Expert Opinion

Vaccines against epidemic and pandemic influenza

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Background: Preventative vaccination is the most effective way to control epidemic and, perhaps, pandemic influenza viral infections. However, the immunogenicity and efficacy of influenza vaccines against epidemic strains are suboptimal among older adults. The risk of serious complications from influenza viral infection is compounded by co-morbid conditions among older adults. Furthermore, despite annual influenza vaccination campaigns, the vaccination rates in high risk populations range from 60.5 – 79.2% only [1]. In addition, H5N1 avian influenza viruses have the potential to cause a pandemic. However, H5N1 vaccines currently licensed in the US are poorly immunogenic in high doses in the absence of an adjuvant even in healthy adults. *Objectives*: In this review, we address the current status of vaccines against epidemic and avian influenza viruses of pandemic potential. *Methods*: We have limited the review to the discussion of technologies and strategies that have progressed to human clinical trials and/or licensure for seasonal and pandemic influenza. *Results/conclusion*: Improving the immunogenicity of vaccines against avian influenza viruses, as well as aggressive programs to vaccinate high risk populations against seasonal and pandemic influenza, are crucial for our public health efforts in minimizing the impact of influenza epidemics or pandemics.

Keywords: H5N1, H9N2, influenza, pandemic, seasonal, vaccination

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1. Introduction

The immunologically naïve young, the immunocompromised and the older adult populations are highly susceptible to illness caused by seasonal influenza viruses. Each year in the US, influenza infects approximately 10 – 20% of the population and results in an average of 36,000 deaths [2]. According to the World Health Organization (WHO), up to 500,000 deaths occur due to influenza annually worldwide, with 90% of those deaths occurring among older adults. While not solely caused by antigenic drift, these deaths associated with seasonal influenza are often a result of secondary bacterial infections due to exacerbation of cardiopulmonary disorders or other chronic diseases, as the rarity of influenza culture confirmation upon admission to hospital masks the absolute diagnosis as influenza [3]. High risk individuals are most vulnerable to the consequences associated with influenza. High risk adults aged 45 – 64 have the same hospitalization and mortality risk as older adults of > 65 years of age [4]. A recent study in the UK suggested that pregnant women have a higher risk for cardiopulmonary events during an influenza season and have a fourfold higher risk of mortality while pregnant during a severe influenza season, as compared to a less severe season [5,6]. However, pregnant women have one of the lowest vaccination rates, with only 14% in 2004, possibly due to obstetricians' reluctance to vaccinate [7-9]. As older adults are a high risk group for influenza-related

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deaths, the goal is to vaccinate 90% of this population [10]. However, recent vaccination rates are stagnant, with coverage still hovering around 65% [11]. Only 43% of healthcare workers, who have the potential to transmit influenza to high risk populations, were vaccinated in 2004 [9]. Racial and ethnic disparities contribute to suboptimal vaccination rates among all populations, including healthcare workers [9]. The disappointing vaccination rates among target populations indicate that more aggressive campaigning needs to occur to ensure that at-risk populations are vaccinated to prevent or minimize complications from influenza infections.

Global influenza surveillance has been monitoring the spread of a novel subtype of avian influenza which is currently showing limited transmission from infected birds to humans, and isolated human-to-human transmission [12-14]. South-East Asia has been implicated as the potential epicenter for its emergence. This novel subtype, H5N1, first appeared in China in 1997 with 18 human cases and six deaths [15,16]. The virus re-emerged in 2003 and has spread to humans in 14 countries in South-East Asia and Africa, with over 385 cases and > 60% mortality (**Figure 1**) [17]. In addition, the H5N1 virus is currently circulating in a number of avian species in over 60 countries in South-East Asia, Europe, the Middle East and Africa (**Figure 2**) [18]. Since 2003, the H5N1 virus has diverged into 10 clades (0 – 9); however only three clades have been isolated from humans [19]. Viruses isolated from humans since 2003 are comprised of two predominant clades that are antigenically distinct: clade 1 viruses have been isolated in Vietnam, Thailand and Cambodia and clade 2 in Indonesia, Turkey and many other countries. Sequence analysis of the clade 2 viruses shows the circulating strains are adapting to selective pressures and have further diverged into five subclades (2.1 – 2.5); however, only subclades 1, 2 and 3 contain viruses isolated from humans.

Low pathogenic avian influenza (LPAI) viruses can also cause disease in birds with mild or no symptoms. H9, as well as H5, viruses are endemic in wild birds and water fowl in South-East Asia and LPAI H9 viruses have been known to cause disease in turkeys in the US since 1966 [20]. LPAI H7 viruses have also been detected in poultry in various regions in the world, including the US, since 1996 [21]. Animal health officials closely monitor the outbreaks of LPAI, as LPAI viruses can evolve into HPAI viruses. Therefore, the culling of infected birds is common practice in LPAI and HPAI outbreaks. In 1998 and 1999, a LPAI virus, H9N2, was transmitted from infected birds to humans and caused eight human cases of non-lethal disease in China [22,23] and one additional case in 2003 in Hong Kong. LPAI H7N2 virus was detected in turkeys in Virginia, West Virginia and North Carolina, resulting in two cases of disease in humans in 2002 and 2003. In 2003, 89 human cases (manifested as conjunctivitis) and one death due to LPAI H7N7 influenza virus transmission from birds was reported in The Netherlands and in 2004 an outbreak in western Canada of LPAI H7N3 in poultry resulted in two laboratory confirmed cases in humans.

Vaccination remains the most effective preventative mechanism to reduce the chance of infection from epidemic or pandemic influenza. The standard assay to assess the immunogenicity of the influenza vaccine is the hemagglutination inhibition (HI) assay and/or virus microneutralization (MN) assay. However, different laboratories consider positive titers ranging from 1:20 to 1:40, depending on their standard operating procedures. Thus, comparison of the results of immunogenicity of each vaccine candidate in clinical trials and drawing conclusions becomes more difficult. Vaccine efficacy can vary depending on the severity of the season, the coverage of the vaccine with the circulating viruses, and the participants' exposure to the virus during the clinical trial. All of these factors play a role in the difficult task of determining the optimal dosing (both the amount of vaccine antigens and number of immunizations), effectiveness of an adjuvant and coverage outside of the vaccine components.

2. Inactivated vaccines

The predominant vaccine against seasonal influenza is a trivalent egg-derived inactivated vaccine composed of 15 μg of antigen from each of three different viruses, typically two components from type A influenza (subtypes H1N1 and H3N2) and a third component from an influenza B virus. Each year, the vaccine strain composition is re-evaluated using global virological and epidemiological surveillance data collected by the WHO network of laboratories. Changes to the composition of the vaccine are considered if the strains in the vaccine are no longer circulating or if the antibodies generated against the vaccine are not capable of offering cross-protection against new variant strains based on human pre- and post-vaccination serological analysis. Vaccine strain selection for the northern hemisphere occurs in February and for the southern hemisphere in September of each year to allow the vaccine manufacturers enough time to manufacture and package the vaccine for the following influenza season. In the US, over 100 million doses of the seasonal influenza vaccine were distributed in 2007 prior to the 2007 – 08 cold and flu season. To produce enough doses of the vaccine, manufacturers generate reassortant strains that contain the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) from the vaccine strain with the other six gene segments from a high growth master donor strain, A/PR/8/34 (A/Puerto Rico/8/34). Since a high-growth B donor strain does not exist, a wild-type strain is used for the B component of the vaccine. Most influenza vaccines are inactivated detergent split-vaccines or subunit vaccines, containing only the HA and NA purified surface glycoproteins. However, recently live attenuated seasonal vaccines have been approved by the FDA for use in humans in the US [24]. Live attenuated vaccines have been used in Russia for many years. Whole virus inactivated vaccines

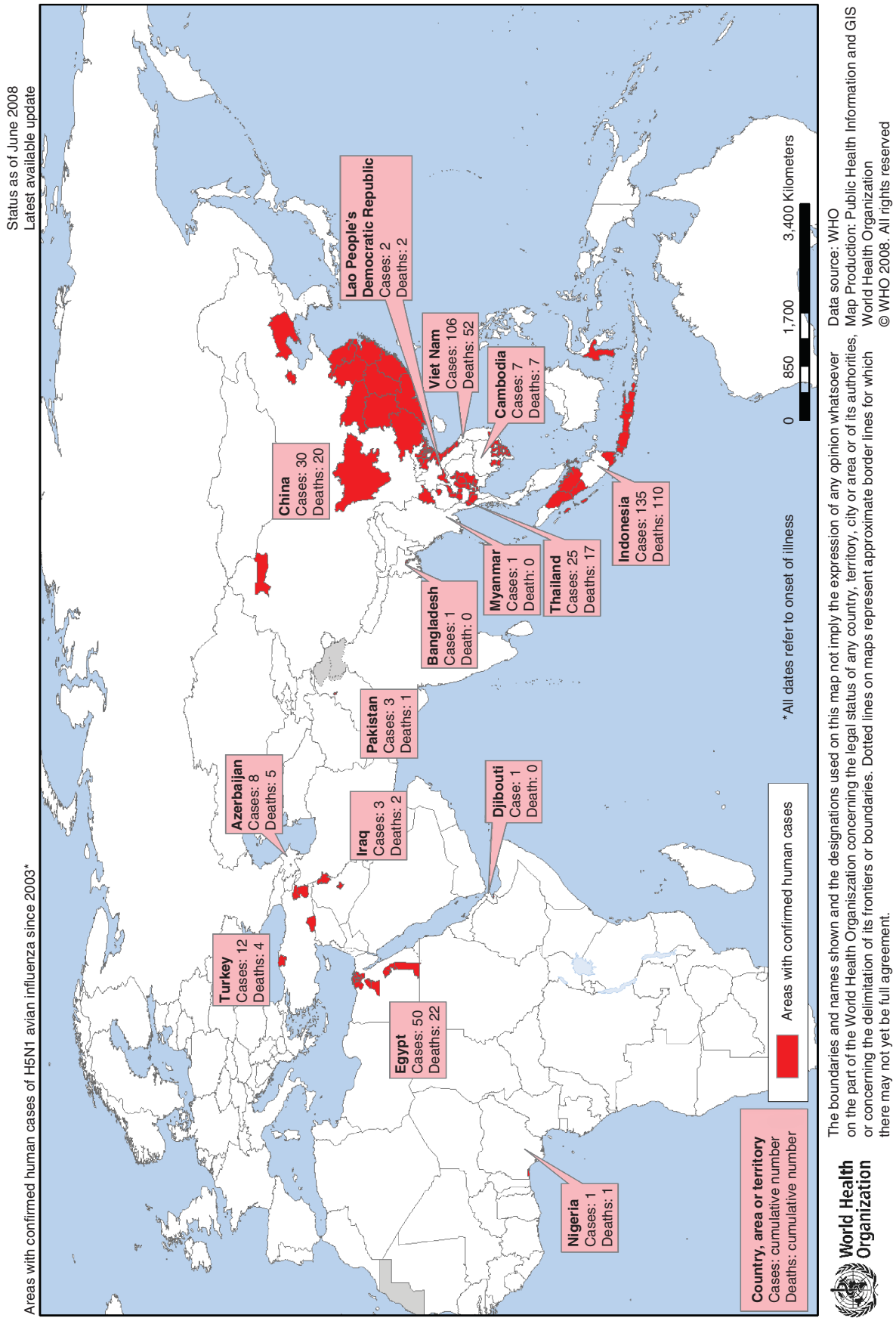

Figure 1. Confirmed human cases of H5N1 since 2003.
Reproduced with permission from The World Health Organization [164].

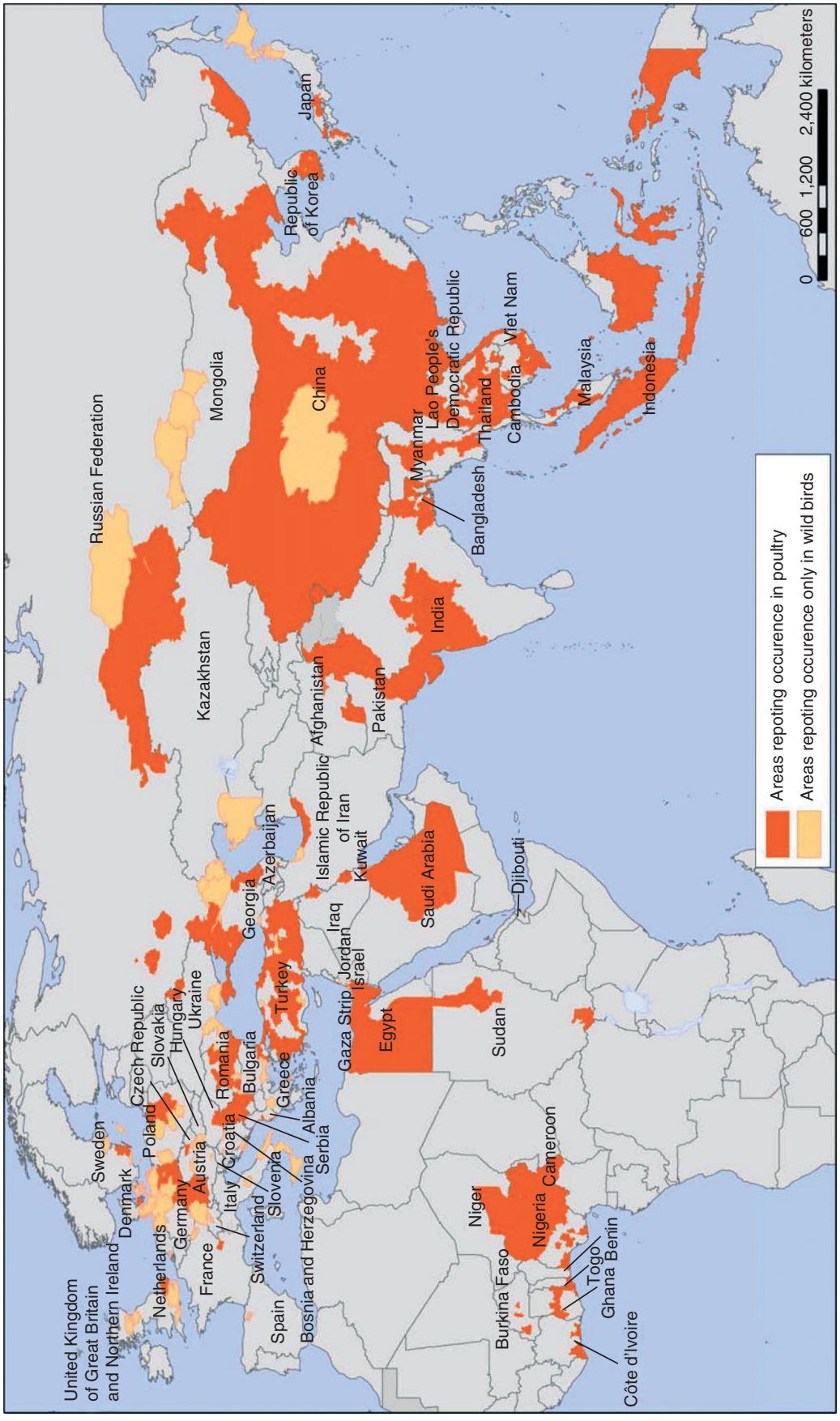

Figure 2. WHO – areas reporting confirmed occurrence of H5N1 avian influenza since 2003 in poultry and wild birds as of 15th of August 2008.
Reproduced with permission from The World Health Organization [165].

against seasonal influenza are used infrequently due to adverse reactions in children to the vaccine.

Intramuscularly administered inactivated vaccines induce humoral immune responses with the production of serum antibodies specific to the HA and offer protection against circulating strains in approximately 70 – 90% of healthy adults under the age of 65 when there is a good match between the circulating strain and the vaccine strain [25,26] and is 50 – 80% effective in preventing hospitalizations and deaths associated with influenza-like illness (ILI) [27,28]. While healthy adults develop protective antibody titers (titers ≥ 1:40) as determined by the hemagglutinin inhibition assay (HI), older adults and immunocompromised populations develop lower titers of hemagglutinating antibodies following vaccination. In addition, it has been shown that the inactivated vaccine is only 30 – 50% effective in these populations in preventing complications from influenza viral infections [28,29].

Inactivated vaccines are safe and recommended for individuals > 6 months of age, including those with chronic illnesses and women who are pregnant. A recent clinical trial evaluated a trivalent inactivated vaccine in infants and determined that two doses of the vaccine were safe and only moderately immunogenic, possibly due to pre-existing maternal antibodies which reduced the antibody response to the vaccine [30]. Inactivated vaccines have not been shown to be very effective during a severe influenza season with a mismatched circulating virus. During both the 1997 – 98 influenza season of the A/Sydney/05/97 (H3N2)-drift and the 2003 – 04 influenza season of the A/Fujian/411/2002 (H3N2)-drift, inactivated vaccines' effectiveness were well below 70 – 90% effectiveness seen during a season with a well-matched vaccine [25,31]. During the mismatched season of 1997 – 98, vaccine effectiveness in older adults fell from 61 to 35% [32]. During the 2003 – 04 influenza season, the hospitalization rates in children rose threefold compared to the following influenza season [33]. This indicates that careful evaluation of the circulating influenza viruses is critical in minimizing the adverse health impact associated with a mismatched vaccine.

3. Adjuvanted inactivated vaccines

Since older adults, one of the target groups for annual influenza vaccination, respond poorly to trivalent inactivated influenza vaccine, improving the immunogenicity of influenza vaccines with adjuvants has been the focus of many researchers. A number of clinical trials have evaluated or are in the process of evaluating adjuvanted seasonal influenza vaccines in healthy adults, followed by subsequent clinical trials to evaluate these promising vaccines in other populations, such as the elderly. Aluminium hydroxide (alum) is the only adjuvant approved in the US for use in humans. However, inclusion of alum as an adjuvant in a subunit monovalent seasonal influenza vaccine did not enhance the immune response in a Phase I/II clinical trial in healthy adults [34]. An influenza vaccine adjuvanted with MF-59, a submicron squalene–water emulsion, has been licensed for use in humans in several European countries and has been shown to significantly increase HI titers to the vaccine in adult and older adult populations with chronic diseases, with only mild local reactions [35,36]. However, another clinical trial reported that only a modest increase in immunogenicity was observed when an MF-59-adjuvanted influenza vaccine was administered to healthy adults [37].

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs have recently been evaluated as an adjuvant in influenza vaccines. CpG ODNs bind to TLR9 on plasmacytoid DC (pDCs) and B cells [38,39] and activate monocytes and macrophages, skewing the priming environment towards a Th1 response, even in the presence of a Th2 adjuvant such as alum [40,41]. A Phase I clinical trial in healthy adults evaluated intramuscular delivery of a CpG ODN(CPG 7909)-adjuvanted trivalent influenza vaccine containing a 1/10th antigen dose of A/Beijing/262/95, A/Sydney/5/97 and B/Harbin/7/94 inactivated viruses. The adjuvanted, reduced-dose vaccine was well-tolerated and induced similar levels of serum HA antibodies as the unadjuvanted full-dose vaccine with no enhancement of antibody titers when the adjuvanted vaccine was given at the full-antigen dose [42]. However, HI titers trended higher in those individuals with pre-existing antibodies of > 1:20 against A/Sydney/5/97 prior to immunization with the 1/10th dose adjuvanted vaccine, suggesting a possible utility as a booster vaccine rather than a priming vaccine. In addition, restimulation of peripheral blood mononuclear cells (PBMCs) collected after two doses of 1/10th dose of vaccine with CPG 7909 with each antigen *ex vivo*, generated significantly higher levels of interferon gamma (IFN-γ) production for all antigens, leading to a Th1 response. These results also suggest that formulation of trivalent influenza vaccines with CpG ODN may allow antigen-sparing without compromising the immunogenicity of the vaccine. Another clinical trial is currently underway to assess the optimal dose of CpG ODN adjuvant (ranging from 10 – 100 μg) combined with a single 0.5 ml dose of subunit influenza vaccine in healthy adults.

Another strategy to enhance the immune response to the vaccine involves reconstitution of the subunit vaccine containing the HA into a phospholipid bilayer to form virosomes [43-45]. Virosomes present the viral surface glycoproteins in a manner similar to that of the intact virus [46]. This intramuscular-administered vaccine, licensed in Europe since 1996, was formulated for intranasal administration with the addition of a powerful mucosal adjuvant, heat labile entertoxin from *Escherichia coli* (also known as LT) but has since been withdrawn from the market due to 46 cases of Bell's palsy reported over a 7-month period [43].

Table 1. Clinical trial status of cell-grown influenza vaccines.

Manufacturer	Cell line	Clinical trial phase
A. Seasonal		
Baxter	Vero	I/II/II
Novartis	Mammalian	I/II/III
Sanofi Pasteur	PER.C6	II
Solvay Pharmaceuticals	MDCK	I
Protein Sciences	Insect	II/III
B. Pandemic		
Baxter	Vero	I/II
Nobilon International BV	Avian embryonic	I/II
Sanofi Pasteur	PER.C6	I
Solvay Pharmaceuticals	MDCK	I
Protein Sciences	Insect	I/II

4. Cell culture-grown influenza vaccines

Vaccine manufacturers produce over 100 million doses nationally and over 300 million doses worldwide of influenza vaccines in embryonated eggs, requiring 1 – 2 eggs per dose. Over 500 million embryonated hen's eggs must be ordered up to 12 months in advance to ensure an adequate supply of vaccine for the upcoming influenza season. Technology shifts to cell culture-based influenza virus growth have been on the horizon for a number of years. To assist in the development of cell culture-based technologies, the US Department of Health and Human Services awarded $1.1 billion to six pharmaceutical companies [47], which also included funds to establish infrastructure for the development of cell culture-based pandemic vaccines. In addition, major pharmaceutical companies have been successful in certifying mammalian and insect cell lines for vaccine production. Mammalian cell lines have posed concerns in the past due to potential microbiological and virological contamination. However, growth of virus in serum-free cell culture media and increased safety testing should result in reduced endotoxin content with minimal contamination as compared to egg-grown viruses. Mammalian cells lines, PER.C6, MDCK and VERO cells, and an insect cell line, are being used in Europe and the US to manufacture seasonal influenza vaccines. Treanor *et al.* have shown an insect cell-derived influenza vaccine to be safe in healthy adults and highly immunogenic as determined by HI titers in groups that received 75 and 135 µg of vaccine for the H1 and H3 components and immunogenic for the B component with 135 µg of HA [48]. Sanofi Pasteur is currently conducting Phase II clinical trials with PER.C6-based influenza vaccine, which has been shown to be well-tolerated in healthy adults [49]. MDCK cell-grown and egg-grown inactivated vaccines have similar immunogenicity [26] and have been shown to be safe for all age groups, from children to the older adults [50]. In addition, viruses grown in cell culture appear to be more similar to human isolates than their egg-grown counterparts [51,52]. Bruhl *et al.* have shown that a Vero cell-grown inactivated vaccine generated humoral immunity and enhanced cellular immunity as compared to that induced by an egg-grown vaccine in preclinical studies [53]. Recently, Baxter International Inc. (Deerfield, IL, USA) evaluated the efficacy of a Vero cell-grown seasonal influenza vaccine in healthy adults [54] and has moved forward with a Phase III clinical trial. A comprehensive list of the status of cell-based vaccines is given in **Table 1**.

5. Live attenuated vaccines

In 2003, the FDA approved the use of FluMist™ (MedImmune Vaccines, Inc., Gaithersburg, MD, USA), a trivalent live attenuated influenza vaccine (LAIV) [24]. This intranasally administered vaccine has been approved currently for non-pregnant healthy persons aged 2 – 49 years in the absence of a history of recurrent wheezing. However, it is not advised for children less than 2 years of age due to increased risk of hospitalizations and wheezing, pregnant women, persons with chronic or acute illness or immunocompromised immune systems, adults ≥ 50 years of age and other groups identified by the Centers for Disease Control and Prevention as having a reported or theoretical risk of an adverse reaction. In addition, LAIV is not recommended for healthcare workers who come in contact with severely immunocompromised patients due to the potential risk of transmission of the replicating live vaccine. The LAIV vaccine has been modified and is based on cold adaptation (ca) of the virus, allowing it to replicate in the lower temperatures (33°C) of the upper respiratory tract, similar to a natural infection, but not in the warmer lower respiratory tract airways, resulting in mucosal antibody responses and cellular responses with the potential to offer some protection against variant circulating strains [55]. This cold-adapted, temperature sensitive trivalent vaccine (CAIV-T) is a refrigerated version of LAIV and was licensed in 2007 for use in the same populations as the frozen LAIV. FluMist is based on a traditional reassortant with ca A/Ann Arbor/6/60 and ca B/Ann Arbor/1/66 as the influenza A and B master donor strains contributing six internal genes and a representative circulating strain donating the HA and NA for a complete 6:2 influenza genome. Five specific mutations in two of the three polymerase genes (PB1 and PB2) and the nucleoprotein (NP) gene result in a cold-adapted replication phenotype that allows for replication at lower temperatures, but an attenuated growth at higher temperatures (39°C) [56]. Klimov *et al.* also characterized a caA/Leningrad/137/17/57 master strain for use in influenza A vaccines in Russia [57]. Doses of 10^7 50% tissue culture infectious doses ($TCID_{50}$)

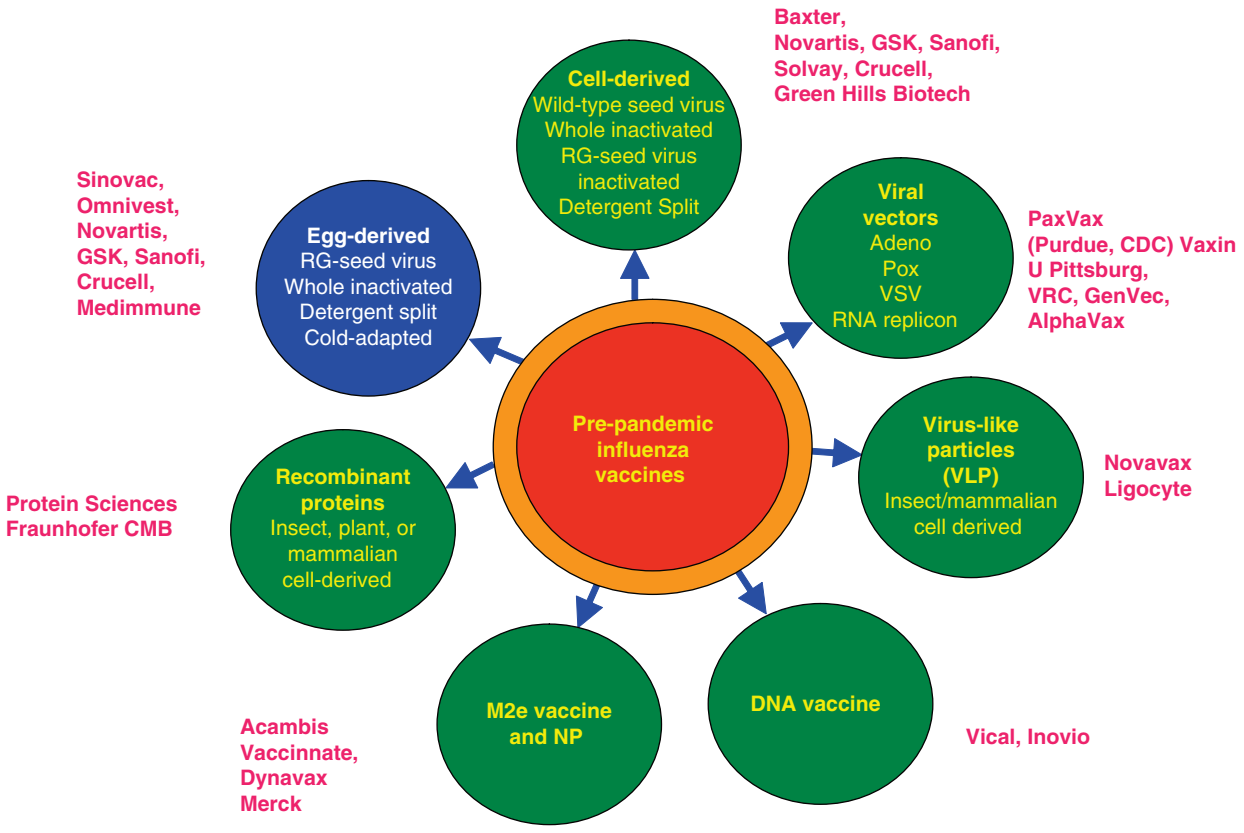

Figure 3. Status of pre-pandemic vaccine technologies.
Adapted from [121].

are safe and immunogenic for all age groups. In contrast, Treanor *et al.* reported no differences in HI titers in healthy adults receiving a CAIV intranasally as compared to those who received a trivalent inactivated vaccine intramuscularly; however, rates of infection were reduced after challenge with the wild-type virus in those participants receiving the CAIV [58]. Moreover, LAIV appears to be less efficacious in healthy adults as determined by culture confirmation of infection against an antigenically distinct virus when compared to an inactivated vaccine [59]. Therefore, similar to inactivated vaccines, LAIV/CAIV must be evaluated yearly for inclusion of surface protein(s) that are antigenically well-matched with the circulating strains.

Despite the disappointing results in healthy adults, LAIV has been shown to elicit a significantly higher immune response in children (age 6 – 59 months) when compared to a trivalent inactivated influenza vaccine [60,61]. The inactivated vaccine group reported 54.9% more cases of culture-confirmed influenza when compared to the group receiving the live vaccine, although higher rates of hospitalizations for any cause, including wheezing, were significantly higher in the live vaccine group (6.1%) when compared to the inactivated vaccine group (2.6%). Three additional studies in children ranging from 2 – 7 years of age indicate there was no increase in wheezing or hospitalizations compared to inactivated vaccine and placebo [62]. In addition, improved efficacy was also observed in children when the circulating virus was a variant from the vaccine strains. One of these clinical trials conducted over a 2-year span (during the time of the emergence of a variant strain – A/Sydney/H3N2) had reported a vaccine efficacy in children of 86% when the circulating strain was a variant virus [55]. However, another 5-year clinical trial comparing a bivalent live attenuated vaccine (minus the B strain) with a bivalent inactivated vaccine and a placebo group did not report a significant difference in vaccine efficacy between the two vaccine groups [63]. Nonetheless, the majority of this evidence suggests that live attenuated vaccines can be very effective in preventing influenza in the absence of pre-existing immunity.

Another Phase I clinical trial is currently underway in Austria to evaluate a live-attenuated, replication-defective influenza vaccine containing a genetically modified influenza virus lacking the non-structural 1 (NS1) gene [64]. NS1 protein, a viral virulence factor, has been shown to suppress the host's innate immune response [65-68] and deletion of the NS1 gene renders the live attenuated virus incapable of causing disease, but recognizable by the host's immune

Table 2. Pre-pandemic vaccines.

Technology	Strain (clade)	Adjuvant	Optimal Dose (µg)	No. of Doses	Assay	Seroconversion	% Seroconversion at optimal dose
Apathogenic [73]	H5 A/DS/97 (ND)	+MF-59	7.5	2	HI MN SRH	≥ 1:40 ≥ 1:20 > 25 mm^2	60% 80% 100%
Apathogenic [74]	H5 A/DS/97 (ND)	+MF-59	7.5	3rd boost	HI MN SRH	Fourfold rise Fourfold rise > 50% increase	67% 100% 100%
RG [80]	H5 A/VN/1203/04 (1)	None	90	2	HI MN	Fourfold rise Fourfold rise	57% 53%
RG [82]	H5 A/VN/1203/04 (1)	None	90/45	3rd boost	HI MN	≥ 1:40 ≥1:40	78%*/67%* 64%*/44%*
RG [83]	H5 A/VN/1194/04 (1)	+alum (-alum)	30	2	HI MN	Fourfold rise Fourfold rise	66% (53%) 41% (27%)
RG [86]	H5 A/VN/1194/04 (1)	+ASO3	3.8	2	HI MN	Fourfold rise Fourfold rise	82% 86%
RG [87]	H5 A/VN/1194/04 (1)	+AFO3	1.9	2	HI MN	Fourfold rise Fourfold rise	72% 92%
Whole/RG [93]	H5 A/VN/1194/04 (1)	Alum†	10	2	HI	Fourfold rise ≥1:40	78% 65%*
Whole/WT [98]	H5 A/VN/1203/04 (1)	+alum (-alum)	7.5/15	2	HI MN SRH	Fourfold rise Fourfold rise > 25 mm^2	35% (47%)/14% (26%) 51% (69%)/46% (68%) 33% (73%)/36% (58%)
Whole/WT [92]	H9 A/HK/1073/99	None	30	2	HI ≤ 32 y.o. (> 32 y.o.) MN ≤ 32 y.o. (> 32 y.o.)	Fourfold rise Fourfold rise	64% (75%)§ 100% (50%)
Subunit [92]	H9 A/HK/1073/99	None	30	2	HI ≤ 32 y.o. (> 32 y.o.) MN ≤ 32 y.o. (> 32 y.o.)	Fourfold rise Fourfold rise	36% (56%)§ 100% (100%)

*%GMT ≥ 1:40.

†Compared to placebo.

§All doses combined.

Strains: A/Duck/Singapore-Q/F119-3/97; A/Vietnam/1203/04;A/Vietnam/1194/04; A/Hong Kong/1073/97.

HI: Hemagglutination inhibition; MN: Microneutralization; ND: Not determined; NR: Not reported; RG: Reverse genetics; SRH: Single radial hemolysis; WT: Wild type; y.o.: Years old.

system. This Vero cell-grown vaccine is being administered intranasally in a single dose.

6. Control measures against pandemic influenza

In anticipation of a pandemic, a wide variety of strategies and technologies are being evaluated to deliver the lowest amount of vaccine in a minimal number of doses possible that will provide protective response(s) that is safe for all ages, regardless of health status, and able to generate cross-protective responses against variant influenza strains (**Figure 3**). Introduction of a novel HA into a relatively naïve population will broadly impact public health, as the spread of the disease in highly mobile populations will be rapid and uncontrollable without proper safe-guards. During this introduction, a higher proportion of deaths will occur in healthy adults < 65 years of age as populations > 65 years of age may possess some level of pre-existing immunity due to circulation of earlier influenza viruses [69]. Although vaccination remains our first line of defense against the spread of a pandemic, other measures, such as the use of antivirals, increased attention to personal hygiene, personal protective equipment (face masks, gloves, etc) and social distancing, including halting air travel and closing schools, may also contribute to controlling the pandemic spread.

6.1 Egg-derived pre-pandemic vaccines

One approach for pre-pandemic vaccines is to use an apathogenic H5 vaccine strain, such as A/Duck/Singapore-Q/F119-3/97 (H5N3), that is antigenically similar to the HPAI H5N1 viruses and can elicit cross-protective antibodies against divergent strains of H5N1 viruses. Lu *et al.* and others have shown that mice vaccinated with inactivated H5N3 virus with adjuvant (delivered intramuscularly) and without adjuvant (delivered intranasally) were protected from lethal challenge with HPAI H5N1 viruses [70-72]. Unlike the conflicting studies with seasonal vaccines adjuvanted with MF-59, a Phase I clinical trial evaluated A/Duck/Singapore-Q/F119-3/97 (A/Duck Singapore/97) adjuvanted with MF59 and has shown a significantly higher seroconversion rate as compared to healthy adults receiving the non-adjuvanted vaccine, while generating cross-protective hemagglutinating antibodies against a variant H5N1 strain [73]. A follow-up vaccination with the same vaccine formulation substantially boosted the antibody titers in those groups receiving the adjuvanted vaccine, but not the non-adjuvanted vaccine group [74]. Sixteen months post-vaccination, HI titers elicited by A/Duck Singapore/97 adjuvanted with MF59 were not detectable until receipt of a booster vaccination with the same apathogenic vaccine, suggesting priming can boost the immune response [75]. **Table 2** provides more specifics on the clinical trials evaluating egg-derived pre-pandemic vaccines.

6.2 Egg-derived pre-pandemic vaccines generated with the reverse genetics approach

Development of vaccines for H5 and H7 viruses pose several limitations to vaccine manufacturers. HPAI viruses are highly lethal to chickens, the source of the embryonated eggs required for virus growth. In addition, the cleavage of the multi-basic cleavage site in HA0, which links HA1 and HA2 and is one of the characteristics of HPAI viruses, is essential for virus infectivity [76]. Moreover, the multi-basic cleavage site has been shown to contribute to the spread of the virus to other organs outside the respiratory tract, leading to its virulence in poultry and the requirement for high containment laboratory facilities [77]. To overcome these limitations, egg- (and cell-) grown reassortant inactivated vaccine candidate viruses have been generated using the backbone of a high growth or ca vaccine donor strain (such as A/PR/8/34 or ca A/Ann Arbor/6/60) and the HA and NA from a HPAI virus using the reverse genetics (RG) approach [78,79]. Replacement of the multi-basic cleavage site with the amino acid sequence from a LPAI virus attenuates the viral virulence but not the antigenicity [76]. Thus, this technology has become the cornerstone of our efforts to develop non-pathogenic pre-pandemic influenza vaccines strains.

6.3 Inactivated split-viral RG vaccines

In a dose-ranging clinical trial with egg-grown RG A/Vietnam/1203/04 × A/PR/8/34, only the highest dose (90 µg) generated protective neutralizing antibody responses in slightly over 50% of the participants receiving two doses [80]. This RG vaccine candidate has recently been approved by the FDA for use as a pandemic vaccine [81]. A follow-up trial in which subjects received a third dose of vaccine revealed that at 28 days after a third vaccination, 78% of the 90 µg dose group and 67% of the 45 µg dose group developed protective neutralizing antibody titers [82].

Another clinical trial evaluated an inactivated egg-grown H5N1 split virion vaccine containing the surface glycoproteins from A/Vietnam/1194/04, with and without alum, and found the vaccine was able to elicit a seroconversion rate of 67% in the group receiving the highest dose (30 µg) of adjuvanted vaccine [83]. However, other studies have demonstrated no effect of alum in the vaccine [84,85]. A proprietary adjuvant (10% oil-in-water based emulsion) with the egg-grown RG A/Vietnam/1194/04 × A/PR/8/34 vaccine candidate was evaluated in a clinical trial and has shown an 86% seroconversion rate at the lowest dose of 3.8 µg [84,86]. Subsequently, evaluation of a 1/2 of antigen (1.9 µg) with this proprietary adjuvant resulted in 72% seroconversion in the group receiving the lowest dose after two immunizations, as compared to those receiving 7.5 µg of the unadjuvanted vaccine [87,88]. The clinical trials reported to date suggest that a unadjuvanted H5N1 vaccine is not very immunogenic and only after the addition of an adjuvant (with the exception of alum) can protective titers be achieved in naïve populations.

6.4 Inactivated whole virus vaccine

Inactivated whole virus vaccines may provide some enhanced immunogenicity [89-91] but can also be more reactogenic. Stephenson *et al.* evaluated an inactivated egg-derived H9N2 whole virus vaccine and found that even though the vaccine was less well-tolerated, with an increased rate of mild side effects over the subunit vaccine, one dose of the whole virus vaccine was able to elicit 100% seroconversion in naïve populations at the highest vaccine dose, whereas 100% of the participants > 32 years of age seroconverted after receiving one dose of the subunit vaccine and not the whole virus vaccine [92]. One explanation for this observation is that H2N2 viruses were circulating prior to 1970 so vaccinees > 32 years in age have detectable reactive HA antibodies to H9N2, which could potentially have a priming effect. Another clinical trial evaluated the addition of alum to 10 μg of an egg-grown H5N1 inactivated whole virus vaccine and found 78% of individuals receiving a prime and boost with the highest dose seroconverted by 42 days post-vaccination, as determined by HI titers [93]. These studies, which are limited in size, suggest that seroconversion of naïve populations may require two doses of adjuvanted subunit vaccine or one dose of whole virus vaccine.

6.5 Attenuated, cold-adapted influenza vaccines

LAIV vaccines are also being evaluated in clinical trials as a potential vaccine strategy against HPAI. A clinical trial was recently completed evaluating the safety and immunogenicity of a non-pathogenic H5N2 LAIV (7:1 HA Reassortant with A/Lenningrad/137/17/57:A/17/Pottsdam/88/92) [94] in healthy adults aged 18 – 25 receiving two doses of the intranasal vaccine [95]. The vaccine was safe and immunogenic with 47% of those participants receiving the LAIV generating a fourfold rise in HI titers as compared to 6% after one dose.

Several other clinical trials have recently evaluated the safety and immunogenicity of recombinant LAIV vaccines (also known as CAIV), H5N1 (A/Vietnam/1203/04 × ca A/Ann Arbor/6/60) recombinant vaccine, H5N1 (A/Hong Kong/213/2003 × ca A/Ann Arbor/6/60) recombinant vaccine, H7N3 (A/chicken/British Columbia/CN-6/2004 × ca A/Ann Arbor/6/60) recombinant vaccine and H9N2 (A/Chicken/Hong Kong/G9/97 × ca A/Ann Arbor/6/60) recombinant vaccine in participants receiving two immunizations. All of the vaccines were well tolerated and while the H7 vaccine trial results are still pending, results from the H9N2 vaccine trial induced ≥ 4-fold rise in HI and virus neutralizing serum antibody titers in 92 and 79% of participants after two doses, respectively, whereas both of the H5N1 pandemic vaccine trials were disappointing with < 10% of the participants having a fourfold or greater rise in HI titers after two doses [96]. The poor results from the H5N1 vaccines may be attributed to poor infectivity due to: i) differences in receptor binding by the HA; ii) over-attenuation of the ca master donor strain; or iii) a combination of both. However, as discussed previously, another CAIV clinical trial reported a similar observation with a seasonal CAIV vaccine when compared to a trivalent inactivated vaccine [58], suggesting that pre-existing antibodies from a prior infection of influenza may potentially lower the infection rates of the vaccine virus [59]. Another possible explanation as reported in a H9N2 clinical trial comparing a whole virus vaccine to a subunit vaccine, where vaccinees > 32 years of age had pre-existing antibodies to an H9N2 subunit vaccine with the suggestion that exposure to H2N2 circulating viruses over 35 years ago might also contribute to the enhanced immunogenicity observed with the H9N2 subunit vaccine [92]. Naive populations appear to generate the most robust immune response to LAIV, specifically children, followed by the adult population, with the elderly exhibiting low or non-detectable response to the live vaccine [58,60,97]. However, caution should be exercised during the administration of live vaccines as further reassortment of the shedding live vaccine strain with circulating wild-type viruses can occur, potentially generating a novel transmissible virus and hindering this approach as a viable strategy for pandemic influenza.

6.6 Cell-based pre-pandemic vaccines

A Vero-cell derived, whole virus, inactivated vaccine from the wild-type A/Vietnam/1203/04 manufactured under BSL3 containment in the Czech Republic has been shown to be immunogenic in animal models [98] and a Phase I clinical trial was completed with alum as an adjuvant [54]. The vaccine was well tolerated and safe and over 60% of the vaccinees developed protective titers of ≥ 1:20 when 7.5 and 15 μg of antigen was delivered with and without alum. Cross-protective antibody titers against A/Hong Kong/156/97 were observed in over 60% of the vaccinees, but cross-protective titers against A/Indonesia/05/05 were significantly lower. A number of other clinical trials with cell-grown pre-pandemic vaccines are ongoing and are listed in **Table 2** [99].

6.7 Recombinant protein-based pre-pandemic vaccines

To assess the safety and immunogenicity of an egg-independent pre-pandemic vaccine, a recombinant baculovirus-expressed purified H5 HA protein (rHA) from A/Hong Kong/156/97 H5N1 strain was used in a clinical trial. In a naïve population, this recombinant HA protein vaccine was well tolerated and generated a 52% seroconversion rate after two 90 μg doses as determined by microneutralization assay [100]. In a subsequent study, 68% of the vaccinees from this earlier rHA study (conducted 8 years earlier) who received one dose of an egg-derived variant vaccine (RG A/Vietnam/1203/04) generated significantly higher levels, neutralizing antibodies against A/Vietnam/1203/04 with titers ≥ 1:40 in 70 – 76% of pre-primed participants, as compared to only 44% of the unprimed participants [101]. Furthermore, cellular responses nine days post-booster

immunization generated similar robust responses against A/Vietnam/1203/04 and A/Hong Kong/156/97 viruses when compared to the placebo groups [102]. These findings suggest that a pre-pandemic priming of the population may be an option to prepare for a pandemic.

6.8 Virus-like particles

Innovative strategies for the generation of safe and immunogenic vaccines for the prevention of influenza have focused on exploiting the assembly and budding features of the virus. Specifically, the design of virus-like particles (VLPs), which have the ability to self-assemble, yet lack the ability to replicate, have shown significant promise in clinical trials. Basically, this strategy involves generation of a transfer vector (baculovirus) containing the HA, NA and matrix protein 1 (M1) genes required for VLP assembly and creation of a recombinant virus in insect cells [103], followed by purification of VLPs from the insect cell supernatant. In addition, the non-pathogenicity and consequent lack of a chemical inactivation step have facilitated the application of this strategy to a mechanistic understanding of the protection resulting from VLP-based prophylaxis. VLPs can provide protection against antigenically similar and distinct strains within the same subtype [104]. An ongoing clinical trial is evaluating the safety and immunogenicity of a 15 or 45 μg dose of an H5N1 A/Indonesia/05/05 VLP vaccine. Preliminary results suggest that the vaccine is safe and able to elicit a fourfold rise in neutralizing antibody titers against the wild-type H5N1 virus in 63% of those that received the highest dose [105]. Cross-protective antibodies have not been evaluated as of yet. Another Phase I/II clinical trial to determine the safety and immunogenicity of three different potencies of an H5N1 VLP vaccine in healthy adults is currently underway.

6.9 Vector-based vaccines

Egg-independent vaccine strategies using viral vectors, such as adeno-, pox- and alpha-viruses, to deliver influenza antigens have been under development for many years. Because of concerns for vaccine safety, replication-competent viral vectors are not considered to be an option for use in humans. However, preclinical and clinical trials have shown that modifications to render the viral vectors replication-deficient still enable the vector to deliver antigen efficiently and generate protective immune responses against influenza [106-113]. The modified vaccinia virus Ankara (MVA) has been evaluated in preclinical experiments for delivery of influenza antigens [114], but it has only been evaluated in clinical trials for delivery of HIV antigens, human papilloma virus, malaria, small pox and some cancers [115-120], where it has been shown to generate humoral and cell-mediated immunity against the antigen delivered. We and others have shown that the human replication-defective Adenoviral 5 (Ad5) vector is able to efficiently deliver influenza antigens in preclinical studies to generate cross-protective humoral and cell-based immune responses protecting against lethal challenge with H5N1 virus [107,109]. In mice, both cell- and humoral-based immune responses were maintained in excess of 12 months and as little as 1×10^6 plaque forming units (pfu) given twice intramuscularly conferred protection against lethal challenge in the absence of detectable neutralizing antibodies [121]. The Ad5 vector delivery strategy is also capable of delivering multiple antigens in a single vaccine construct to generate protective immune responses against multiple influenza antigens [122]. Influenza antigens have been shown to be delivered safely and effectively by Ad5 vector in a clinical trial of 24 healthy adults; 83% of those immunized intranasally with two immunizations of 10^8 viral particles of an Ad5 vector expressing the HA from A/PR/8/34 resulted in a fourfold rise in HI titers compared to those receiving a topical application of 10 – 1000-fold higher vaccine viral particles, with a fourfold rise in HI antibody titers ranging from 33% of participants for 10^9 and 10^{10} viral particle groups to 67% of participants receiving the vaccine of 10^{11} viral particles [108]. A replication-defective alphavirus vector has also been shown to effectively deliver seasonal influenza antigens and generate robust humoral and cellular immune responses in preclinical studies in mouse, rhesus macaque and rabbit models [112]. One bilateral footpad immunization (5×10^5 infectious units) of the alphavirus construct expressing an influenza virus HA gene was capable of generating HI antibody titers > 1:100 in a mouse model. A Phase I/II clinical trial was recently completed evaluating two concentrations of a one- or two-dose intramuscular or subcutaneous delivered vaccine composed of an alphavirus vector expressing the HA gene from A/Wyoming/03/2003 [123]. Protective HI titers were induced in 77% of the group that received a single dose of low concentration vaccine and in 80% of the group that received a single dose of high concentration vaccine, with no significant difference detected by route of administration. Administration of a second dose of vaccine slightly elevated the percentage of responders in both vaccine dose groups that had protective titers, and extended the durability of the T-cell responses when compared to responses generated against a single dose of vaccine [123]. Additional clinical trials to evaluate the effectiveness of vector-based pandemic vaccines are currently in the planning stages.

6.10 DNA vaccines

DNA vaccines have attracted much attention since the first report by Ulmer and colleagues in 1993 that a DNA vaccine can protect mice from influenza infection [124]. Subsequent preclinical studies have established that DNA vaccination is efficient in inducing long-lasting serum antibodies and/or strong helper and cytotoxic T cells specific for a variety of influenza virus-derived antigens in a number of animal models, including rodents, chicken, ferrets and non-human primates [125-128]. Most importantly, the vaccinated animals were completely protected against lethal viral challenge.

Despite these successes in animals, the initial results from a Phase I clinical trial were disappointing, as DNA vaccines failed to induce serum antibodies against the surface glycoproteins of influenza A viruses in humans after intramuscular delivery of the vaccine. However, using particle-mediated epidermal delivery technology, where plasmid DNA-coated gold beads are injected into the recipients' skin using high pressured helium gas [129], Drape and colleagues have shown in a Phase I study that epidermal delivery of a monovalent DNA vaccine encoding HA of an H3N2 influenza A virus resulted in induction of protective levels of antibody titers after three immunizations [130].

New focus has been placed on developing a 'universal' influenza vaccine that can confer protection against different subtypes of influenza A viruses, including the HPAI H5N1 viruses, by targeting the highly conserved proteins such as NP, M1 and matrix protein 2 (M2) [129,131-135]. Initial results in a preclinical trial using a mouse model have shown that DNA immunization using NP, M1 or M2 led to efficient generation of antigen-specific CD4 and CD8 T cells and/or broadly cross-reactive serum antibodies and conferred protection against lethal challenges with variant influenza A viruses, including H5N1 [134,136,137]. Two clinical trials are underway to assess the effectiveness of intradermal H5N1 DNA vaccines. One clinical trial is evaluating the intradermal injection of multiple plasmids (HA, M2, and NP) from A/Vietnam/1203/04 with and without adjuvant (Vaxfectin™, Vical Incorporated, San Diego, USA) [138], while the other clinical trial is evaluating a three-dose regimen of an intradermal HA DNA vaccine from A/Indonesia/05/05 in 45 healthy adults [139]. Results of safety and immunogenicity testing are pending.

6.11 Universal vaccines based on conserved proteins

Influenza vaccines and antivirals targeting the HA and NA surface glycoproteins, respectively, have been the focus of research since these surface glycoproteins are critical for virus infectivity and are primary targets for humoral host responses. However, these surface glycoproteins are subject to antigenic drift and a mismatch in a vaccine could result in suboptimal protection. Targeting vaccines to more conserved proteins may facilitate cross-protection against variant viruses and reduce morbidity and mortality.

Vaccines targeting the NP, M1 and M2e, the ectodomain of an ion channel protein (M2), tend not to succumb to antigenic drift. M2 is a small transmembrane protein of influenza viruses, consisting of only 97 amino acid residues. It forms tetramers in its natural form and functions as an ion channel to regulate the pH of the viral core. M2 has a 24 amino acid-long, non-glycosylated ectodomain (M2e), which is highly conserved among different human influenza A virus isolates [140]. The conserved nature of the M2 protein makes it an attractive vaccine candidate for novel vaccine approaches to induce broadly cross-reactive immune response against different subtypes of influenza viruses. Although a primary immune response is not mounted against M2e in a natural influenza infection in mice and humans [141,142], M2e antibody is detected at low levels in humans after multiple influenza infections [143]. M2e induces a substantial antigen-specific serum antibody response in a number of experimental animals when delivered in a vaccine [142,144-147]. Although the immunized animals were not completely protected from infection, M2-specific serum antibodies inhibited viral replication and reduced morbidity and mortality. So far, a wide range of M2e-based vaccine approaches have been evaluated in animal models, including M2e recombinant proteins [145,146], M2e genically fused to a carrier protein [144], M2e-derived synthetic multiple antigenic peptide covalently linked to a helper T-cell determinant [142], recombinant protein comprised of toll-like receptor 5 (TLR5) ligand fused to four copies of M2e [148] and DNA plasmids encoding M2e [149]. In preclinical studies, M2e-specific serum antibodies, the correlate of M2e vaccine-induced protection, could be detected and partial protection was demonstrated in most of the preclinical studies. Results from a Phase I/II clinical trial are also encouraging.

It is noteworthy that the M2e-specific serum antibodies may not always be as broadly cross-reactive as expected under certain experimental conditions. For example, serum antibodies raised against M2e conjugated to keyhole hemocyanin or *Neisseria meningitides* outer membrane protein failed to cross-react with the M2e of a pathogenic H5N1 virus [150], suggesting that immune responses induced by M2e vaccines derived from a circulating human influenza virus may only provide limited cross-reactivity to H5N1 and H9N2 viruses, which share over 90% amino acid ectodomain sequence homology with human isolates [151]. In this regard, Tompkins and colleagues have shown that using a conserved M2 sequence can overcome this limitation [136]. Mice immunized with a DNA vaccine encoding a full-length M2 consensus-sequence generated M2-specific antibodies against both human and avian M2 sequences and the animals were protected against lethal H5N1 challenge. One potential safety concern associated with M2e-based vaccines is that unlike mice, pigs immunized with a DNA vaccine encoding M2-NP fusion protein demonstrated exacerbated disease after challenge with influenza virus [149]. A recent clinical trial of 79 healthy volunteers has just been completed and preliminary results show that M2e vaccines are well-tolerated and immunogenic [152]. Results from the studies so far indicate the potential of M2e-based immunity as one component that can potentially broaden the protective effect of a 'universal' influenza vaccine.

7. Antigen limiting strategies

Skin is a dynamic physical and functional barrier that maintains the physiological integrity of the individual

by preventing the entry and colonization of potential pathogens. As the skin is reinforced by numerous innate defense mechanisms cooperating intimately with adaptive immunity, it provides a highly responsive immune environment for vaccine delivery. Langerhans cells (LCs) are professional antigen-presenting cells (APCs) found in the skin at a frequency of 500 – 1000 cells/mm^2. It is presumed that the activation of LCs in the skin results in their migration to the draining lymph, thereby making them extremely attractive targets to stimulate systemic immune responses [153-155]. Non-adjuvanted, antigen limiting or dose limiting approaches have been evaluated in healthy adults and have shown that 1/5th of the traditional intramuscular dose of seasonal vaccine given intradermally was equally as immunogenic as the intramuscular route, with only mild but frequent local reactions [156]. Belshe *et al.* reported that a 60% reduction in antigen by intradermal administration was enough to generate a comparable or increased immune response when compared to a full dose intramuscular injection in healthy adults; however, in adults > 60 years of age there was a decreased immune response to the intradermal vaccine compared to the intramuscular vaccine [157]. Dose limiting clinical trials have not been reported in children.

A dose ranging clinical trial of traditional routes of immunization has also shown that a reduction of antigen dosage can result in immune responses similar to a full dose vaccine. Treanor *et al.* has shown that a half dose trivalent vaccine administered intramuscularly yields similar immune response as an intramuscularly delivered full dose vaccine [158]. This indicates that in a given year with a vaccine shortage, whether due to egg supply or manufacturing issues, strategies can be implemented to stretch the current vaccine supply [159].

8. Adjuvants/formulations

Immunostimulatory compounds derived from plant, animal and insect products including manufactured pharmaceutical compounds, have been combined with influenza vaccine antigens and evaluated in preclinical trials to assess their safety and immunogenicity in animal models. However, only a limited number of adjuvants have advanced to clinical trials. Currently, only alum-adjuvanted vaccines are licensed for use in the US in human vaccines, but alum-adjuvanted, pre-pandemic influenza vaccines have shown mixed results thus far, necessitating the need to consider other effective adjuvants for influenza vaccines. The addition of adjuvants to a vaccine, with the exception of alum, has been shown to reduce the amount of influenza antigen required to generate a protective immune response when evaluated in clinical trials [86,160]. Influenza adjuvants under evaluation in preclinical trials have been reviewed previously and will not be covered in this review.

9. Conclusion

Vaccination still remains our best defense against epidemic influenza and a potential influenza pandemic. As far as pre-pandemic H5N1 vaccines are concerned, currently the US Department of Health and Human Services (DHHS) has stockpiled 12 million doses, enough to protect at least 6 million people without an adjuvant. DHHS's 5-year goal is to have enough vaccine stockpiled to ensure that all residents of the US can be vaccinated within 6 months of the onset of the pandemic. Although oil-in-water and inactivated whole virus vaccines have been demonstrated to induce broadly cross-reactive neutralizing antibodies, the level of cross-protection generated by the administration of the stockpiled vaccine against a variant pandemic strain remains to be seen. Other vaccine strategies that target more conserved influenza proteins could also offer protection in the event of a pandemic. Results from previous clinical trials have shown that non-adjuvanted vaccines are poorly immunogenic and reliance on egg supply and high containment facilities to manufacture pandemic vaccines could potentially delay the supply of an antigenically matched vaccine. Alternative egg- and adjuvant-independent strategies are also being evaluated in preparation for increased demand globally for a vaccine. Global production capacity for pandemic vaccines must be expanded, possibly through the use of new cell-based production technologies. Novel adjuvants and novel delivery and dose-limiting strategies that engage the innate immune system are other approaches to extend the limited supply of vaccine. However, these adjuvants and strategies must undergo rigorous safety testing to ensure maximum immunogenicity is obtained with minimal and acceptable reactogenicity. Recent advances in vaccine technology, as well as increased understanding of the immune system, should lead to the development of new and improved influenza vaccines in the 21st century.

10. Expert opinion

Vaccinating the global population against seasonal and pandemic influenza vaccines requires not only dose-sparing and alternate delivery strategies, but also formulations with novel adjuvants, as current manufacturing capacity does not meet the global demand for influenza vaccines. Furthermore, the relatively poor immunogenicity of some vaccines, for example, avian influenza vaccines in healthy adults, as well as seasonal vaccine in older adults, warrants research investment in identifying novel adjuvants and delivery technologies. We have come a long way from the days of a mystery brew with ill-defined adjuvant systems to defined chemical entities. An ideal adjuvant should be simple to manufacture, safe, stable and whose mechanism of action is known. Adjuvant systems need to engage the innate immune system to facilitate the mobilization, activation, differentiation, maturation and migration of antigen-presenting cells and to

stimulate humoral and cellular immune functions to facilitate dose-sparing and maintain the stability and potency of the vaccine. Activation of innate immune systems is key to priming or boosting adaptive immune responses. Hence, understanding the innate immune system could lead to the identification of novel molecular adjuvants and their mechanism(s) of action. In addition, improvements in the vaccination rates of the seasonal vaccine for recommended groups need to occur to ensure that at-risk populations are vaccinated to prevent or minimize complications from influenza infections. Such strategies could entail detailed educational programs, convenient locations and worksite vaccination settings, and/or a yearly reminder card program [161-163].

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None of the authors in this report declares a conflict of interest. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control or the funding agencies.

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