in mammalian cell lines results in fewer mutations than in eggs, with up to two mutations in the hemagglutinin of H3N2 strains after three passages in MDCK [128–130] or MDCK-SIAT1 [56] but no genotypic changes reported after 10 passages of avian influenza using human colorectal adenocarcinoma cell line, Caco-2 [131]. These examples suggest that alternative methods for influenza propagation are expanding and optimization could ensure more consistency and efficacy.

In 2013, the FDA approved the first MDCK cell-culture influenza vaccine (CCIV) called Flucelvax®, originally developed by Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA [115] but acquired by CSL Limited, Melbourne, Australia, as of December 2015 [132], which is tolerable and safe, and demonstrates comparable, if not greater, protection to that of traditional egg-based technologies [133–137]. Furthermore, trials involving trivalent preparation of influenza viruses through growth in green monkey kidney cell line, Vero, is another new alternative to egg-based propagation [138]. Although not yet FDA approved in the United States, the Vero cell-based technology licensed as Vepacel® from Baxter AG, Vienna, Austria, is a pre-pandemic whole, inactivated virus H5N1 vaccine designed to elicit strong immunological responses in healthy, as well as immunocompromised populations [139]. Also in 2013, the FDA approved a recombinant HA (rHA) only influenza vaccine (Flubok®) from Protein Sciences Corporation, Meriden, CT, USA [118]. Flubok is generated from insect cells using a baculovirus expression system to produce high yield rHA vaccine against each seasonal influenza subtype. Further studies are still needed to assess cost-effectiveness in transitioning the current platforms of egg-derived influenza virus to mammalian cell culture. Modifications to standard method of virus propagation have economical and practical challenges, but federal initiatives suggest divergence from traditional egg-based approaches.

4. Alternatives to current influenza vaccine strategies

A disadvantage of mammalian cell culture is the need for regulated growth conditions and reagents, which hinder immediate production and increase cost; however, advancements in high-throughput bioreactors are ongoing [140]. However, the same types of technologies involved in mammalian cell culture virus propagation may further advance novel alternatives to eggs. Virus-like particles (VLPs) are non-replicating, self-assembling nanostructures that mimic virus surface protein presentation and are synthesized through co-expression of recombinant DNA in insect or mammalian cell culture [141–147]. Multiple advantages are associated with VLP over standard methods [141], including [1] they are non-infectious material and therefore not a biohazard risk and inactivation is not required, [2] VLPs self-assemble into proper conformation and multivalency, and [3] influenza VLPs have enhanced stability and HA potency up to 12 months with no degradation [148]. Several investigators have utilized this platform for evaluating immunogenicity of influenza HA [149–151], NA [144], M2 [152], and combinations of viral proteins. A mammalian VLP derived in Vero cells, H5N1-VLP (RG-14) [153], was composed of four influenza virus structural proteins and protected against a lethal H5N1 challenge in mice, yielding H5-specific IgG1 antibodies with a dose as low as 2.5 µg in a prime-boost regimen. Similarly, baculovirus-derived VLPs expressing H3N2 protected against a lethal challenge of mouse-adapted influenza H3N2 A/Hong Kong/68 [149], with intranasal instillation providing the greatest HA-specific antibody titers and protection as compared to intramuscular administration or two doses of sublethal intranasal challenges. The baculovirus/insect cell expression systems generate high yields of VLPs, and trivalent vaccine preclinical studies in mice and ferrets demonstrate the baculovirus-derived VLPs effectively elicit serum HAI antibodies against H1N1, H3N2, and influenza B, although cross-reactivity to heterologous strains was poor [154]. Importantly, side-by-side comparison of baculovirus-derived VLPs versus whole virion vaccine or recombinant HA suggests VLPs elicit comparable, if not higher, IgG titers, but also yielded antibodies that cross-react to a broader panel of non-homologous influenza viruses [146]. The baculovirus-derived VLPs have been fully characterized by Novavax, Inc. from Rockville, MD, USA, for the HA content, NA activity, stability, and VLP purity [155], and safety and reactogenicity were evaluated in two clinical trials with H5N1 and H1N1 baculovirus-derived VLPs [156, 157].

Proprietary application of recombinant RNA bacteriophage Qbeta by Cytos Biotechnology Ltd., Switzerland, offers an alternative strategy for conformational antigen presentation on the surface of Qbeta-derived virus-like particles through covalent chemical linkage [158]. Since presentation on Qbeta-derived VLPs does not involve post-translational modifications from the VLP-producing cell, potential cell type-specific alterations to the viral surface glycoprotein are avoided [159]; moreover, chemical linkage of antigens to Qbeta-derived VLPs overcomes the steric hindrance associated with masked immunodominant epitopes and efficiently elicits B cell responses in the absence of an adjuvant [160]. Importantly, specific sites or domains can be exclusively presented on the Qbeta-VLPs, such as the globular head of influenza hemagglutinin to induce hemagglutination inhibiting antibodies [161]. Consequently, this approach advanced to Phase I clinical trial in Singapore through sponsorship of the manufacturer Cytos

Biotechnology; the findings suggest that the Qbeta-VLP is tolerable and elicits HAI-specific antibodies comparable to the standard influenza vaccines [162]. However, as with any new approach, insect-, mammalian-, or Qbeta-VLP manufacturing processes still need to undergo more thorough safety evaluation and quality control; therefore, marketing and production are still a few years away.

Modern recombinant technology offers alternatives to live virus preparation. Synthetic or subunit viral peptide vaccines stimulate the immune response in similar mechanism as split inactivated vaccines and may be formulated with pattern recognition receptor agonists or emulsions to enhance adjuvant activity. Plasmid DNA encoding an immunogenic viral protein can be electroporated [163], administered intranasally in conjugation with nanoparticles [164], or delivered with a biolistic system. Particle-mediated epidermal delivery of DNA encoding influenza HA was recently optimized in an experimental ferret model and, when administered to the abdomen or tongue, yielded HAI titers greater than 1:40 [165]; sublingual route of vaccine administration has potential to elicit mucosal immunity and mount protective secretory immunoglobulin A (sIgA) [166, 167]. Previously in preclinical studies, delivery of recombinant IgA protects against infection in the experimental animal influenza model [168] while elicitation of IgG controls disease severity, thus elicitation of IgA by any universal vaccine candidate would be highly beneficial. In the effort to reduce infant morbidity associated with influenza infections, ongoing studies will test the safety, efficacy, and tolerability of vaccination of pregnant mothers and their newborns [169–171]; subsequent protection through maternal IgG and sIgA lasting up to 6 months could reduce infection within influenza virus [172].

In addition to the HA, DNA vaccination of the neuraminidase N1 from H1N1, A/New Caledonia/20/99, also partially protects against heterologous challenge with H5N1, A/Vietnam/1203/04, in the mouse model; similarly, passive immunization of heterologous anti-N1 sera in naïve mice provided modest protection against H5N1 challenge [173]. NA-DNA vaccination also conferred crossprotection against heterologous H3N2 strains in a lethal H3N2 influenza challenge [174], further suggesting neuraminidase may be an additional target for eliciting protection against drift variants within a influenza subtype. In addition to the neuraminidase, viral targets such as the nucleoprotein (NP), matrix 1 and 2 (M1 and M2), and polymerase (PB1) are different immunogens for vaccine candidates, resulting in varied immunological responses. Internal NP is the predominant target antigen for cytotoxic T lymphocyte (CTL) activity and is well conserved among influenza A viruses; peptide-based and DNA vaccination strategies have demonstrated promising protection against homologous and heterologous challenge with influenza A viruses. However, as with epitope-specific T cell responses, binding and affinity between epitope and human leukocyte antigen (HLA) could be restricted to highly polymorphic HLA alleles and could reduce efficacy among particular populations [175]. Consequently, promiscuous T cell epitopes that bind sufficiently to a wide range of HLA alleles remain to be identified. Recent studies also suggest cooperative T helper cell assistance to mount B cell immunological responses to promiscuous epitopes on the ectodomain of M2 protein, M2e [176]. Modified M2e, termed M2e multiple antigenic peptides (M2e-MAPs), appears to protect against influenza challenges among mice of various genetic

background, providing proof of concept that M2e-MAPs are not necessarily MHC allele-restricted. Multiple efforts remain ongoing to test the feasibility of M2e-MAP-based vaccine strategies against influenza.

Clinical Trials	Immunogen	Principle	Immune response target	Phase	ClinicalTrials.gov ID	Sponsor	Route	Ref
VAX102	Adjuvanted recombinant antigen	M2e fused to TLR5 ligand S. typhimurium flagellin	M2e	1, 2	NCT00603811 ; NCT00921947	VaxInnate Corporation	I.M. & S.C.	
MVA-NP+M1	Viral Vector	Attenuated vaccinia virus prime-boost regimen	NP and M1	1	NCT00993083 ; NCT00942071	University of Oxford	I.D.	[177]
MVA-NP+M1 and ChAdOx1 NP+M1	Viral vector	Attenuated adenoviral and vaccinia virus prime-boost regimen	NP and M1	1	NCT01818362	University of Oxford	I.M & I.M.	
FLU-v	Synthetic polypeptides	Lyophilized polypeptides T- cell epitopes	M1, M2, NP and PB1	1	NCT01181336	PepTcell Limited	S.C	
ACAM FLU-A	Recombinant antigen	Recombinant M2e	M2e	1	NCT00819013	Sanofi	I.M.	
NIBRG-14	Reassortant, inactivated influenza virosome	A/Vietnam/1194/2004HA (Head/ (H5N1) and A/PuertoStalk) Rico/8/34 (H1N1) (PR8) with alum or ISCOM-like Matrix M™ adjuvant		1, 2	NCT00660257 ; NCT00535665	Sinovac Biotech Co., Ltd	I.M.	[150], [151]
H5N1 VLP-1	Baculovirus VLP	A/Indonesia/05/2005 VLP	HA, NA, and M1	1,2	NCT00519389	Novavax	I.M.	[157]
H1N1 VLP	Baculovirus VLP	A/California/07/2009 VLP	HA, NA, and M2	1,2		Novavax	I.M.	[158]
gH1-Qbeta	Qbeta VLPs	A/California/07/2009 VLP	HA	1	CIRB Ref: 2012/906/E	Cytos Biotech.	I.M.	[163]

Table 2. Clinical trials ongoing the US testing novel influenza vaccine or antiviral approaches.

Since monovalent HA reactivity is thought to limit broad use of an influenza vaccine, alternative strategies aimed at less conventional tactics aside from full-length, wild-type HA. Directed reactivity to either the globular head or stalk have been examined in mice, ferrets, and human clinical studies [150, 151, 156]. Broad protection against heterologous influenza virus strains is thought to rely on elicitation of well-conserved, HA2, stalk- or stem-specific antibodies [177]. Cross-protection against heterologous strains and crossreactivity to various subtypes appears the most rational direction toward a universal influenza vaccine. Immunization with recombinant HA2 construct that maintains a neutral pH conformation conferred protection against homologous H3N2-type virus strains, such as A/Philadelphia/2/82 and A/HK/68, but not heterologous H1N1-type viruses [178]. Importantly, prime-boost immunization of chimeric hemagglutinin expressing mismatched globular HA heads on homologous HA stalk (cH6/1, cH9/1, and cH5/1) elicited stalk-reactive, neutralizing antibodies and conferred protection against lethal H1N1 challenge [179]. Protection, however, remained limited within a particular influenza group; therefore, further investigation remains to overcome such large diversity of influenza viruses. In an attempt to address the vast diversity of globular HA, computationally optimized broadly reactive antigens (COBRA) have been utilized in mammalian VLP platforms against H5N1 infection in mice, ferrets, and nonhuman primates [180–182]. Through multiple layers of consensus sequences of influenza hemagglutinin, COBRA methodology avoids database input bias associated with influenza surveillance systems; constructs generated have been validated to stimulate greater immunological breadth than monovalent vaccine strategies while successfully eliciting seroconversion, yielding hemagglutination-inhibiting antibody titers more than fourfold. **Table 2** is a summary of several clinical trials that investigated novel influenza vaccine strategies with various targets and approaches for eliciting protective immunity. In conclusion, the efficacy of the immunogen, route of administration, and types of immune responses elicited will further determine whether novel approaches could replace current vaccination methods.

The ClinicalTrials.gov database provides latest information regarding past and current clinical trials and the status toward future applications. Unlike the conventional influenza vaccines that dominant to HA-mediated immune responses, recent clinical trials test antiviral therapies targeting influenza matrix (M and M2e, matrix ectodomain) or highly conserved nucleoprotein (NP). These strategies come from various technologies, including virus-like particles (VLPs) and platforms, which do not rely on the egg-based vaccine manufacturing processes. The routes of delivery also vary, including intramuscular (IM), intradermal (ID), and subcutaneous (SC).

5. Concluding remarks

The pursuit of a universal vaccine against influenza is a constant battle between influenza virus evolution and current technologies. Since influenza infections remain ubiquitous among humans, as well as wildlife and domestic livestock, reassortment of genomic segments can still yield combinations that the human species has not previously encountered; alternatively, gradual antigenic variation of the hemagglutinin also results in poor cross-reactivity and

protection against heterologous strains. As such, protective measures such as effective vaccination and more thorough understanding of HA evolution are needed to prevent forthcoming pandemics. Current practices rely on relatively traditional technologies for vaccine selection and manufacturing, and as witnessed during the pandemic H1N1 outbreak of 2009, it took months to identify and manufacture a new vaccine [183, 184]. In addition, inadequate quality control led to sterility breaches of millions of doses Fluvirin®, which at-time was manufactured by Chiron, acquired first by Novartis Vaccines and Diagnostics Limited, Speke, Liverpool, UK [185, 186] but now recently under Seqirus™, a CSL Limited company. During such shortages, it was evident that influenza vaccine resources were insufficient given the substantial health risks. To minimize future damages, both in morbidity and economical losses, the international community must disseminate new findings rapidly and cooperatively strive for more effective and readily available preventative actions.

In addition to the current vaccine approaches, innovative strategies are being tested for feasibility, safety, and the elicitation of broad immunogenicity to influenza strains and subtypes. Overcoming the antigenic diversity remains the greatest challenge to the efforts of designing a universal influenza vaccine. Moreover, changes in manufacturing and rapid implementation are critical for vaccine efficacy and may require divergence from the currently accepted methodologies. The recombinant technology and nonconventional vaccine platforms aforementioned demonstrate potential and may rival the current standard of care. As our understanding of immune responses to influenza and antigenic variability furthers, improvements to influenza vaccine strategies are expected to prevent significant morbidity and unnecessary disease through preventative measures.

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