Vaccine development for new influenza A (H1N1) and vaccine candidates for future pandemics

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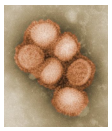

Picture used on the front page:

Negative stain EM image of the 2009 H1N1 Influenza A/CA/4/09.

C. S. Goldsmith and A. Balish, Centre for Disease Control.

http://www.cdc.gov/media/subtopic/library/diseases.htm

Vaccine development for new influenza A (H1N1) and vaccine candidates for future pandemics

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ABSTRACT
On the 11th of June 2009, the first influenza pandemic of the 21st century was announced by the WHO. This pandemic H1N1 influenza A virus appeared to be a result of reassortment of classical swine H1N1, human H3N2, Avian, and Eurasian swine influenza viruses over the years. Since it has not circulated in humans before, the population is immunologically naïve and the virus can easily spread globally. On the 25th of October 2009, over 440,000 confirmed cases have been reported worldwide, of which >1% was fatal. Vaccination is considered to be the best defensive strategy against an influenza outbreak. Conventional influenza vaccines contain hemagglutinin and vaccine production can only start when the consensus virulent strain is identified. During a pandemic outbreak, time is sparse and therefore dose and time sparing strategies as well as vaccines leading to cross-protection and/or heterosubtypic immunity are desirable. These options for novel influenza vaccines are discussed in this paper.

INTRODUCTION

The World Health Organization (WHO) announced the appearance of a new influenza A (H1N1) virus late April and the virus had caused nearly 30,000 confirmed cases in 74 countries at the time the WHO declared it to be pandemic on the 11th of June 2009. This H1N1 strain has not circulated in humans before and was noted as a contagious virus which can easily spread globally[1]. On the 25th of October 2009, over 440,000 confirmed cases and 5700 deaths had been reported worldwide, which might be even higher since many countries stopped counting milder individual cases[2]. At present this pandemic is of moderate severity and mortality rates are comparable to seasonal influenza outbreaks, however of all European cases, nearly 80% is under 30 years of age whereas seasonal influenza virus affects mostly elderly people and infants[3]. Considering the threat of mutations and/or reassortments of the virus that might increase severity of the new H1N1 influenza stain, and the threat of pandemics caused by other more virulent influenza viruses e.g. avian-flu H5N1, it would be valuable to obtain potent pandemic vaccines[4].

Influenza viruses

Influenza viruses are enveloped, negative sensed segmented single-stranded RNA viruses and belong to the family of *Orthomyxoviridae*[5,6] his family consists of the influenza A virus, influenza B virus, influenza C virus, Thogotovirus, and Isavirus genera[6]. Influenza virus types A, B, or C, is determined by the genetic composition, nucleoproteins (NPs) that bind the RNA, and ion channels in the membrane which are also known as M2 proteins[5,7-9]. Human influenza viruses are denoted as Subtype/Geographic origin/strain number/year of isolation (HA and NA subtype). For example the H1N1 virus strain used in the forthcoming H1N1 vaccines is A/California/7/2009(H1N1)[10].

Influenza A structure

Influenza A is composed of eight helices of RNA, encoding 11 proteins of the virus. The virions are generally spherical with a diameter of approximately 80-120nm but it has also been shown they can be pleomorphic[8,11]. The envelope is composed of a lipid bilayer containing the viral hemagglutinin (HA), neuraminidase (NA), and ion

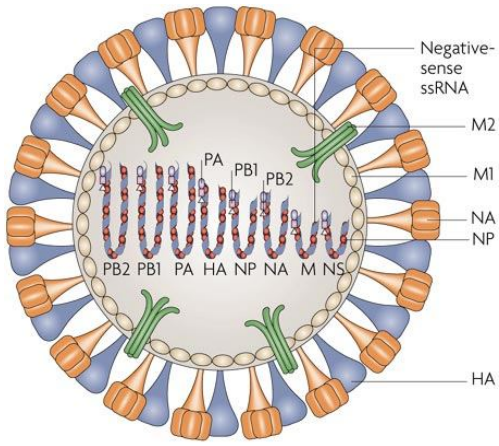

Figure 1 | **The structure of influenza A virus.** The envelope is composed of HA, NA, M1 and M2 proteins. Other proteins and the eight segmented (-) ssRNA strains are present in the core of the virion. Figure derived from Nelson *et al.*[12].

channel (M2) proteins, and underneath a layer of matrix protein (M1) can be found. The remaining proteins are located inside the virion (Figure 1)[12] where the nucleoprotein (NP) bound to the viral RNA (vRNA) strands along with nuclear export protein (NEP) and the polymerase proteins PBI, PB2, and PA, that build up the viral ribonucleoprotein (vRNP)[5,8,9].

HA, M2, and NA function in viral replication

Where many viruses fuse with the host cells at the plasma membrane, influenza viruses undergo fusion within an endosomal compartment. The virus attaches to the cell via binding of the viral HA glycoprotein to the sialic acid receptor on the host cell. Sialic acids can be 2,3- or 2,6-linked to galactose resulting in a specific conformation recognized by the HA. In the human upper respiratory tract, 2,6-linkages are abundant which are preferred by human influenza viruses, whereas 2,3-linkages, preferred by avian influenza viruses, are present in avian species and in human lung epithelia[13]. The virus is internalized by receptor-mediated endocytosis. The M2 ion channels play an essential role in decreasing the pH in the late endosomes. This leads to conformational changes of the HA, resulting in fusion of the endosomal and viral membranes and release of the vRNP into the host cell[9,14].

After transcription in the nucleus of the host cell and translation in the endoplasmatic reticulum (ER), proteins and RNA segments assemble at the membrane. Budding of the membrane results in virus particles that need to be detached from the host cell. Neuraminidase plays a critical role in this process by cleaving the sialic acids and thus is crucial for spreading and transmission of the influenza virus[5].

Immune responses

Humoral and cellular responses both play an important role in viral clearance upon infection. B cells encountering an antigen will expand and differentiate into antibody (Ab) producing plasma cells and memory B cells. These latter ones enhance a rapid response to secondary infections and secretion of neutralizing antibodies[15,16]. Especially HA, but also NA can be recognized by neutralizing antibodies, making them commonly used vaccination targets by blocking respectively virus binding to the host sialic acids and prevention of cleavage of the sialic acids and thus inhibition of viral spreading[5,17]. However, also M2 can be neutralized by antibodies by ionchannel blockage resulting in inhibition of membrane fusion (for review see Fu *et al.*[18]).

Since HA and NA are highly variable, more conserved viral proteins (e.g. NP and matrix proteins) are promising targets to induce heterosubtypic immunity. Cytotoxic T lymphocytes (CTLs) can recognize these NP and M1 epitopes and upon recognition, CTLs destroy virus-infected cells by granzyme and perforin release or by Fas/Fas-ligand interaction. A summary of the correlates of protection for the different responses described above, and their characteristics is denoted in Table 1[17].

Cross-protection

Americans born before 1950 showed in 34% of the cases immunity against 2009 H1N1 compared to 4% of the people born after 1980. It is suggested this effect is due to the National vaccination campaign in the U.S. after the outbreak of swine influenza H1N1 virus in 1976, since the 2009 H1N1 HA has greater similarities to the A/New Jersey/1976 strain used in that vaccination campaign compared to seasonal influenza H1N1 viruses used in annual vaccination[19]. Yu *et al.* showed 90 year old survivors of the 1918 influenza pandemic were protected for life by possession of highly functional, virus-neutralizing antibodies against the 1918 influenza strain. Also cross-reaction with the HA genetically similar 1930 swine H1N1 influenza strain was shown. Vaccination can thus induce life-long immunity and can protect against different virus strains that are antigenically similar[20]. Cross-protection between influenza strains of the same type but from different clades (e.g. H1N1 clade 2A and H1N1 clade 2B) is called intra-subtypic immunity. Cross-protection between influenza strains of different subtypes (e.g. H1N1 and H5N1) is called inter-subtypic cross-protection or heterosubtypic immunity[21]. The development of vaccines able to induce broad protective immunity is desirable to protect against pandemic viruses.

Table 1 | **Potential correlates of protection against pandemic influenza.** Adapted from Rimmelzwaan *et al.*[17].

Correlate of protection	Remarks	Vaccine type
HA specific antibodies	Vaccine should match outbreak strains	Conventional subunit, split virion or whole inactivated virus with adjuvant and novel vaccine candidates
NA-specific antibodies	Preferable matching NA, but intra-subtypic cross-reactivity exists	Recombinant protein, vector vaccine, DNA
M2-specific antibodies	Conserved protein, broad reactive	Recombinant protein, vector vaccine, DNA
Virus-specific CTL	Many conserved epitopes, broad-reactive	Live vaccine, vector vaccine, DNA, particulate

CONVENTIONAL VACCINATION STRATEGIES

There are two types of influenza vaccines currently in use, inactivated virus vaccines and life attenuated virus vaccines. The content of these trivalent vaccines are evaluated annually and three target strains (H1N1, H3N2, and influenza B) are recommended for the northern and southern hemisphere by the WHO. For the 2009-2010 season the northern seasonal vaccine is directed against the A/Brisbane/59/2007(H1N1), A/Brisbane/10/2007(H3N2), and B/Brisbane/60/2008 virus. Current vaccines contain a dose of 15 µg of HA of every influenza strain[10].

Inactivated virus vaccines

Viruses can loose their pathogenicity when inactivated or killed after growth in embryonated chicken eggs[22]. Three vaccine formulations of inactivated virus are used: whole virus vaccine inactivated with formaldehyde or β-propiolactone to destroy the viral envelope, in split virus vaccines the viral envelope of the virus is disrupted by further treatment with a detergent (e.g. ether or tributyl phosphate), and subunit vaccines consist of highly purified HA and NA which are obtained through additional centrifugation steps. Lower doses of whole virus vaccine can be used compared to split or subunit vaccines, however, it was shown that this type of vaccine might cause an increased incidence of side effects compared to the subunit vaccines which are considered to be safest[23].

Live attenuated virus vaccines

Live attenuated vaccines have the potential to induce a strong long-lasting immune response and are considered to be safe (for reviews see Murphy *et al.*[24] and Ambrose *et al.*[25]). Different approaches have been developed to generate life attenuated influenza virus vaccines. Serial passage of an influenza virus at sequentially lower temperatures results in a virus that grows at low temperatures (approximately 25-33°C)[26]. Flumist® is a cold-adapted live attenuated virus vaccine licensed for the use in humans and can be administered intranasally[27].

A novel strategy to generate live attenuated influenza vaccines is based on modified NS1. Deletions in the NS1 encoding gene cause expression of truncated NS1 proteins, resulting in a replication deficient virus. Vaccination with these NS1-modified live attenuated influenza viruses, have shown to induce a protective and even cross-neutralizing response in a broad range of animals and appears to be a promising strategy. However safety and efficacy tests are still needed (for review see Richt *et al.*[28]).

Classical reassortment

Since the influenza viral genome is segmented, exchange of viral genes between two viruses can

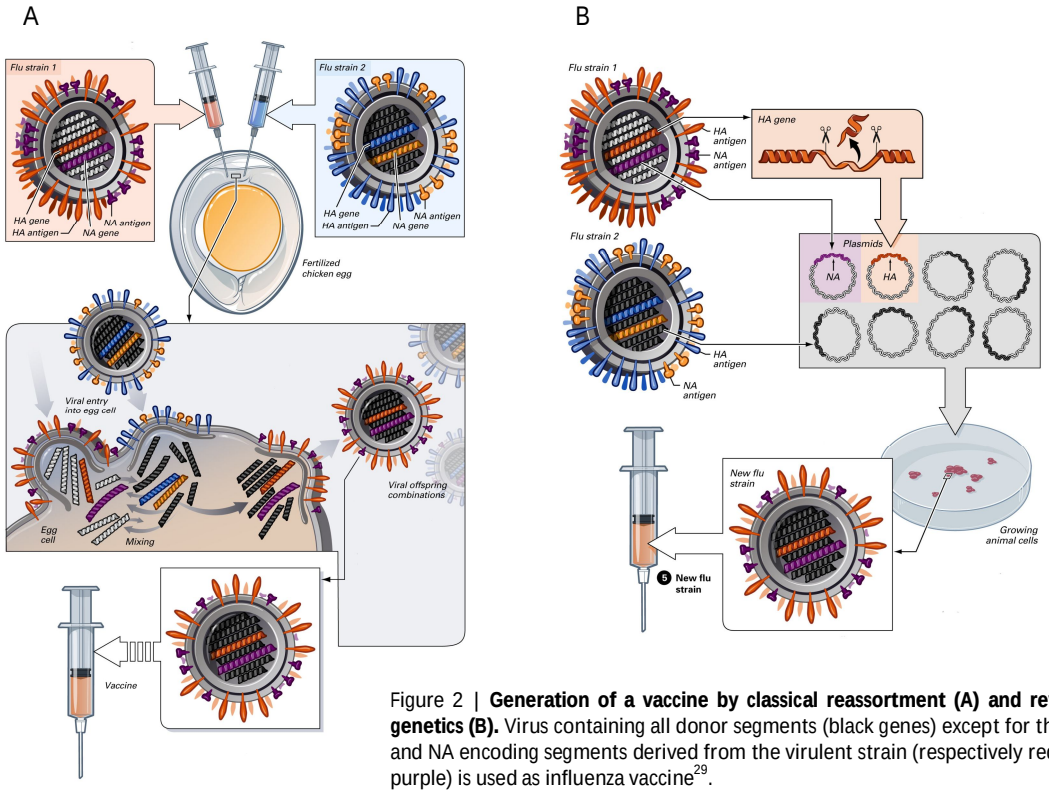

Figure 2 | **Generation of a vaccine by classical reassortment (A) and reverse genetics (B).** Virus containing all donor segments (black genes) except for the HA and NA encoding segments derived from the virulent strain (respectively red and purple) is used as influenza vaccine[29].

take place in co-infected cells. Injection of both the virulent virus strain and a live attenuated donor strain will lead to reassortment of the gene segments (Figure 2A)[29]. The generally used donor strain is A/Puerto Rico (PR)/8/34 which is safe in humans and has a high growth rate in embryonated eggs. Two viruses both containing 8 segments can theoretically result in 254 different rearranged progeny viruses. Virus containing only the segments coding for HA and NA of the virulent strain and all other segments from the donor strain need to be selected to create the new vaccine with (Figure 2A)[29]. Altogether, vaccine production by classical reassortment is a very time consuming process[22,24,29].

Reverse genetics

Reverse genetics uses plasmids encoding the HA and NA of the circulating virus which can be modified to remove virulence motives. The other 6 plasmids are derived from the donor strain. All 8 plasmids are transfected in mammalian cells and subsequently the virus containing all donor segments except for the HA and NA encoding segments derived from the virulent strain, is isolated (Figure 2B)[29,30].

Eggs vs. cell based production

Before the licensure of MDCK cells for vaccine production in Europe, all influenza vaccines were produced in eggs, requiring at least one egg per vaccine dose. The main advantage of cell based vaccines compared to production in eggs is the manufacturing time. The cell manufacturing process will be weeks shorter where in eggs it can take 6-9 months and a short production time can be crucial during a pandemic. When an influenza pandemic occurs and especially when arisen from an avian source, the production of embryonated eggs might be insufficient or even stopped due to high mortality under chickens[31-33]. Other advantages of cell-cultured vaccines are that production can be easily upscaled when high amounts of vaccines are needed, the risks for allergic reactions to egg components and microbial contamination is decreased, and cell based vaccines show better antigen matching than egg based vaccine strains[33,34]. In Europe only MDCK cells are licensed for vaccine production, the Vero cell line is currently only used for one viral vaccine licensed in the U.S.[33,35]. Those cell lines together with PER.C6 are considered by the WHO to be commercially viable and are in development to use in vaccine production[35].

Adjuvants

To improve the immunogenicity of vaccines, adjuvants can be used. They prolong the exposure time of antigen to the immune system, enhance the delivery to antigen presenting cells (APCs), or provide immunostimulatory signals. This way, lower vaccine doses can be used which might be valuable, especially during a pandemic[36,37]. However, adjuvants are often debated for the possibility to induce (delayed) side effects[38]. Adjuvants used in influenza vaccines are alum, MF59 and AS03 which are discussed below. Multiple adjuvants are currently under investigation (for review see Atmar *et al.*[36]) and a promising novel adjuvant based on immune-stimulating complexes (ISCOMs) is also discussed here.

Aluminum

The most widely used and only adjuvant currently used in the U.S. is alum (aluminum phosphate or hydroxide)[37,39]. The working mechanism of alum remains still unclear. It has been suggested that alum stimulates the Th2 response through uptake by Dendritic Cells (DCs), results in a slow release of the antigen by depot formation, or induces inflammation resulting in activated APCs. Research from Kool *et al*. suggests alum is able to induce necrosis of cells, resulting in uric acid release triggering DC activation[39].

Oil-in-water emulsions

In Europe other adjuvants for influenza vaccines that are approved are MF59 and AS03. Both are oil-in-water emulsions (o/w) consisting of small microvesicles: oil surrounded by a detergent monolayer. MF59 consists of Squalene, a natural cell membrane component, the surfactant sorbitan triolate (Span 85), and Polysorbate 80 (Tween 80) which is widely used as emulsifier in foods, cosmetics and pharmaceuticals[40]. MF59 has been administered to more than 20 million individuals and is considered to be a safe and potent adjuvant (for review see Kreijtz *et al.*[21]), reducing the normal HA dose of 15 µg to 7.5 µg HA per dose[41]. AS03 is composed of DL-α-tocopherol, squalene, and Tween 80[41]. AS03 adjuvanted vaccines have shown to induce more local and general adverse effects compared to non-adjuvanted vaccines, however since no severe adverse effects were reported and the HA dose can be reduced to 3.75 µg HA per dose, this adjuvant is now approved to be used in Europe[38,42].

Table 2 | **Projected formulations for new influenza A(H1N1) vaccine.** Derived from Collin *et al.*[46].

	Total	Whole virion	Split virion	Sub-unit	Live attenuated	Recombinant protein
Vaccines	33	9	14	4	5	1
Adjuvanted	12	6	3	3	–	–

PANDEMIC H1N1 INFLUENZA

Point mutations in genes coding for HA and NA cause influenza strains to change frequently. This antigenic drift results in annual update of seasonal influenza vaccines since neutralizing antibodies in individuals are unable to recognize the altered surface of these drifted strains[13,43].

In a cell infected with both an animal and human virus, reassortment can take place. When a human virus adapts an HA molecule which has not circulated in the human population before, this antigenic shift could lead to a pandemic since the population is susceptible to this virus[13,43].

The pandemic H1N1 influenza A virus appeared to be a product of reassortment of classic swine H1N1, human H3N2, Avian, and Eurasian swine influenza viruses over the years (Figure 3)[44]. Segments of the 2009 human H1N1 virus coding for the polymerases, HA, NP and non-structural (NS) proteins show high similarities with the H1N2 influenza A North American swine virus which was isolated in the late 1990s. The segments coding for NA and matrix proteins are thought to be related to the swine virus isolated in Europe in 1992 (Figure 3)[44].

Pandemic H1N1 vaccines

Since influenza viruses constantly change, and since no vaccine is available that is protective against all influenza strains, production of a (pandemic) influenza vaccine can only start when the virulent strain is apparent in the population. Since there is not enough time to perform long-term safety tests on the vaccine in a pandemic setting, mock-up vaccines provide a solution. These vaccines mimic future pandemic vaccines and make it possible to test and predict for production, properties and safety of the vaccine. When a strain becomes pandemic in the population, the mock-up virus strain is replaced by the actual pandemic strain and since a lot of testing on the vaccine already has been done, authorisation for the use of the vaccine can be obtained in a limited amount of time[45].

Collin *et al.* describes a survey carried out by the WHO among influenza vaccine manufacturers on their views and planned seasonal and pandemic vaccine production. The plans for new influenza A(H1N1) vaccine production are summarized in Table 2[46]. Thirty-three products were reported and most were based on whole virion or split vaccines. Twelve vaccine formulations would contain an

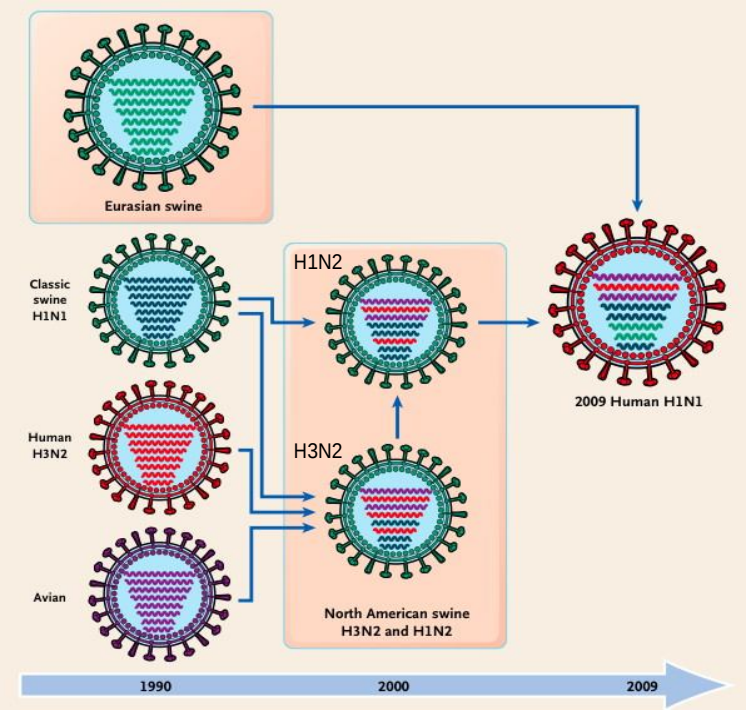

Figure 3 | **History of Reassortment Events in the Evolution of the 2009 Influenza A (H1N1) Virus.** The eight segments shown within each virus code for the following proteins of the influenza A virus (top to bottom): polymerase PB2, polymerase PB1, polymerase PA, hemagglutinin, nuclear protein, neuraminidase, matrix proteins, and nonstructural proteins. The segments of the human 2009 influenza A (H1N1) virus have coexisted in swine influenza A virus strains for more than 10 years. The ancestors of neuraminidase have not been observed for almost 20 years. The mixing vessel for the current reassortment is likely to be a swine host but remains unknown. Adapted from Trifonov *et al.*[44].

adjuvant, in most cases alum, or an oil-in-water emulsion[46].

H1N1 vaccines in the Netherlands

For the 2009 H1N1 pandemic, the Dutch government has ordered Pandemrix™ and Focetria® to vaccinate the entire population of the Netherlands twice. Both vaccines are mock-up vaccines and eventually will contain the A/California/7/2009(H1N1)v-like virus reference strain as recommended by the WHO, since the genetic and antigenic properties of this strain is closely related to H1N1 circulating in at least 28 countries[10,47,48].

Pandemrix™ is a mock-up vaccine developed by GlaxoSmithKline (GSK). This inactivated split influenza virus contains adjuvant AS03 and is propagated in eggs. The mock-up influenza A/VietNam/1194/2004 (H5N1) like strain (NIBRG-14) is replaced with the influenza A/California/7/2009(H1N1)v-like strain (X-179A)[38].

The Focetria® vaccine, developed by Novartis, is generated using classical reassortant methods. The gene segments coding for HA, NA, and PB1, were derived from the influenza A/California/7/2009(H1N1) strain, the remaining genes were taken from the influenza A/PR/8/34 virus. The virus was propagated in Madin-Darby Canine Kidney (MDCK) cells and MF59 adjuvant is used to improve the immunogenicity[34,41].

VACCINE CANDIDATES FOR FUTURE PANDEMICS

Fast and effective vaccine production is of great importance during a pandemic outbreak, however, current vaccines can only be generated when the consensus virulent strain is identified. It is necessary to develop novel vaccine strategies which are time and dose sparing, and induce a broad protective immunity. Recognition of conserved epitopes by the CR6261 antibody, DNA vaccines, Viral vector vaccines, and ISCOMs are promising strategies in influenza vaccine and are discussed in this chapter.

CR6261 antibody

Thorsby *et al.* recovered human monoclonal antibodies (mAbs) from memory B cells obtained from individuals recently vaccinated against (seasonal) influenza. The mAbs showed neutralizing activity against H1, H2, H5, H6, H8, and H9 influenza subtypes[49]. This cross-reactivity is due to antibody binding to a conserved helical domain of HA. Binding of the antibody (Ab) to that HA domain blocks the conformational changes the HA protein needs to undergo for membrane fusion between the virus and host cell to take place[50]. The IgG CR6261 antibody shows to have both a prophylactic and therapeutic effect in mice challenged with lethal doses of influenza virus. Immunization with 2 mg/kg CR6261 resulted in 100% survival when challenged with H1N1 influenza virus 24 hours later. Mice challenged with H5N1 influenza virus showed the same effects with 5 mg/kg (Figure 4)[49]. Mice therapeutically treated at day 3, 4, or 5 post infection (p.i.) with H5N1 virus resulted in a higher survival, less loss of body weight, and the clinical score improved compared to mice treated at day 6 p.i. or inoculation with control mAbs 4 days after challenge (Figure 4)[49]. The capability of the human antibody CR6261 to induce both a prophylactic and therapeutic immunity might be of great importance in case of a pandemic threat, when individuals showing symptoms also can be treated. Based on these results it would be desirable that further research would be done to examine the possibilities of a CR6261 antibody-inducing vaccine.

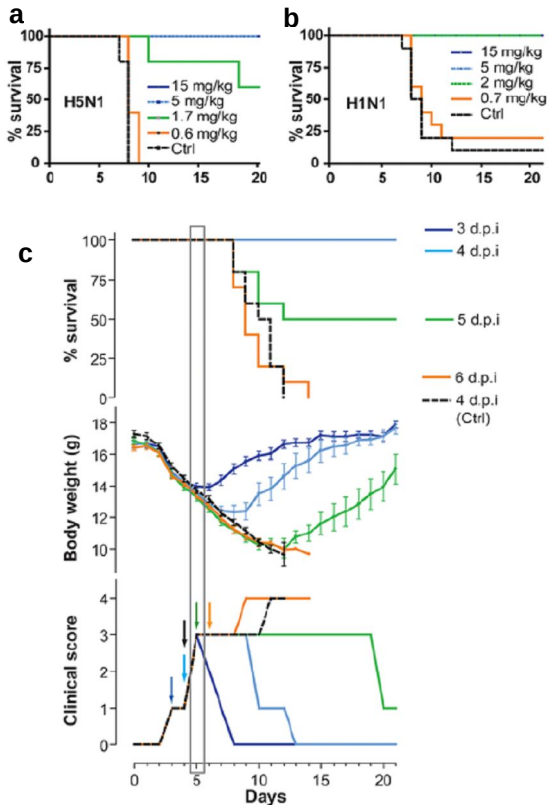

Figure 4 | ***In vivo* protective activity of CR6261 against wild-type H5N1 and H1N1 strains.** Kaplan-Meier survival curves of BALB/c mice were injected (i.p.) with CR6261 or irrelevant control (15 mg/kg) then challenged 24h later (i.n.) with (a) 10 LD_{50} of A/Vietnam/1203/04 (n=5) or (b) 25 LD_{50} A/WSN/33 (n=10). (c) Survival (upper panel), mean ±s.e.m. body weight (middle panel), and median clinical signs (bottom panel) of mice (n=10) challenged with 25 LD_{50} A/Hong Kong/156/97 and injected i.v. with 15 mg/kg CR6261 IgG1, 3 (dark blue), 4 (light blue), 5 (green) or 6 (orange) days, or control mAb (black dotted line) 4 days after challenge. The day five time point at which therapeutic efficacy is lost is indicated by a grey box[49].

DNA vaccines

A DNA vaccine consists of a DNA plasmid encoding for a viral gene that can be expressed in host cells. When administered to the host, DNA can be taken up by DCs resulting in MHC-presentation to T cells and an antibody-mediated immune response[26]. Advantages of DNA vaccines are that they do not require embryonated chicken eggs or mammalian cell cultures and can be manufactured quick and cheap. A relatively low dose seems to be sufficient in inducing a long-lasting response and no adjuvants are needed but might be used to improve immunization. However, there might be a risk when plasmid DNA would integrate in the host genome what might result in mutagenesis, autoimmune responses, and inducing of tolerance[7,51].

DNA vaccines against NP, MP1, NS1

The conservation of NP, M1 and M2 proteins was investigated in five host-specific influenza lineages including avian, classic swine (H1N1), and human strains. Nucleotide sequencing showed the maximum amino acid difference between NPs was 10.8%[52], M1 showed a 24.6% divergence, M2 showed the highest variability with 48.5%[53]. Of these viral proteins, the NP and M1 proteins are thus best conserved and will therefore provide a certain level of cross-protection between multiple virus strains[54]. Darapaneni *et al.* showed that the NS1 and NS2 proteins also contain highly conserved regions[55]. Mice immunized with a combination of DNA plasmids encoding for the conserved influenza NP (pNP), M1 (pM1), and NS1 (pNS1) from H1N1 strains resulted in an enhanced survival after challenge with a lethal doses of an H5N2 influenza virus strain compared to separate use of these plasmids, empty vector treated, or untreated mice (Figure 5A)[54]. To test whether this protective effect could be observed in a different host and against an antigenically unrelated influenza strain, chickens were vaccinated with pNP+pM1 or with pNP+pM1+pNS1 and afterwards challenged with lethal doses of H5N3 avian influenza virus. Chickens vaccinated with pNP+pM1+pNS1 showed an improved survival compared to the pNP+pM1 and placebo vaccinated chickens (Figure 5B)[54].

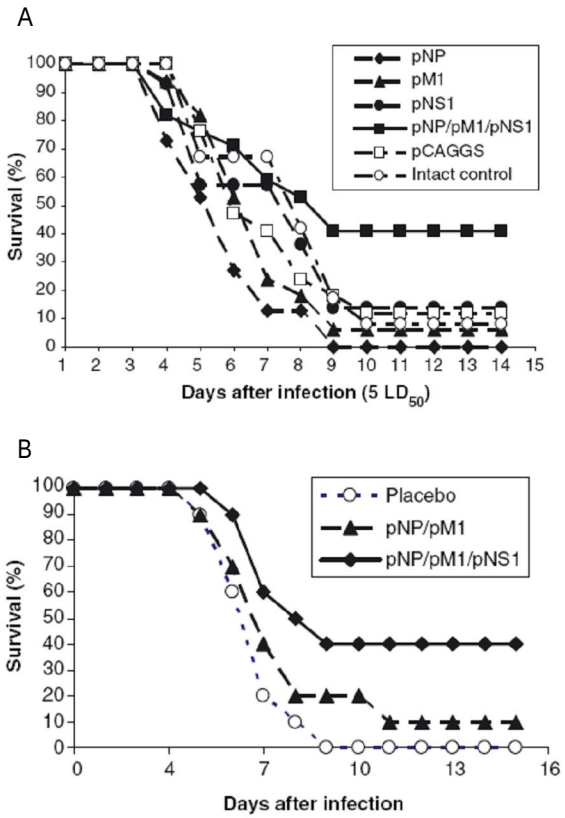

Figure 5 | **Survival after vaccination.** (A) Mice vaccinated with the combination of pNP, pM1, and pNS1 after challenge with 5 LD_{50} of influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2). (b) Chickens vaccinated with pNP/pM1 or pNP/pM1/pNS1 were challenged with lethal doses of avian influenza A/Tern/SA/61 (H5N3) virus[54].

Viral vector vaccines

Incorporation of a gene encoding a viral influenza antigen into a non-pathogenic viral vector results in expression of these antigens in cells infected with the vector. Upon vaccination this will result in immunization of the host by antigen presentation of the major histocompatibility (MHC) class I and II, leading to both a humoral and CTL response. Since a non-pathogenic vector is used, viral vector vaccines are predicted to be safe in humans[56,57]. Pre-existing antibodies to the vector might limit the immunogenicity of the vaccine with multiple doses or when the same vector is used for different vaccines. Intensive and promising research is performed on vectors of Newcastle disease virus, vesicular stomatitis virus, Venezuelan equine encephalitis virus, poxvirus, adenovirus, and Modified Vaccinia virus Ankara (MVA)[56]. Adenoviral and MVA based vaccines will be discussed here.

Adenovirus vaccine

Recombinant adenoviruses are replication-defective due to the deletion of the E1 gene and thus are incompetent to spread from the original infected cells. This in combination with their ability to accommodate foreign DNA segments makes them suitable targets to use for viral vector vaccination strategies[58].

Price *et al.* conducted an experiment where mice were primed by DNA vaccination against NP and M2 proteins and boosted to enhance the

responses to these priming antigens. Boosting with cold adapted (ca) virus (intranasally, i.n.) was compared to boosting with recombinant adenovirus (rAd) (intramuscular, i.m.) or i.n., NP from an influenza B virus, and unboosted naïve mice. Mice were challenged with lethal doses of an influenza A H1N1 virus 2 months or 8 months after boosting. Survival, weight loss, and virus titers were measured and only the results after 8 months are depicted in Figure 6[51]. Boosting with rAd both i.m. and i.n. resulted in a 100% survival and for ca i.n. 70% eight months prior to H1N1 viral challenge (Figure 6A). rAd i.n. boosting resulted in minimal weight loss in mice (Figure 6B) and the virus titer was at both day 3 and 5 significantly decreased compared to the controls. rAd i.m. and ca i.n. boosting showed a significant diminished virus titer at day 5 (Figure 6C)[51].

Challenging with an influenza A H5N1 virus showed comparative result (Price *et al.*). Parallel experiments were performed in ferrets, an animal model resembling human influenza more closely, and significant results were obtained without any additional delivery systems which were needed in previous studies to enhance minimal protection. This are promising results for future rAd vaccines and protection properties in human, but further research should be done[51].

Modified vaccinia virus vaccine

Modified vaccinia virus Ankara (MVA) is a replication deficient poxvirus vaccine strain that proved to be safe in humans as smallpox vaccine[57]. Cross-protection induced by a MVA-based influenza vaccine, between influenza H1N1 and H3N2 strains was shown by Bender *et al.* Mice immunized with recombinant MVA virus expressing HA and NP genes of influenza H1N1 (MVA HA-NP), produced increased amounts of anti-H1 IgG and IgA antibodies compared to control MVA wild type vaccinated and naïve mice (Bender *et al.*)[59]. When challenged with influenza virus A/Port Chalmers/1/73(H3N2), nasal and lung viral titers were diminished in MVA HA-NP immunized mice compared to control mice (Figure 7)[59].

Kreijtz *et al.* showed recombinant MVA viruses expressing HA genes of influenza A H5N1 viruses could induce cross-protective immunity against H5N1 viruses from different clades[60]. MVA virus vaccines expressing HA genes of the influenza A/Hongkong/156/97 (HK) or A/Vietnam/1194/04 (VN) strains were used to immunize C57BL/6J mice. Control mice were inoculated with PBS, whole-inactivated NIBRG-14 virus (positive control) or wild type (wt) MVA. Mice were challenged with influenza A/HK/156/97 (clade 3), A/VN/1194/04 (clade 1), or A/IND/5/05 (clade 2.1) virus. Mean

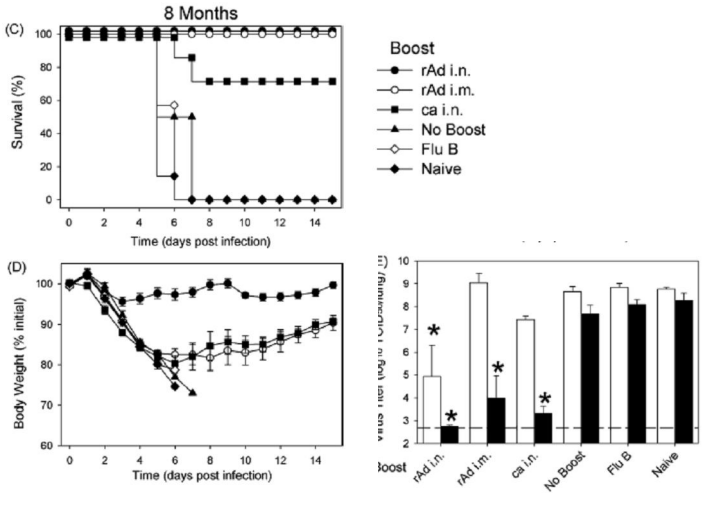

Figure 6 | **Morbidity and mortality after H1N1 challenge following prime-boost immunizations**. Groups of seven mice were primed and boosted as described. Mice were challenged with ~100 LD50 of H1N1 virus two months (not shown or eight months after boosting. (a) Survival and (b) weight loss after challenge 8 months post boosting. The dashed line shows the limit of detection. * indicates a statistically significant difference ($P < 0.01$) in titer compared to influenza B and naive animals at the same time point[51].

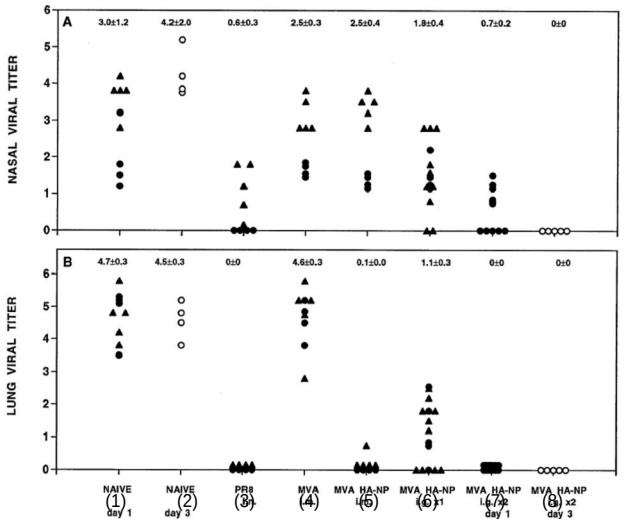

Figure 7 | **Nasal and pulmonary influenza virus titers of mice challenged with influenza H1N1 virus (PR8).** Mice inoculated twice with MVA HA-NP were challenged with H1N1 (PR8) and sacrificed after 1 day (closed symbols) or 3 days (open symbols). (1) Naïve day 1. (2) Naïve day 3. (3) PR8 i.n. (4) MVA i.m. (5) MVA HA-NP i.m. (6) MVA HA-NP 1x i.g. (7) MVA HA-NP 2x i.g. day 1 (8) MVA HA-NP 2x i,g. day 3[59].

weight loss in days post infection is depicted in Figure 8. Mice inoculated with MVA-HA-VN/04 were protected against viruses from all three clades[60]. This cross-protection was also demonstrated in cynomolgus macaques that were immunized twice with MVA-HA-VN/1194/04 and challenged with influenza virus A/VN/1194/04 or A/IND/5/05. Animals vaccinated with either influenza strain all tested negative whereas PBS or wild-type MVA (wtMVA) vaccinated animals had high lung virus titers (Figure 9)[61]. These primates showed the same pathogenesis after infection with H5N1 virus as was observed in humans, so protection of macaques by MVA based vaccination suggests this vaccine could be very promising and clinical evaluation seems warranted[61].

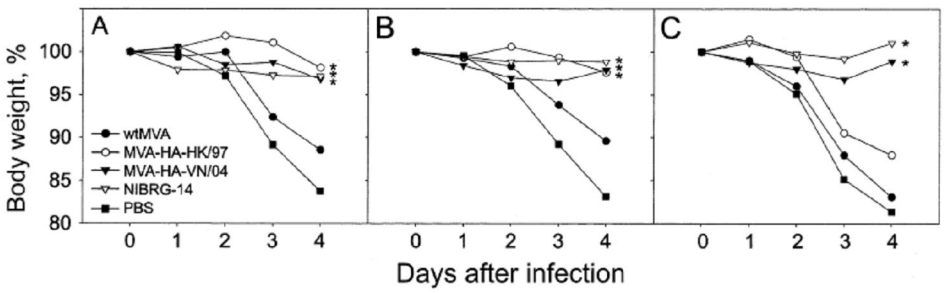

Figure 8 | **Weight loss in mice i.n. infected with 10^3 TCID50 of influenza virus A/HK156/97 *(A)*, A/VN/1194/04 *(B)*, or A/IND/5/05 *(C)*.** Mean weight loss is expressed as the percentage of the original weight before infection. *Statistically significant difference (P <0.05)[60].

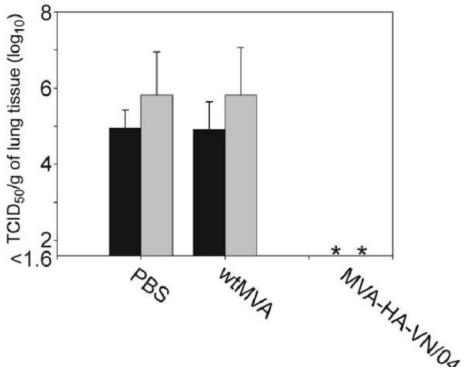

Figure 9 | **Virus titers in the lungs after infection with H5N1 influenza viruses.** Virus titers were determined on day 4 after infection in the lungs of animals that were infected with either A/Vietnam/1194/04 *(black bars)* or A/Indonesia/5/05 *(gray bars.* *All animals had negative results of virus isolation, resulting in a mean virus titer below the cut-off value[61].

ISCOMs

Immunostimulatory complexes (ISCOMs) are antigen delivery systems composed of antigen, cholesterol, phospholipids, and the build-in saponin adjuvant (Quil A). ISCOMatrix have the same composure except they lack the antigen incorporation[62]. ISCOMs and ISCOMatrix are able to induce immunity against a wide range of antigens and due to successive clinical trails in animals, they are now approved for veterinary use (see for review Sun *et al.*[62]). In influenza studies it is shown that ISCOM vaccines can reduce the HA dose needed to induce an immunogenic response ten fold (Figure 10)[63].

Sambhara et al. showed that influenza H1N1 vaccine formulated as ISCOM (flu-ISCOM) resulted in an enhanced recovery and clearance of virus in mice challenged with different heterosubtypic virus strains[64]. Vaccination with flu-ISCOM resulted in cross-protection not only between H1N1, H2N2, and H3N2 (results not shown), but also between H1N1, H5N1, and H9N2 influenza strains (Table 3). Mice were immunized twice with a non-adjuvanted sub-virion H1N1 vaccine, ISCOMatrix (ISCOM without influenza proteins), and flu-ISCOM. Mice were challenged with H1N1, H5N1, or H9N2 influenza strains and the HI titers and viral lung titers were determined (these latter is depicted in Table 3)[64]. Both flu-ISCOM and H1N1 vaccinated

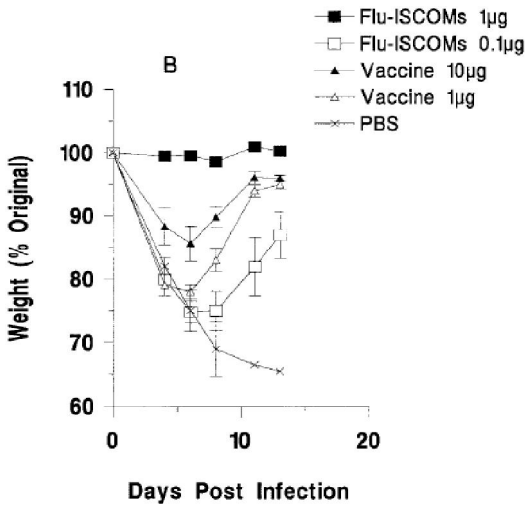

Figure 10 | **Weight loss following virus challenge for mice immunized with flu-ISCOMs or subvirion vaccine.** Groups of 6 BALB/c mice were immunized twice with A/Taiwan/1/86 (A/Taiwan) flu-ISCOMs, subvirion vaccine containing 0.1–10 mg of influenza HA, or PBS and then challenged with 5 LD50 of mouse-adapted A/Taiwan virus. Mortality in PBS control and immunized groups was 50% and 0%, respectively, 14 days after challenge[63].

mice showed significant reduced viral titers after H1N1 challange. ISCOMatrix or H1N1 vaccine immunized mice did not show viral clearance in mice challenged with H5N1 and H9N2 virus, whereas flu-ISCOM resulted in a reduction in viral lung titers of respectively $3.3\log_{10}$ and $3.2\log_{10}$. These data were consistent with the H2N2 and H3N2 results and suggest that ISCOM based vaccines induce heterotypic immunity[64].

Table 3 | **FLU-ISCOM facilitate viral clearance when challenged with H5 and H9 Viruses.** (Adapted from Sabhara *et al*.[64]).

	Viral titers in lungs ($\log_{10}$ EID_{50}/ml + SD)*		
Immunogen	H1N1	H5N1	H9N2
ISCOMatrixFlu	6.4 ± 0.7	6.9 ± 0.9	5.4 ± 1.0
Vaccine	1.2 ± 0.0	7.1 ± 0.6	5.3 ± 0.8
FLU-ISCOM	1.2 ± 0.0	3.6 ± 1.6	2.2 ± 1.0

* The sensitivity of the assay is 1.2 $\log_{10}$. EID_{50}, 50% egg infectious dose.

Virus-like particles

Virus-like particles (VLPs) mimic the structure of a virus particle without requiring the containment of infectious genetic material[65]. Most influenza VLPs are created using baculoviral vectors encoding for the influenza HA, NA, and M1 proteins. SF9 insect cells infected with the baculoviruses produce the VLPs. Advantages of this system are the reduced risk of pathogenic effects upon vaccination in humans and the large vaccine production rate in eukaryotic cells which are easy to scale-up[65].

VLP vaccinated mice and ferrets showed a better immune response compared to vaccination with whole formalin-inactivated wild-type influenza virus or recombinant HA. Animals vaccinated with VLP vaccine were already protected when vaccinated with a dose of 3 µg HA[66].

Quan *et al.* showed mice immunized (i.n.) with VLPs containing HA and M1 from the influenza PR8 (H1N1) strain, had higher and long-lived antibody titers (results not shown) and had significantly lower lung viral titers when challenged with influenza PR8 or A/WSN/33 (H1N1) virus (Figure 11), and thus showed cross-protection between these two influenza H1N1 virus strains[67]. Mice immunized with VLPs consisting of only M1 proteins showed viral lung titers comparable with the naïve unvaccinated mice when challenged with influenza PR8 or A/WSN/33 virus, showing that immunization with only M1 protein does not reduce viral lung titers upon infection (figure 11)[67].

Taken together that VLP vaccines showed cross-protection, is relatively safe in humans, has a high production rate, and has large dose-sparing properties, makes it a very promising strategy in pandemic influenza vaccine development.

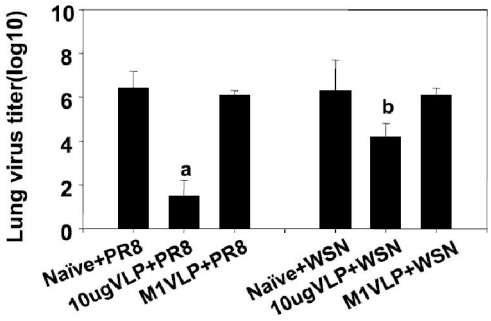

Figure 11 | **Virus titers in lungs.** Lung samples from individual mice in each group (n=6) were collected on day 4 post challenge with a lethal dose of PR8 or WSN. Naïve denote unvaccinated mice, VLP contain both PR8 HA and M1 proteins, M1VLP only consist of PR8 M1 proteins. PR8 and WSN denote a challenge infection with respectively PR8 and WSN. Statistical significance is indicated between groups of mice immunized with VLPs and HA-negative M1 VLPs (a, $P < 0.01$; b, $P < 0.05$)[67].

CONCLUSIONS AND DISCUSSION

A lot of research has been done on the improvement of influenza vaccines and the development of new vaccine strategies, especially during the last decennium when pandemic threats of H5N1 (avian) and H1N1 (swine) led to an increased interest and investments into pandemic vaccine developments. Table 4 summarizes examples of influenza vaccination strategies in use or in a developmental stage for the use in humans.

Reassortment of the influenza viruses and the threat of the introduction of avian influenza viruses in humans makes it challenging to generate a protective vaccine prior to appearance of a virus strain in the population. In a pandemic setting, lack of time is one of the most important limitations. Developing a safe and effective vaccine against a pandemic influenza virus should therefore be time and dose sparing, and ideally induce broad protective immunity.

A vaccination strategy against a future pandemic is the search for conserved epitopes and the creation of vaccines against these epitopes, leading to cross-protection or even to heterosubtypic immunity. When such a vaccine is established, one single vaccine could protect humans against various influenza viruses. But until then, a lot of research has to be done.

Concerning the new H1N1 influenza, the Health Council of the Netherlands advised the Dutch government to start vaccination of the

following risk-groups: individuals at medical risk conform the seasonal influenza vaccination indications, all persons aged 60 and above, pregnant women, healthcare staff, and family members/(informal) carers of individuals at high risk[47,68]. In the last update of the 30th October 2009, it appeared people infected with new H1N1 virus were abundantly under the age of 65. Normal hospitalized patients and hospitalized intensive care patients were predominantly aged respectively 0-4 and 10-15 years. Of all IC hospitalized patients, 38,5% was considered healthy upon new H1N1 infection[69]. Therefore it was decided to vaccinate young children as well as individuals aged 60 and above[70]. CDC already advised earlier to vaccinate persons between the age of 6 months and 24 years against this influenza virus, as well as individuals who live or care for children younger than 6 months of age[71]. In the recommendation of the American Academy of Pediatrics (AAP) for annual influenza vaccination in the U.S. was denoted that all children from 6 months up to 18 years of age should be vaccinated since this would not only reduce hospitalization of children, but also transmission of the virus[72]. Where the new H1N1 flu first seemed to be rather mild and a lot of discussion took place about the amount of vaccine doses ordered by the Dutch government, not enough vaccines were available to vaccinate both elderly and younger individuals from the start[73]. In the media it appeared that many people have concerns about the safety of the vaccines and its contents, since the actual vaccines are not yet tested in clinical trails and multiple studies seem to contradict each other[74-76] what might afflict the vaccination coverage.

In the decision-making of vaccination programs not only for current but also for future vaccination campaigns, multiple aspects have to be considered. Will vaccination improve the national health, reduce the incidence and/or mortality in the population? What will be the social and economical consequences of mass vaccination and would people want to get vaccinated, even when there is no direct threat? Will the benefits outweigh the costs? When decided to (mass) vaccinate, a good information campaign could stimulate the population to get vaccinated. However, in my opinion it is important never to force people to get vaccinated, even if it might be seen as national importance.

Fortunately until now the new pandemic H1N1 influenza virus appeared to be rather mild, however, mutations or reassortments could result in an increased pathogenicity of the virus. Also the threat of a new pandemic to arise remains, and so the search for safe and effective (pandemic) influenza vaccine continues.

Table 4 | **Summary of influenza vaccine strategies.** Examples, advantages, disadvantages, and stage of development for human use are denoted.

Vaccine strategy	***Examples***	***Advantages compared to traditional vaccines***	***Disadvantages***
Inactivated vaccines	Whole virus vaccines* Split virion vaccines* Subunit vaccines* With or without adjuvant: - Alum* - Oil-in-water (MF59, AS03)* - ISCOMS	 Dose-sparing Dose-sparing Dose-sparing / Cross-protection	No or limited cross-protection / Time intensive / Safety
Live attenuated vaccines	Cold adapted* Modified NS1	Long lasting immunity	Safety / Time intensive
Conserved epitope (recognized by Abs)	CR6261	Prophylactic & therapeutic immunity / Cross-protection	
DNA vaccines	NP, MP1, NS1	Heterosubtypic immunity / Cheap and quick production	Risk of DNA integration into host cells
Viral vector vaccines	Recombinant Adenovirus Modified vaccinia virus Ankara	Cross-protection / Heterosubtypic immunity	Possible negative effect of anti-vector immunity
Viral-like particles	HA, NA, M1	Time and dose-sparing / Cross-protection	

* influenza vaccines of this types are already in use in humans

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