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Reincarnations of Influenza Virus and Challenges with the Development of a Robust Influenza Vaccine

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ABSTRACT

While some viruses occasionally pop-up and over-powers the immune system and cause deadly diseases Influenza virus is not one among them. In contrast, Influenza infections are much common with clinical manifestations such as common cold however they cannot be over sighted since they can cause uncommon consequences and even death if untreated. In this context prevention of Influenza infection is of primordial issue. Federal governments and pharma and biotech industries spend billions of dollars every year to develop novel yet robust anti flu vaccines but unfortunately we cannot shirp yet due to the fact that the immunity offered by them is temporary thanks to their very high rate of mutability. Currently these influenza vaccines are produced using reassortment genetical method in embyonated eggs. Cell culture based vaccines have also been developed however their efficacy is *in par* with inactivated influenza vaccines and live attenuated influenza vaccines only. Developing a universal influenza vaccine is the need of the hour and the future influenza vaccines are moving in these direction. With the advent of such universal vaccine influenza pandemics would be certainly a past history.

Introduction

Influenza viruses belong to orthomyxoviridae and they are the major causal agent of respiratory disease related morbidity and mortality world-wide[1]. Influenza has been responsible for millions of deaths every year. Mutability and high frequency of genetic reassortment and the resultant antigenic changes in the viral surface glycoproteins make influenza virus formidable challenges for control efforts. There are 3 immunologic types designated as A, B and C. The virion is spherical, pleomorphic, 80-120 nm in diameter, helical nucleocapsid. The composition of the virus is RNA (1%), protein (73%), lipid (20%), and carbohydrate (6%). Influenza possess single stranded RNA, negative sense with a overall size of 13.6 kb which made up of 8 segments. The virus produces 9 structural and 2 non-structural proteins. The envelope of influenza has two important proteins namely hemagglutinin (HA) and neuraminidase (NA)[2] (Fig.1)[3].

The HA protein of influenza virus binds virus particles to susceptible cells and is the major antigen against which neutralizing antibodies (protective) are directed. HA protein is a glycoprotein found on the surface of the influenza viruses. It is responsible for binding the virus to cells with sialic acid on the membranes, such as cells in the upper respiratory tract or erythrocytes. It is also responsible for the fusion of the viral envelope with the endosome membrane, after the pH has been reduced. The name "hemagglutinin" comes from the protein's ability to cause agglutination with RBC[4]. The primary sequence of HA contains 566 aminoacids[2]. HA protein cleaves into HA1 and HA2 which are linked by strong disulphide bridge. The NA spike on the virus particle is a tetramer, composed of 4 identical monomers[5]. A slender stalk is topped with box shaped head. NA functions at the end of replication cycle. NA is a sialidase enzyme that removes sialicacid from glycoconjugates. It facilitates release of virus particles from infected cell surfaces during budding process. It is speculated that NA helps the virus negotiate through the mucin layer in the respiratory tract to reach the target epithelial cells. Both HA and NA mutate tremendously and cause antigenic drift (mild changes) or antigenic shift (major change resulting in development of new sub type)[2]. Based on the hemagglutinin (HA) subtype, influenza A viruses are further divided into two phylogenetic groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15)[6].

The virus replicates in the nucleus. Upon infection it shuts down the host cell within 3 hours. This permit selective translation of only viral mRNA. New progeny viruses are produced within 8-10 hours. The virus attaches to the cell surface sialic acid via the receptor site located on the top of the large globule of the HA. The viruses are then internalized by receptor mediated endocytosis. The fusions of viral envelop and cell membrane occurs and lead to uncoating of the virus. The low pH in the endosome facilitates the release of ribonucleo protein (RNP) into cytoplasm. Acid pH causes a conformational change in the HA structure to bring the HA2 fusion peptide in correct contact with the membrane. The M2 ion channel protein present in the virion permits the entry of ions from the endosome into the virus particle, triggering the conformational change in HA .Viral nucleocapsid are then released into the cell cytoplasm. Then genome goes to nucleus and initiates transcription, translations, assembly and release. Eventually the viral shedding starts and virion seeks next cell to infect and the cycle continues. Thus the viral replication is a dynamic event and this process is

spontaneous and the mutations are beneficial to the virus, unfortunately. Because of rapid mutations development of anti influenza vaccine is much challenging.

There are three antigenically distict influenza viruses prevail and they are Influenza types A, B and C. Type A is antigenically highly variable and responsible for most of the epidemics. Types B and C are milder in terms of variability and causing epidemics than type-A. Due to antigenic shift type- A virus undergo major shuffling of antigens and these types are called subtypes[2] and the pandemics caused by these subtypes are shown in Fig.2.

It is generally believed that influenza that infect birds and humans exist as separate entities but when they both replicate in swine host where they reborn as swine flu and infect human beings which is depicted in the Fig.3.

Influenza vaccines

The real ice breaker in discovery of the vaccine was the magnificent work done by Edward Jenner (1796) against small pox. It took almost two centuries to develop an effective vaccine, global campaigns in administration of small pox vaccine and subsequently eradication of the virus from the world on 8th May, 1980, thanks to the highly coordinated work done by World Health Organization (WHO). Currently there are 12 successful vaccines against infectious diseases and they are the vaccines against smallpox, diphtheria, tetanus, yellow fever, pertussis, *Haemophilus influenzae* type b disease, poliomyelitis, measles, mumps, rubella, typhoid and rabies. Development of vaccines against influenza viruses dates back to 1931 when E.W. Good pasture from Vanderbilt University grown influenza virus in embryonated Hen's egg[7]. This work was further followed by Macfarlane Burnet, Wilson Smith, Thomas Francis and Jonas Salk which lead to the

development of early influenza vaccine. Later, US Army developed a fully approved version of influenza vaccine to protect its soldiers during second world war[8]. Embryonated hen's eggs were continued for the production of viruses that are used in influenza vaccines. However several others have improved its purity reducing the egg proteins that causing hypersensitivity reactions[9]. The problems with influenza vaccines for that wild type virus continue to mutate and at a given time point the vaccine strain was obsolete. Another tumbling block was these vaccines failed to cause effective protection among elderly and children. Also these inactivated vaccines did not induce mucosal IgA. To overcome those challenges, clinical isolates from each flu season was isolated and used for the large scale vaccine production[10].

Inactivated Influenza Vaccines (IIV) (Conventional Influenza Vaccines)

Inactivated influenza vaccines (IIV) have been extensively used in clinics world-wide. These vaccines consisted of purified virus that was chemically inactivated with formalin or β-propiolactone. In the recent days influenza infections are caused by both influenza type A and type B. Hence, the current vaccines against influenza epidemics contain two influenza A subtypes namely H1N1 and H3N2 and one or two variants of influenza B virus[11]. The composition of the trivalent vaccine consists of two influenza A subtypes namely H1N1 and H3N2 and one influenza B strain that were isolated from recent flu season. In general, the vaccine strain of influenza a subtype are adapted to grow in embryonated hen's eggs or developed by genetic reassortment method in which the vaccine strain and current season's wild type clinical strain is allowed to recombine. In this recombinant vaccine strain the backbone of the vaccine strain containing all the other proteins namely

polymerase basic protein PB-1 (PB-1), PB2, polymerase acidic protein (PA), nucleo-protein (NP), M1-M2 and NS which encode the internal proteins from A/Puerto Rico/8/34 (PR8) (H1N1) virus fused with HA and NA from wild type virus (11). There are 3 types of inactivated influenza vaccines. They are (i) whole virus vaccines, (ii) split virus vaccines and (iii) subunit vaccines[12].

Although whole-virus vaccines are still in use in a few countries most vaccines manufactured since the 1970s are either split virus vaccine[13] or subunit vaccines and these vaccines are produced in embryonated hen's egg. These vaccine efficacy vary due to many factors such as age of vaccine recipient, antigenic homology between the vaccine strain and clinical strain, etc. Studies indicated that efficacy of influenza vaccine was 55-86% among non-elderly adults and 70% among adults[14]. In a South Korean study, the efficacy of the influenza vaccine was found to be 46.5-50.8% in patients aged 19-49 years and 58.7-63.3% among those aged 50-64 years during the 2010-2011 influenza season[15]. The low effectiveness of influenza vaccine in the elderly was noticed which accounted for only 39% and there was a 30-40% reduction in hospitalization[16-18]. These studies are suggestive of inactive vaccines having much higher efficacy among elderly persons.

Vaccine strain mismatch can affect the overall efficacy of the influenza vaccine. In the influenza season 1997-1998 there was a poor match between vaccine strain (A/Wuhan/359/59 (H3N2)) and circulating influenza strain (A/Sydney/05/97) was noticed[19]. Even though this vaccine prevented 35-39% mortality in the 1997-1998 season, prevention was much better in the previous year's 1996-1997 (60-61%)[20]. From this study it is also noted that not all antigenic drift will evade the immune system completely. Tricco, AC *et al*. (2012) compared Tri valent inactivated vaccine (TIV) with live vaccine and reported that both vaccines offered comparable protection[21]. However the limitations of inactivated influenza vaccines are inconsistant efficacy, short term protection and inability to induce powerful local immunity and IgA production.

Adjuvanted vaccines

To overcome the shortcomings with IIV several modification of IIV were employed. One such approach was the adjuvanted influenza vaccines which was first introduced in the 1950s and in this method IIV is adminstered along with an adjuvant. Salts of Aluminum have been widely used as adjuvants[22] and it is thought that this approach would increase attraction of antigen presenting cells, uptake and activation of the inflammatory cells[23]. The commonly used aluminum salts are aluminum hydroxide, aluminum phosphate and potassium aluminum sulphate (also referred as alum). These studies showed that aluminum salts induced a profound antibody promoting Th2 response against influenza while it had less activity in inducing Th1 response[24]. While alum was studied enormously in USA mineral oil preparation was tried extensively in Europe.

The vaccine formulations included the emulsification of antigens with mineral oil[25,26]. The essential role of adjuvants is dose sparing and enabling a stronger and broad immune response and expansion of the antibody response[27] and this method was to target older adults. These vaccines induced greater and more sustained antibody responses and enabled antigen sparing[28-30]. However, usage of mineral oil adjuvanted vaccines was hampered by the elicitation of unwanted local reactions such as cysts and sterile abscesses[31]. MF59 is a micro fluidized, oil-in-water emulsion containing squalene. The oil is stabilized by adding a water-soluble surfactant (polysorbate 80,

Tween 80) and an oil-soluble surfactant (sorbitan trioleate, Span 85)[32]. MF59 stimulated an infiltration of inflammatory cells and establishes a localized immunostimulatory environment and enhanced antibody production[33]. TIV containing oil-in-water emulsion was first licensed in Europe in 1997. Fluad (Novartis, Basel, Switzerland) is a seasonal MF59-adjuvanted IIV had been shown to have enhanced protection against influenza infections[26]. Another oil-in-water emulsion, AS03, contains α-tocopherol, squalene and polysorbate 80[34]. AS03 showed an increased efficiency against influenza A/H3N2 strain[35].

Intradermal (ID) influenza vaccines

Sanofi Pasteur introduced Fluzone Intradermal was first introduced during the flu seasons of 2011-2012. This intradermal flu vaccine require very little antigen. Intradermal (ID) immunization triggers antigen migration through lymphatic ducts and consequent stimulation of resident dendritic cells. Activation and migration of dermal dendritic cells lead to potent T cell activation[36,37]. The first available ID influenza vaccine, Intanza (Sanofi Pasteur, Lyon, France), also known as IDflu, was licensed in 2009 in Europe. The doses adminstered are 9 μg HA per strain for adults aged 18-59 years and 15 μg HA per strain for adults aged ≥ 60 years[38,39]. Same vaccine is given at a dose of 9 μg HA per strain was licensed in the US for use in adults aged 18-64 years[40].

High-dose (HD) HA vaccines

The standard dose (SD) influenza vaccine contains 15 μg of HA for each strain. Studies have shown that High-dose (HD) influenza vaccine containing 60 μg of HA for each influenza strain was inducing better protection[41]. In 2009, FDA approved *Fluzone High Dose* (Sanofi Pasteur) for the prevention of influenza in persons aged ≥ 65 years[42].

Cell-culture influenza vaccines

Cell culture based production of vaccines has several advantages over egg based methods. Cell culture influenza vaccine (CCIV) method avoids the dependency on chicken egg supply, egg cultivation facility and the risk of contamination of eggs. Manufacture of CCIV is faster and can be easily scaled up in comparison to egg based vaccines. In addition, the initial purity of the vaccine is higher with CCIV. The introduction of CCIV helped in the improvement of generating global stockpiles of pandemic influenza vaccines. Several cell lines have been used for influenza vaccine manufacture[43]. E.g. Madin Darvy Canine Kidney (MDCK) and Vero cells. Studies have reported that CCIV has a comparable immunogenicity and safety to egg based influenza vaccines[44-46].

DNA vaccine against influenza virus

Although some advances in improving the speed of conventional vaccines it is time to rewrite the algorithm utilizing the experience of the past and newer generation technologies. Though DNA vaccine field is relatively new it is shown to be a remarkable system. One of the advantages of DNA vaccines is its ability to induce both humoral and cell mediated immunity at the same time. This type of vaccination can prime both MHC class I and Class II antigen presentation which can trigger both CD4 and CD8, respectively. An epidermally injected DNA vaccine for influenza was found to be more immunogenic among humans[47]. Intramuscular influenza HA DNA vaccines have been shown to be immunogenic in preclinical studis[48]. DNA vaccines expressing various combinations of the viral

HA or NA as well as other viral genes have been shown to be protective in animal models[49-51]. Our group adds on remarkable efficacy of DNA vaccine protecting against Herpes virus[52-54]. Production of these vaccines is relatively safe and economic and potentially rapid. The historical concern with DNA vaccines in humans is their poor immunogenicity, although clinical studies utilizing plasmids expressing H3 HA proved effective at very low doses such as 4 µg[55]. More clinical studies are required to fully understand the safety, immunogenicity, and effective and long lasting protection of DNA vaccines in human influenza infections.

Live Attenuated Influenza vaccine (LAIV)

Even though inactivated vaccines had been used globally it had the limitations listed above. This necessitates to seek for alternative methods such as using live attenuated influenza strains. Live attenuated vaccines are attractive since the virus replicates in the host and thus both MHC class I and Class II presentation of antigen will occur in the natural way. This will result in elicitation of both humoral immunity and cell mediated immunity. One such LAIV vaccines is Flu Mist, a nasal spray vaccine. The virus in this preparation is attenuated, temperature sensitive and cold adapted. LAIV is a reassortant virus possessing internal proteins of a master donor virus and surface proteins (both HA and NA) from the wild-type influenza virus (Scheme shown in Fig.4). The strains of A/Ann Arbor/6/60 and B/Ann Arbor/1/66 were developed as master donor viruses which acquired the *ca*, *ts*, and *att* phenotypes as a result of multiple mutations in the gene segments that encode internal viral proteins[56].

There are five seasonal LAIV backbone strains that currently approved by FDA and they are A/Len/134/17/57, A/Len/134/47/57, A/Ann Arbor/6/60, B/USSR/60/69, and B/Ann Arbor/1/66. Except A/Len/134/47/57 strain, all others are currently used as master donor strains in the production of seasonal LAIV vaccines. In USA and Canada, LAIV is licensed under the trade name Flumist and in Europe as Fluenz. Previous animal experiments suggested a 'replication-deficient vaccine', new class of vaccines that could be developed in the future, and it is speculated that this vaccine would possess the advantages of both LAIV and the inactivated vaccines[57]. LAIV was first licensed to use in USA[58]. Upon inhalation the LAIV strains replicate in epithelial cells of nasopharynx.

This is followed by strain specific IgA development and good protection[59]. In a study conducted using LAIV it was found that children of age group 2 to 6 years the efficacy was just above 90% and only a very few (16-33%) side effects such as febrile otitis media[60-62]. Thus LAIV had out performed non-adjuvanted TIV[63] however it needs to be admitted that its efficacy was poor among adult LAIV recipients. This could be due to development of immunity against LAIV[64]. This vaccine was approved for the use among healthy individuals of aged 2-49 years and its usage among pregnant woman and children below 2 year (risk of wheezing) was prohibited. For the age groups of above 50 years inactivated split vaccines are recommended. LAIV is not indicated in young children who are under 2 years of age because of the risk of wheezing[65]. Current influenza vaccines are with moderate efficacy (effective among healthy individuals of 2-49 years of age), good safety, and acceptable tolerability. Another concern is the hypersensitive reactions to egg proteins and thus there is a need of egg-free, quickly produced, safe, efficacious, and protective influenza vaccines[66,67].

Quadrivalent influenza vaccines

The circulating influenza viruses in LAIV are either Yamagata strain or Victoria strain. Studies showed that only about 2% match was found with Victoria strain and 17% match with Yamagata strain. To improve the probability vaccine manufacturers have been working on a Quadrivalent vaccine that possess four strains of influenza (to enhance the efficacy during mismatching) containing two influenza subtype A strains (H1N1 and H3N2) and two type B strains (Victoria, and Yamagata strains). The first Quadrivalent LAIV vaccine was Med Immune's nasal spray vaccine called Flumist Quadrivalent and it was licensed to use by Food and Drug Administration (FDA). New Quadrivalent vaccines in addition to the trivalent influenza vaccines are available in the market now[68].

Universal influenza vaccines

There is a great interest among the scientific community to develop a universal vaccine that encode for the conserved regions of the influenza and induce solid protection against all strains of influenza. Until now it is only a fantasy since influenza virus is not a stale virus. On the contrary it is a virus with monumentus mutation at the HA region which makes the proposition of anti influenza vaccine algorithms futile.

A reliable universal vaccine should be able to protect against all influenza a virus subtypes and both lineages of influenza B viruses. However, it would be extremely difficult to develop such a universal vaccine. Due to more variants of influenza a types in both humans and animals, developing universal vaccines have been focused on influenza a viruses. It would be highly promising to develop a vaccine that is broadly cross-protective compared to currently licensed influenza vaccines[65]. The concept behind developing universal vaccines is to utilize the highly conserved antigenic target and to make it immunogenic sufficient enough for inducing protective immunity. At present universal influenza vaccines are at the experimental stages such as HA-M2 protein based M2e conjugate vaccine and M2e VLP vaccines however their full clinical success is yet to be known. Until a much better blue-print laid to a successful influenza virus, the concept of universal influenza vaccine is still in the horizon[69,70].

Conclusion

As of today, most licensed influenza vaccines are manufactured by methods that were established nearly 50 years ago despite recent scientific advances both conceptually and technologically in vaccinology. Newer influenza vaccines to improve the span of protection would be plausible but significant changes in technical, regulatory, and logistical grounds is inevitable. It will provide highly informative insight into developing novel anti influenza vaccines. In addition understanding the underlying immunological mechanisms, exploiting the cross protection mechanisms, inclusion of appropriate immune enhancing agents would pave way for the discovery of novel and robust influenza vaccines. Such vaccines would stop the reincarnations of influenza virus which threatens the global human community.

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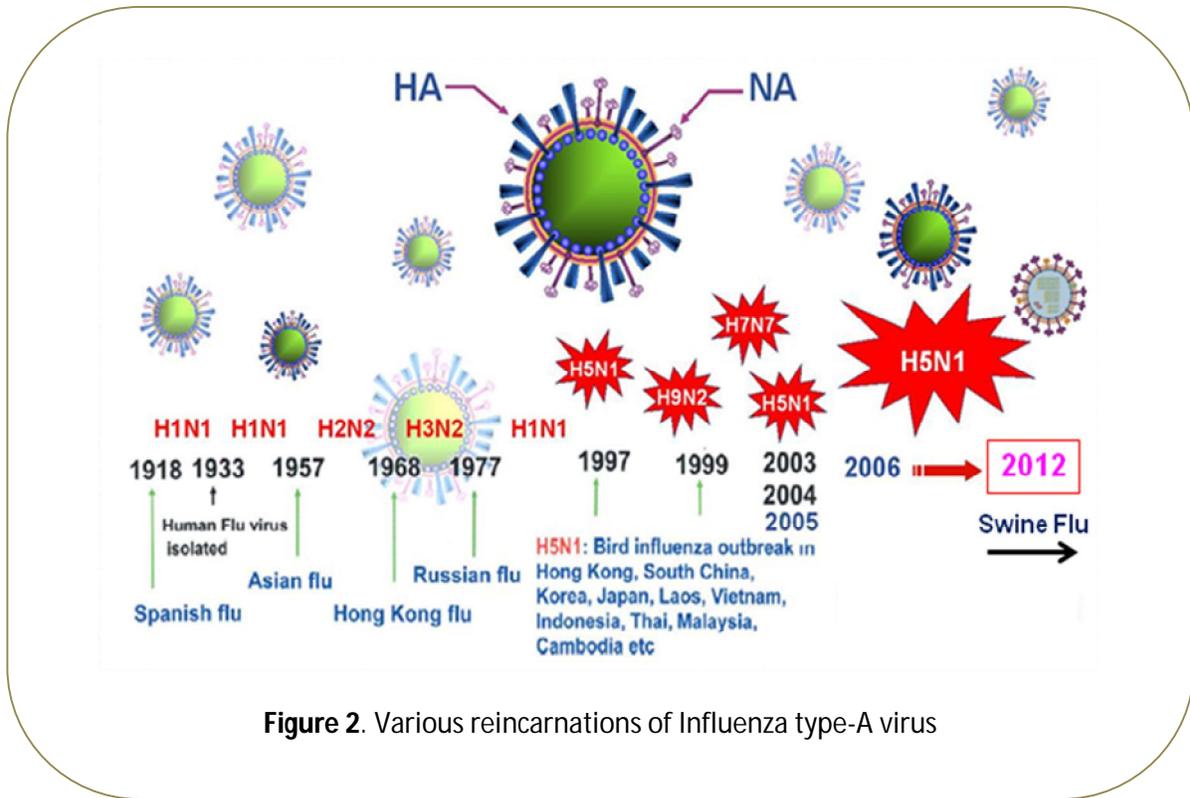
Figure 1. Structure of Influenza virus
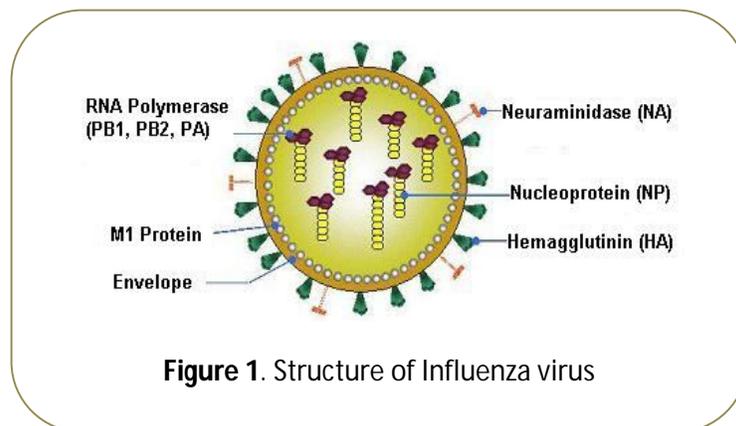
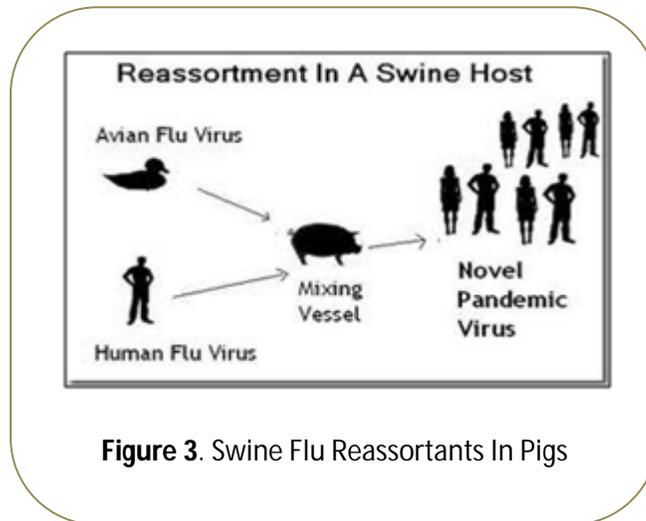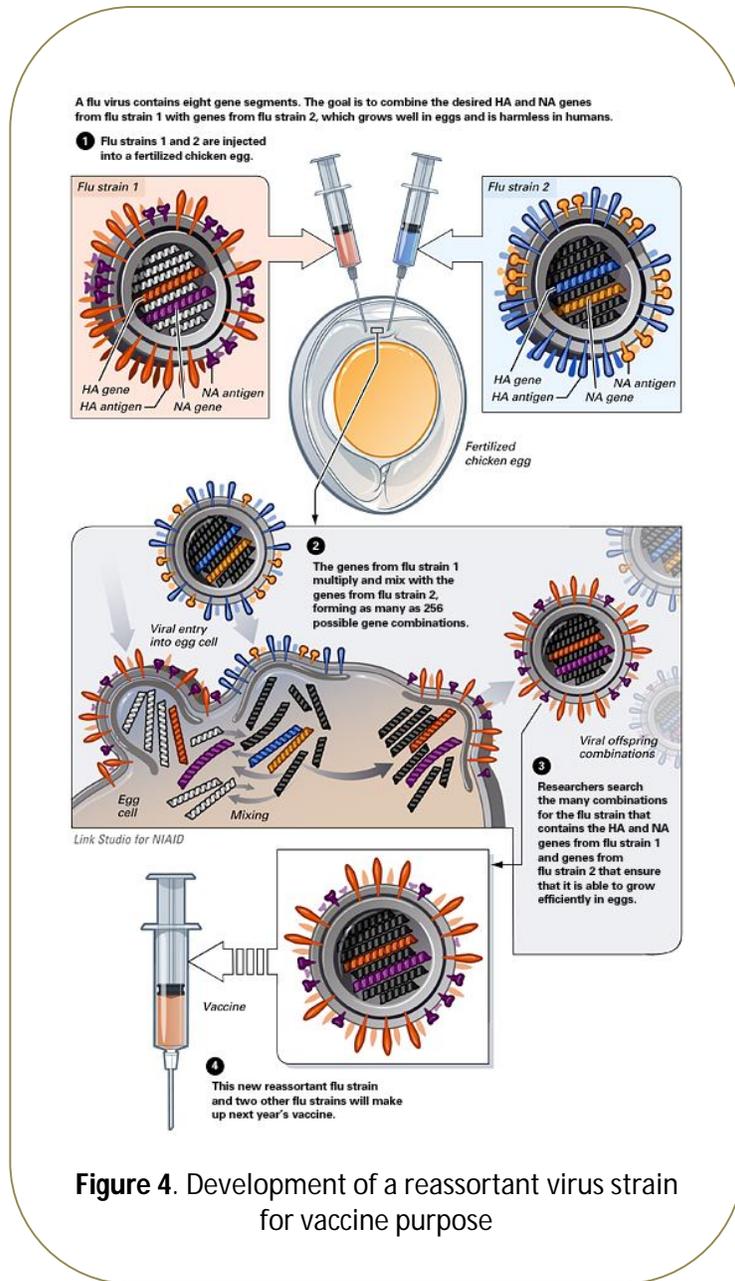**Figure 2**. Various reincarnations of Influenza type-A virus

Figure 3. Swine Flu Reassortants In Pigs

Figure 4. Development of a reassortant virus strain for vaccine purpose