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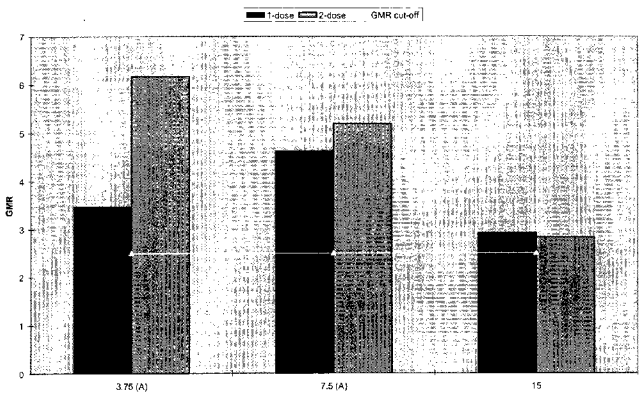

Figure 1

(57) Abstract: The present invention relates to novel process for purification of influenza viruses and the highly pure influenza virus obtained by such process. The invention is based on the premise that specific modification of viral purification processes can lead to increases in the yield, purity and robustness. The invention also provides an improved, low dose, stable, safe and efficacious compositions for easy administration to subjects and their use in augmenting immune responses to influenza antigens.

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IMPROVED INFLUENZA VACCINE

FIELD OF THE INVENTION

This invention is in the field of vaccines for protecting against influenza virus infection. In particular, the present invention relates to a novel purification process for influenza viruses, highly pure influenza virus obtained by such process, their improved formulations and their use in augmenting immune responses to influenza antigens.

BACKGROUND OF THE INVENTION

The influenza virus and its variations are the cause for a contagious respiratory illness commonly referred to as "influenza" or the "Flu". In humans and animals this can cause mild to severe illness, and at times can lead to death. Influenza viruses have been with mankind for at least 300 years, causing epidemics every few years and pandemics every few decades. They result in 250,000 - 500,000 deaths, and about 3-5 million cases of severe illness each year worldwide, with 5-15 % of the total population becoming infected (WHO 2003).

There are three main types of influenza viruses: influenza A, influenza B, and influenza C. Within each type of influenza and within influenza A in particular there are many different subtypes which differ based upon certain proteins expressed on the surface of the virus, specifically the hemagglutinin (HA) and the neuraminidase (NA) proteins. Till date sixteen HA subtypes and nine NA subtypes of influenza A virus have been reported from avian isolates. Many different combinations of HA and NA proteins are possible, and each combination represents a unique subtype. "Human influenza virus" usually refers to those subtypes that spread widely among humans. There are three known influenza A subtypes currently circulating among humans: H1N1, H1N2, and H3N2, each of which include various individual influenza viral strains.

Since influenza A viruses can constantly undergo mutations, reassortments, genetic "drift and shift," and the like, host specificities are changing alarmingly. Influenza viruses can be spread among various animal species, and infection from non-human species (such as avians, porcines, primates, and other animals) to human hosts leads to new influenza subtypes that

can adapt over time to infect and spread more rapidly or thoroughly among human populations. Examples that have been widely reported in recent years include, for example, H5, H7, and H9 subtypes.

Pandemic viruses typically emerge as a result of a process called "antigenic shift," which causes an abrupt or sudden, major change in a virus, e.g., influenza A virus. In the process of antigenic shift, two or more different strains of a single virus (or of different viruses) combine to form a distinctly new subtype that expresses a unique combination of surface antigens found in the strains that originally combined. While antigenic shift has been reported in various viral species, it is most widely observed in influenza virus, thus representing the most common form of genetic reassortments that gives rise to a phenotypic change in the resultant strains.

A new strain of influenza virus, officially named the "new H1N1", first identified in April 2009, and commonly called "Swine flu" initially spread in Mexico and then globally by transmission. Genetic analyses of this virus have shown that it originated from animal influenza viruses and is unrelated to the human seasonal H1N1 viruses that have been in general circulation among people since 1977. It is thought to be a mutation of four known strains of the influenza A virus, subtype H1N1: one endemic in (normally infecting) humans, one endemic in birds, and two endemic in pigs (swine).

In India as of February 24 2010, 1357 confirmed deaths from H1N1 have been reported, and 29,583 confirmed cases of H1N1 have been reported. It is likely that the true count of cases is much higher, because some patients may never seek medical attention. The emergence of the 2009 pandemic influenza A (H1N1) virus demonstrates the unpredictable nature of influenza. The virus has the potential to cause disease, death, and socio-economic disruption. Hence the development of effective vaccines is a public health priority. Vaccination programs for 2009 influenza A (H1N1) are under way, but the optimal formulation is unknown.

Vaccination remains the cornerstone of influenza prevention and numerous vaccines capable of producing a protective immune response specific for different influenza viruses/virus strains have been produced for over 50 years. The different types of vaccines in use today for influenza can be divided into killed virus vaccines and live virus vaccines. Killed virus vaccines can be divided into whole virus vaccines, and split or subunit vaccines. Whole virus

vaccines were the first to be developed. Split vaccines are prepared by fragmentation of whole influenza virus, either infectious or inactivated, with solubilizing concentrations of organic solvents or detergents and subsequent removal of the solubilizing agent and some or most of the viral lipid material. Split vaccines generally contain contaminating matrix protein and nucleoprotein and sometimes lipid, as well as the membrane envelope proteins. Split vaccines will usually contain most or all of the virus structural proteins although not necessarily in the same proportions as they occur in the whole virus.

Subunit vaccines consist of purified HA and NA proteins, with the other viral components removed.

Split and subunit vaccines cause fewer local reactions than whole virus vaccines, and a single dose produces adequate antibody levels in a population exposed to similar viruses .Compared to whole cell vaccines, split vaccines are preferred because of their better characterization, low ovalbumin content and lesser reactogenicity.

However, subunit vaccines have been reported to yield variable results in the past. For example, a sub-unit influenza vaccine adjuvanted with the adjuvant MF59, in the form of an oil-in-water emulsion is commercially available, and has demonstrated its ability to induce a higher antibody titer than that obtained with the non-adjuvanted sub-unit vaccine (De Donato et al. 1999, Vaccine, 17, 3094-3101). However, in a later publication, the same vaccine has not demonstrated its improved profile compared to a non-adjuvanted split vaccine (Puig-Barbera et al., 2004, Vaccine 23, 283-289).

Manufacturing of influenza vaccine is based on technologies using embryonated hen's eggs, which were first developed in the 1940s and provides approximately 350 million doses per year globally. However, these many doses do not cater to the requirement of global vaccination programme. A critical requirement for increasing the production and meeting the time line is a robust, scalable and commercially viable process and the availability of appropriate analytical methods. At present the production of human influenza vaccine is experiencing a renaissance with significant effort to increase productivity. The key to increase the productivity is to develop an efficient purification process that not only brings down the cost and time but also minimises the loss during the process. Purification of influenza virus has classically been approached in two ways; (1) non-specific concentration

of the virus from allantoic fluid by preparative ultra-centrifugation, (2) specific adsorption to and elution from the ligand/exchanger coupled to matrix such as cellulose.

Various methods for purification and concentration of influenza virus have been published over the last 50 years from chicken egg culture. In early 50's influenza virus purification was done by adsorption to rabbit erythrocytes and eluting the adsorbed virus from erythrocytes [F.W. Sheffield et al., 1954]. Adsorption of virus on column of aluminium phosphate and elution by small shifts in salt concentration and pH was reported by H.K. Miller and R.W. Schlesinger, 1955, M. Klembala and I. Szekacs, 1965. These reports were followed by reports on the use of density gradient centrifugation with zonal ultra-centrifuge [C.B. Reimer et al., 1967] and affinity purification with disulfide linked immunosorbent prepared from gamma globulin of rabbits [C. Sweets et al., 1974]. In the late 1970s chromatographic techniques were introduced to purify influenza virus using controlled pore glass beads in size exclusion and adsorption mode [S. Brester et al., 1975; J.T. Heyward et al., 1977]. Today a large variety of influenza vaccines and vaccine candidates exist, but the processes of purification of the influenza virus largely rely on the zonal ultra-centrifugation. Zonal ultracentrifuge has been considered as the major breakthrough in influenza purification so far.

In 1980, Goyal et al, reported chromatography purification of influenza virus. Human influenza virus from allantoic fluid was adsorbed to positively charges filters and eluted with 1 M sodium chloride. Purification of influenza virus from allantoic fluid by adsorption method tried by Goyal et al, had shown that the best adsorption occurs at pH 6 and the best elution occurs at pH 11 in presence of saline with virus recovery >100%. However, the method was not applied to a large scale purification of influenza virus. Purification of poliovirus and simian rotavirus by similar method had been reported before [S.R. Farrah et al., 1978], where these viruses were purified from tap water using membrane adsorption technology. It was shown that the adsorption of the virus to the membrane was pH dependent indicating involvement of ionic interaction that exists between the membrane and the virus surface protein. Use of ion exchange membrane was described for the purification of bacteriophage PRD1 from bacterial culture [L. Walin et al., 1994]; and alpha herpes virus from cell culture supernatant of porcine kidney [A. Karger et a., 1998]. J. Eveleth et al. [2000] had carried out one step ion-exchange chromatography to purify Sendai virus using source Q 15 resins. Another example is the purification of densonucleosis virus using

Sartobind anion and cation exchange membranes depending on the pH of the feed [R. Specht et al., 2004].

The known processes of purification of influenza viruses have had their share of limitations in the past. The conventional methods of purification of virus involves several steps such as inoculation and propagation of influenza virus in hen's egg, harvest of allantoic fluid after incubation, centrifugation to remove the cell debris, chemical refinement of the supernatant, zonal ultra-centrifuge to separate the virus, inactivation of the virus, splitting of the virus, removal of splitting agent and final polishing . These current methods for the purification of influenza viruses are cumbersome and do not easily allow fast large scale production of virus stocks for vaccines of required purity. Further, if the purification process is not efficient there may be presence of impurities and aggregates in vaccines which can elicit undesired immune responses. Some contaminants are of concern for quality and reproducibility, other contaminants would be harmful in a final vaccine, and so their removal is primarily a safety concern. Especially in a situation where influenza vaccines have to be produced in an emergency situation on large scale, (e.g. after a pandemic outbreak) pressures on manufacturers might inadvertently result in the release of vaccines that may not be very pure.

None of the prior known methods disclose the purification of influenza virus from allantoic fluid of hen's egg by a chromatography process using strong anion exchanger quaternary ammonia bound to regenerated cellulose membrane. The novel process of the current invention is a single step influenza virus purification process that can be easily scaled up to meet process requirement. Further the current invention incorporates a step of hydrophobic interaction chromatography after treatment with splitting agent which results in a highly pure virus preparation for formulation as a vaccine.

Once a highly pure influenza virus is obtained according to the present invention, the formulation techniques employed for such highly pure influenza virus, specifically a highly pure influenza split virus should be such that they provide a low dose, stable, safe and efficacious dosage form.

In the past, use of adjuvants to enhance the immune response to influenza vaccines is well known. For many years the only adjuvants approved for human use were aluminium salts, but vaccines containing the MF59 adjuvant have been approved in some countries, including Italy (in Chiron's FLUAD product). Further adjuvants include mixtures of cholesterol and

saponins, ISCOMs, MPL.TM. AS04, virosomes, SBA4 etc. A common feature of some alternatives to aluminium salts is the presence of fatty components. For example, the MF59 adjuvant includes squalene oil). Interferences can occur between such fatty components and antigens and the components may not be compatible leading to stability issues. Certain prior studies also report surfactant induced antigenic disaggregation. Hence, so far the general practice has been to package antigen and adjuvant components separately and make an admixture just before administration to patients. For example, recently approved seasonal vaccine HUMENZA and pandemic vaccines PANDEMRIX, AREPANRIX are supplied in two vials.

Further, emulsions may also contain compounds such as lecithin or saponin known to have ionic surfactant properties. Problems of stability can be observed with emulsions used as vaccine adjuvants, in particular during storage or transport. This is particularly true when these compositions contain concentrated immunogens, especially non-purified concentrated immunogens.

In practice, it is usually difficult to generate a vaccine that satisfies the requirements of purity, safety, stability, ease of administration, efficacy and the cost-effectiveness. This difficulty is inherently associated with the process of attenuation and formulation. More recently, although highly purified, better characterized split influenza vaccines have been combined with various adjuvants (US20090304742, US20090263422, US20090220541, US20080014217), in spite of significant increases in immune responses in mice, a number of approaches using new generation adjuvants have not proved possible to confirm in man. Further, the supply of antigen and adjuvant as different units makes the process cumbersome and less user-friendly.

Stable and safe vaccines with an improved immunogenicity and prepared by a fast and highly efficient purification process are therefore still needed. The purification process of the invention is suitable for commercially useful quantities of influenza virus. The invention advantageously avoids problems associated with existing methods of purifying influenza virus and relies on chromatographic techniques which enable simple scale up of the process.

Further formulation of improved highly pure influenza virus, specifically split virions with potent adjuvants, in a single dosage unit is a possible approach for enhancing immune

responses to influenza. The present invention aims to overcome the above difficulties and provides a highly pure, low dose, stable, safe, efficacious and simple vaccine dosage form.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method by which influenza virus can be purified in a simple manner to produce a vaccine against influenza.

In a further specific aspect, the purification process of the present invention is applied to an inactivated virus.

It is yet another specific object of the invention to provide a method for the purification of an influenza virus, which comprises subjecting a solution containing the influenza virus to a chromatography process using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane.

In yet another aspect, it is an object of the invention to purify a virus preparation pre treated with a splitting agent by passing through hydrophobic interaction chromatography.

It is a still further object of the invention to provide highly pure influenza virus/ virions.

In a second aspect of the present invention fully liquid, safe, stable, efficacious, low dose, easy to administer formulations of the improved highly pure split influenza virus are provided as single vial oil in water emulsions.

In a third aspect, safe, stable, efficacious, easy to administer formulations of the recombinant influenza virus are provided as single vial, oil in water emulsions.

In the most preferred embodiment the present invention provides an improved purification process for influenza virus comprising the steps of

a) propagating candidate influenza virus strains, in embryonated hens' eggs
b) providing a harvested mixture of cultured influenza virus in allantoic fluid
c) optionally adding a salt to the allantoic fluid

d) optionally carrying out one or more clarification steps or filtration steps to separate whole virus from non-virus material
e) optionally carrying out one or more concentration steps
f) carrying out column chromatography using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane.
g) splitting of the whole virus using a suitable splitting agent
h) carrying out hydrophobic interaction chromatography.
i) optionally carrying out one or more further purification and/or concentration steps

In another most preferred embodiment the present invention provides a process for providing an infuenza vaccine formulation comprising the steps of

a) propagating candidate influenza virus strains, in embryonated hens' eggs,
b) providing a harvested mixture of cultured influenza virus in allantoic fluid,
c) optionally adding a salt to the allantoic fluid,
d) optionally carrying out one or more clarification steps or filtration steps to separate whole virus from non-virus material,
e) optionally carrying out one or more concentration steps,
f) carrying out column chromatography using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane,
g) splitting of the whole virus using a suitable splitting agent,
h) carrying out hydrophobic interaction chromatography,
i) optionally carrying out one or more further purification and /or concentration steps,
j) providing a pure fully liquid low dose antigenic component,
k) providing a fully liquid oil in water emulsion component,
l) optionally providing other carriers, excipients, adjuvants and diluents

wherein, the components of steps j) to l) are mixed during manufacture and formulated in a single unit.

In a preferred embodiment the current invention provides a safe, stable, low dose, fully liquid immunogenic composition comprising

a) an highly pure, low dose antigen component, comprising one or more split influenza virus antigens,

b) an adjuvant component, comprising an oil-in-water emulsion,

c) optionally other carriers, excipients, adjuvants and diluents

wherein, the components a) to c) are mixed during manufacture and formulated in a single unit.

In another preferred embodiment the current invention provides a fully liquid composition comprising

a) one or more highly pure, low dose split influenza virus antigen component(s)

b) an emulsion comprising squalene

c) Sodium chloride

d) Potassium chloride

e) Potassium dihydrogen phosphate

f) Disodium hydrogen phosphate

g) Magnesium chloride

h) Thiomersal

wherein, the dose of HA is less than 8 micrograms and wherein components a) to h) are mixed during manufacture and formulated in a single unit.

In yet another preferred embodiment the current invention provides a fully liquid composition comprising

a) one or more highly pure low dose split influenza virus antigens

b) an emulsion comprising squalene

c) Sodium chloride

d) Potassium chloride

e) Potassium dihydrogen phosphate

f) Disodium hydrogen phosphate

g) Magnesium chloride

h) Thiomersal

wherein, the dose of HA is less than 4 micrograms and wherein components a) to h) are mixed during manufacture and formulated in a single unit.

In another aspect, there is provided a method of vaccination of a subject with an immunogenic composition comprising an improved influenza virus preparation or antigenic preparation thereof and an oil-in-water emulsion adjuvant, as hereinabove defined.

DETAILED DESCRIPTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the examples included therein.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific antigens or to particular methods, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

DEFINITIONS:

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "comprising" means "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "adjuvant" refers to any substance that assists or modifies the immunological action of a pharmaceutical composition, including but not limited to adjuvants that increase or diversify the immune response to an antigen.

"Oil" as defined herein, is a liquid that is immiscible with water.

By "antigen" is meant a molecule that contains one or more epitopes capable of stimulating a host's immune system to make a cellular antigen-specific immune response or a humoral antibody response when the antigen is presented in accordance with the present invention.

As used throughout, by "subject" is meant an individual. Preferably, the subject is a mammal such as a primate, and, more preferably, a human. The term "subject" includes domesticated animals, such as cats, dogs, etc., livestock (for example, cattle, horses, pigs, sheep, goats, etc.), laboratory animals (for example, ferret, chinchilla, mouse, rabbit, rat, gerbil, guinea pig, etc.) and avian species (for example, chickens, turkeys, ducks, pheasants, pigeons, doves, parrots, cockatoos, geese, etc.). The subjects of the present invention can also include, but are not limited to fish, amphibians and reptiles.

As used herein, the term "treat" (or "treating", "treated", "treatment", etc.) refers to the administration of an immunogenic composition to a subject who has influenza, a symptom of influenza or a predisposition toward influenza, with the purpose to alleviate, relieve, alter, ameliorate, improve or affect the influenza, a symptom or symptoms of influenza, or the predisposition toward influenza. In some embodiments, the term "treating" refers to the vaccination of a subject.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

As used herein, the term "immunogenic" means capable of producing an immune response in a host animal against a non-host entity (e.g., an influenza virus). In some embodiments, this immune response forms the basis of the protective immunity elicited by a vaccine against a specific infectious organism (e.g., an influenza virus).

An “immunological response” to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the antigen or composition of interest. For purposes of the present invention, a “humoral immune response” refers to an immune response mediated by antibody molecules, while a “cellular immune response” is one mediated by T-lymphocytes and/or other white blood cells.

The terms “effective amount” or “pharmaceutically effective amount” or “immunologically effective amount” of the adjuvant/antigen composition, as provided herein, refer to a sufficient amount of the adjuvant/antigen composition to treat a condition of interest.

The term "excipient" refers to substances that are commonly provided within finished dosage forms, and include vehicles (water, saline, buffer solutions, glycerol, polyethylene glycol, hyaluronic acid, ethanol, etc.), binders, disintegrants, fillers (diluents), lubricants, glidants (flow enhancers), compression aids, colors, sweeteners, preservatives, suspensing/dispersing agents, film formers/coatings, flavours and printing inks.

An "anion exchange chromatography" refers to a separation technique that employs a solid phase resin that is positively charged, and thus can bind negatively charged molecules in an aqueous solution passed over or through the solid phase

The term "hydrophobic interaction chromatography" (HIC) refers to a technique that separates biomolecules, under relatively mild conditions by working in a more polar and less denaturing environment than reverse phase chromatograph. In HIC, hydrophobic interactions are promoted by salts so that the biomolecule, e.g., protein, is adsorbed.

The term "highly pure" refers to the improved purity of the virus obtained by following the anion exchange chromatography of the invention when compared to virus obtained from conventional techniques like zonal ultra-centrifugation. The amount of egg protein found in the conventional zonal centrifugation is between 800-1000 ng /ml, whereas, in the new chromatography process the amount of egg protein has been reduced to 200- 400 ng /ml.

The term 'Emulsion Adjuvant' refers to an oil in water emulsion.

The term "low dose" refers to a dose of haemagglutinin antigen (HA) which is equal to or less than 7.5 µg and most preferably equal to or less than 3.75 µg.

The term 'about' refers to a value +/ - 10 %, of the value specified.

The term 'SAS version 9' refers to asoftware for statistical analysis. SAS (pronounced "sass", originally Statistical Analysis System) is an integrated system of software products provided by SAS Institute Inc, which enables programmers to perform statistical analysis. Software version 9 of SAS software delivers a variety of procedures and enhancements, which are motivated by methodological advances in statistics.

The term 'Seroprotection' refers to the proportion /percentage of subjects achieving an HI antibody titer ≥ 1:40 that meets or exceed 70%.

The term 'Seroconversion' refers to proportion /percentage of subjects with a 4-fold rise in HI titer (pre HI <1:10, post ≥1:40, pre HI ≥1:10, post ≥4x pre) that meets or exceed 40%.

The term 'HI assay' refers to a procedure for the measurement of serum antibodies directed against a hemagglutinating virus. The basis of the HI assay is that antibodies to influenza virus will prevent attachment of the virus to red blood cells. Therefore hemagglutination is inhibited when antibodies are present. The highest dilution of serum that prevents hemagglutination is called the HI titer of the serum. If the serum contains no antibodies that react with the H1N1 strain, then hemagglutination will be observed in all wells. Likewise, if antibodies to the virus are present, hemagglutination will not be observed until the antibodies are sufficiently diluted.

The present invention provides an improved method by which influenza virus can be purified to produce vaccine against influenza. The invention is based on the premise that specific modification of viral purification processes can lead to increases in the yield, purity and robustness. The invention also provides an improved, low dose, stable, safe and efficacious compositions for easy administration to subjects.

A specific embodiment of the invention is that the virus is first inactivated and then purified using the improved method of the invention and is still able to yield more virus than conventional techniques like zonal ultra-centrifugation. The present invention relates to a simplified technique for obtaining inactivated whole virus from allantoic fluid. The invention is applicable to any virus having antigen producing protein attached in a hydrophobic/hydrophilic manner to the remainder of the virus.

The present invention is based on the premise that anion exchange chromatography column with quaternary ammonium adsorbed in the regenerated cellulose membrane anion has a specific affinity with influenza virus as a whole and the surface antigens (HA and NA proteins). This column is effective for isolation and purification of such virus or virus proteins from a material containing the same.

Thus, according to the present invention, there is provided a method for the purification of influenza virus which comprises subjecting an impure or partially purified influenza virus containing preparation to an anion exchange chromatography column with quaternary ammonium adsorbed in the regenerated cellulose membrane

The method of the present invention can be employed at any stage in the purification of influenza virus proteins. For example, the method of the present invention can be applied directly to an allantoic fluid or a culture broth infected with an influenza virus, so that the influenza virus as a whole can be specifically separated from the other substances contained in such fluid or liquid. The method of the present invention can also be applied to a solution which has undergone some purification process (es) and / or inactivation beforehand, in order to further purify influenza virus. For example, a solution after the detergent/ether-treatment, which has been cleared of lipids but may contain various proteins and nucleic acids as well as HA and NA proteins, can be subjected to the method of the present invention so as to specifically isolate the HA and NA proteins from the other substances contained in the solution. Of course, two or more of such purification procedures can be combined, optionally with conventional separation techniques (e.g. ultra-centrifugation or other ion exchange chromatography), so as to obtain influenza virus purified as highly as possible, particularly, with respect to HA and NA proteins. It should also be noted that the present invention is applicable to a solution containing influenza virus of any type or any origin. Thus, the method of the present invention can be employed in purifying virus of type A or type B influenza as well as the other types of influenza.

In an especially preferred embodiment the anion exchange chromatography is carried out after salt treatment of the allantoic fluid.

Buffers and solvents used for equilibration, washing and elution of the chromatography columns used in the invention and well known in art and are within the skill of a person having ordinary skill in art.

In another preferred embodiment, splitting of the virus is carried out after anion exchange chromatography e.g., by using Triton X-100 and the treated solution is passed through a hydrophobic interaction chromatography like Toyopearl Phenyl/Hexyl 600 M (HIC) column.

According to one preferred embodiment of the invention, the process of the invention comprises of clarification of the allantoic fluid, capturing of the virus on the anion exchange column, splitting of the influenza virus, followed by concentration of the same.

According to one more preferred embodiment, the allantoic fluid is subjected to inactivation using an inactivating agent, in presence of a suitable buffer system. The sample is then subjected to clarification on adjusting its conductivity to not more than 22 mS/ cm. The clarified sample is applied to an anion exchange column, which is then washed and the elution of the HA containing fractions or fractions containing any other protein of interest, is affected using a suitable buffer system. Further the HA containing fractions (or the fractions containing the any other protein of interest) are subjected to splitting. The fractions are diluted to adjust the conductivity to around 2 mS/ cm and concentrated. An appropriate splitting agent is added to the virus pool and incubated for specific time interval at appropriate temperature. The splitting agent is then removed using suitable method such as buffer exchange, chromatographic techniques, etc. The split virus containing sample is then subjected to concentration and purification and is stored appropriately.

According to another more preferred embodiment, the allantoic fluid is subjected to buffer exchange and clarification. The conductivity of the sample is adjusted to about 13.5±1.5 mS/cm^2. The clarified sample is then subjected to anion exchange chromatography. The washing and the elution of the HA or any other protein of interest containing sample is done using appropriate buffer system. The conductivity of the eluted fractions is then adjusted to around 2 to 2.5 mS/cm and the sample is subjected to concentration. An appropriate splitting agent is added to the virus pool and incubated for specific time interval at appropriate temperature. The splitting agent is then removed using suitable method such as buffer exchange, chromatographic techniques, etc. This sample is then subjected to inactivation using appropriate inactivating agent for appropriate time and at a particular temperature. The inactivated sample is subjected to buffer exchange and is concentrated.

According to one particularly preferred embodiment of the invention, the allantoic fluid is subjected to inactivation by an inactivating agent, prior to the step of chromatography.

Another specifically preferred embodiment of the invention uses a process which comprises inactivation step by an inactivating agent, after the step of chromatography.

The inactivating agent used according to one of the embodiment of the invention is most preferably formalin, at a preferable concentration of 0.005 – 0.1%, more preferably at 0.01-0.05%, and most preferably at 0.02%.

According to the invention, any anion exchange column may be used. According to especially preferred embodiment of the invention, Sartobind Q single step chromatography is used for capturing the clarified virus.

One specifically preferred embodiment of the invention deals with the use of the allantoic fluid for chromatography, wherein the conductivity of the fluid is adjusted to be not more than about 22 mS/ cm.

The washing of the columns and the elution of the protein of interest during the chromatography may most preferably be affected in phosphate based buffer systems.

Triton X- 100 is the splitting agent specifically used according to one most preferred embodiment of the invention. The concentration of Triton X- 100 used may be more preferably in the range of 0.1- 1.0% and most preferably 0.5%.

The sample to be treated with the splitting agent does not have a conductivity of more than about 3 mS/ cm, according to a particularly preferred embodiment of the invention.

According to one preferred embodiment of the invention, the Triton X- 100 may be removed using hydrophobic interaction chromatography. Toyopearl Phenyl 600 M (HIC) column is preferred for the purpose according to a particularly specific preferred embodiment of the invention.

By following the process of the current invention it is possible to highly purify the viral suspension resulting in obtaining higher amount of HA antigen. According to the purification method of the present invention, influenza virus can be purified to a high degree, i.e. to several times the purity thereof in the starting solution. The improved process results in easy elimination of contaminants like Tween, chloroform, other splitting agents employed, egg proteins and other components, lipids, as well as a portion of the virus disruption products.

The purification method of the present invention can be carried out on an industrial scale without need for expensive equipment and give the desired purified influenza on an industrial scale with lower cost. Besides, the membranes used herein are very stable, and the product thus obtained has no impurities as occasionally observed in the conventional products.

The modified membranes used in the current process can achieve high product recoveries and contaminant reduction. Due to a fast binding kinetic membrane adsorbers enable to operate the capturing process at an increased flow rate. Hence, the productivity can be significantly enhanced making them a valuable choice for industrial influenza vaccine production processes

Hence, a further object of the invention is the products so obtained, and particularly purified virus, specifically a split virus that can advantageously be used for the preparation of vaccines.

The process of invention enables the use of the purified viral antigen for formulation of an immunogenic composition or vaccine formulation. The antigen obtained by the process of the invention, may be adjuvanted or may not be adjuvanted, before the formulation into a vaccine composition. The vaccine thus obtained, may be effective in inducing immunological response in a subject, by administering immunologically active amount of the virus. Such a vaccine may be effective on administration of single or double or more doses to the subject.

According to one important and preferred aspect of the invention, the purification process of the invention, results in a purified antigen giving more than about 50 % seroconversion values.

According to another more preferred aspect of the invention, the seroconversion values obtained are more than about 60- 70%.

One most preferred aspect of the invention deals with the process of purification of viral antigen, according to the invention, which results is purified viral antigen giving more than about 80 % seroconversion.

The inventors have also found an improved oil in water formulation for the improved split virus which is stable, safe, efficacious, low dose and easy to administer.

These improved formulations will advantageously be effective to target the humoral and/or the cell-mediated immune system in order to increase responsiveness against various influenza strains.

The improved influenza compositions according to the invention may have several advantages like

- improved immunogenicity and improved seroprotection
- improved cross-protection profile
- persistent immune response against influenza related disease
- reduced antigen dosage
- Less cumbersome dosage form and easy administration
- Meeting the seroprotection and seroconversion levels or efficacy criteria as required by various countries

Influenza strains

Influenza virus strains for use in vaccines may change from season to season. In the inter-pandemic period, vaccines typically include two influenza A strains (H1N1 and H2N3) and one influenza B strain, and trivalent vaccines are typical. The invention may be used to prepare seasonal vaccines. The invention may also use viruses from pandemic strains (i.e. strains to which the vaccine recipient and the general human population are immunologically naïve), such as H2, H5, H7 or H9 subtype strains (in particular of influenza A virus), and influenza vaccines for pandemic strains may be monovalent or may be based on a normal trivalent vaccine supplemented by a pandemic strain. Depending on the season and on the nature of the antigen included in the vaccine, however, the invention may protect against one or more of influenza A virus HA subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16. The invention may protect against one or more of influenza A virus NA subtypes N1, N1, N3, N4, N5, N6, N7, N8 or N9.

Suitable pandemic strains are, but not limited to: H1N1, H1N2, H5N1, H9N2, H7N7, H2N2, H7N1, H7N2, H7N3, H10N7, H3N2, and H5N2.

The influenza virus may be a reassortant strain, and may have been obtained by either classical reassortment or by reverse genetics techniques.

A particularly preferred strain for use in the invention includes the reassortant strain A/California/7/2009 (H1N1) v-like strain used (X-179A).

Said influenza virus or antigenic preparation thereof may be egg-derived or cell-culture derived. For example, the influenza virus antigen or antigenic preparations thereof according to the invention may be derived from the conventional embryonated egg method, by growing influenza virus in specific pathogen free (SPF) embryonated eggs and purifying the harvested allantoic fluid.

In a preferred embodiment, virally infected allantoic fluid from chick embryos is prepared according to guidelines currently established for vaccine production. Embryonated eggs may be obtained 9-12 days after fertilization and candled to locate the air sac. The egg may then be pierced under aseptic conditions, and the seed- virus inoculated into the air-space with a syringe. The procedure may be carried out manually or automatically by machines. The inoculated egg may then be incubated for approximately two to three days in a humidified atmosphere. At the end of this period, the egg can be maintained at approximately 4°C if desired in order to terminate the embryo and aid clarification of the allantoic fluid. The top of the egg may then be removed, the membrane pierced, and the allantoic fluid collected. Again this can be achieved manually, or by automated machinery. The contents of allantoic fluid can be divided into four groups;

(A) desirable influenza virus,
(B) undesirable particulate matter in the size range greater than virus,
(C) undesirable dissolve solids, and
(D) water.

The allantoic fluid may be clarified, for example, by centrifugation to remove cell debris and/or subjected to further purification.

The virus infected allantoic fluid contains a high concentration of live virus, but much of the virus is associated (aggregated) with fibrous or granular debris. Some salts may be added to the allantoic fluid to accomplish dissociation of virus from the aggregated debris.

One or more steps of filtration or centrifugation may be carried out, virus-containing supernatant or filtrate may be then processed as desired to further enrich for virus. Influenza

virus solutions can be concentrated by well-known techniques without losing appreciable viral potency/activity.

In the most preferred embodiment the virus containing allantoic fluid is subjected to a chromatography process using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane.

The principle behind ion exchange membrane separation is the reversible interaction between target protein and membrane functional groups (i.e., charged functional groups that are covalently attached to the membrane surface). Anion exchange membranes have positively charged functional groups such as quaternary ammonium and will bind viral components which are negatively charged. The rate of association between target proteins and functional groups in ion exchange membranes is very rapid, unlike the slow rate of diffusion through conventional packed columns. The fast convective flow combined with negligible pressure drop limitations exhibited by the thin membranes mean that processing times are dramatically reduced compared with conventional packed columns.

The affinity of the virus for the exchanger depends on both the electrical properties of the external coat of the virus, and the relative affinity of other charged substances in the solvent. Hence, bound virus can be eluted by changing the pH, thus altering the charge of the virus, or by adding competing materials like salts. Because different substances have different electrical properties, the conditions for release vary with each bound molecular species. In general, to get good separation, the methods of choice are either continuous ionic strength gradient elution or stepwise elution. For an anion exchanger, either pH is decreased and ionic strength is increased or ionic strength alone is increased. The actual choice of the elution procedure is usually a result of trial and error and of considerations of stability of the virus being purified.

It is to be appreciated that the present method can also be applied along with any other fractionation or separation processes useful to obtain purified virus from allantoic fluids, including those utilizing size exclusion chromatography, centrifugation, filtration, solvent extraction, ion-exhange chromatography, and the like. Moreover, any one or more of the resulting fractions can be rendered non-isotonic, to improve the virus recovery process, in that virus is rendered substantially monodisperse in solution.

Alternatively, the virus may be derived from any of the new generation methods using tissue culture to grow the virus or express recombinant influenza virus surface antigens. Suitable cell substrates for growing the virus include for example dog kidney cells such as MDCK or cells from a clone of MDCK, MDCK-like cells, monkey kidney cells such as AGMK cells including Vero cells, suitable pig cell lines, or any other mammalian cell type like hamster, cattle, primate (including humans and monkeys) and dog cells which may be suitable for the production of influenza virus for vaccine purposes.

The most preferred embodiment is exemplified in specific examples; however these are non-limiting examples.

The virus thus obtained may be processed to make whole virus vaccines, split virus vaccines, surface antigen vaccines (subunit vaccines). Split vaccines are the most preferred according to the present invention and are further elaborated below.

The Split Influenza Antigen

General methods of splitting influenza viruses are well known in the art (e.g. see WO02/28422, WO02/067983, WO02/074336, WO01/21151, WO02/097072 and WO2005/113756). Splitting of the virus is typically carried out by disrupting or fragmenting whole virus, whether infectious or non-infectious with a disrupting concentration of a splitting agent. The disruption results in a full or partial solubilisation of the virus proteins, altering the integrity of the virus.

Traditionally split flu was produced using a solvent/detergent treatment, such as tri-n-butyl phosphate, or diethylether in combination with Tween TM (known as "Tween-ether" splitting) and this process is still used in some production facilities. Other splitting agents now employed include detergents or proteolytic enzymes or bile salts, for example sodium deoxycholate as described in patent no. DD 155 875, incorporated herein by reference.

Bile acids that may be used for the practice of the current invention include cholic acid, deoxycolic acid, chenodeoxycolic acid, lithocholic acid ursodeoxycholic acid, hyodeoxycholic acid and derivatives like glyco-, tauro-, amidopropyl-1-propanesulfonic-, amnidopropyl-2-hydroxy-1-propanesulfonic derivatives of forementioned bile acids, or N,N-

bis(3 D Gluconoamidopropyl) deoxycholamide A particular example is sodium deoxycholate Na DOC.

Preferred splitting agents are non-ionic and ionic {e.g. cationic) surfactants e.g. alkylglycosides, alkylthioglycosides, acyl sugars, sulphobetaines, betains, polyoxyethylenealkylethers, N,N-dialkyl-Glucamides, Hecameg, alkylphenoxy-polyethoxyethanols, quaternary ammonium compounds, sarcosyl, CTABs (cetyl trimethyl ammonium bromides), tri-N-butyl phosphate, Cetavlon, myristyltrimethylammonium salts, lipofectin, lipofectamine, and DOT-MA, the octyl- or nonylphenoxy polyoxyethanols (e.g. the Triton surfactants, such as Triton X-IOO or Triton N l01), polyoxyethylene sorbitan esters (the Tween surfactants), polyoxyethylene ethers, polyoxyethlene esters, etc.

The preparation process for a split vaccine may include a number of different filtration and/or other separation steps such as ultracentrifugation, ultrafiltration, zonal centrifugation and chromatography (e.g. ion exchange) steps in a variety of combinations, and optionally an inactivation step eg with heat, formaldehyde or β-propiolactone or U.V which may be carried out before or after splitting. The splitting process may be carried out as a batch, continuous or semi-continuous process.

When produced in eggs, the split virus may be depleted from egg-contaminating proteins, or when produced in cell culture, the split virus may be depleted from host cell contaminants. A split virus antigenic preparation may comprise split virus antigenic components of more than one viral strain.

In the most preferred embodiment the current process employs a method which involves the steps of selective capturing and purification of virus in the anion exchange membrane in appropriate buffer system, splitting of the virus using detergent, removal of detergent by hydrophobic interaction chromatography and concentration of the virus using tangential flow filtration (TFF) membrane. In TFF, viruses in solution are passed through hollow fiber filter tubes or across plates of filter material. As opposed to normal flow filtration wherein the feed flow and pressure are in the same direction, TFF relies upon pressure that is perpendicular to the feed flow. Thus, in TFF, the filtrate passes through the membrane-containing walls of the tube while the retentate flows down the path of the tube. During this process, solution volume can be reduced as desired.

Membranes for use in TFF of virus-containing solutions are commercially available (e.g., MILLIPORE, Billerica, Mass, Sartorius). Hereto, the particular TFF membrane selected will have a pore size (300 kDa, 100 kDa, 10 kDa) sufficiently small to retain virus but large enough to effectively clear impurities. Selection of the appropriate membrane cut off range is within the skill of a person having ordinary skill in art. The membrane composition may be, but is not limited to, regenerated cellulose, polyethersulfone, polysulfone, or derivatives thereof. The membranes can be flat sheets (also called flat screens) or hollow fibers.

The process of invention specifically involves capturing the virus in an anion exchange membrane of pore size 3 μ. The anion exchanger is quaternary ammonium adsorbed in the regenerated cellulose membrane.

Oil in water emulsions

The adjuvant composition of the invention contains an oil-in-water emulsion adjuvant; preferably said emulsion comprises metabolisable oil in an amount of 5% to 15% of the total volume.

Oil-in-water emulsions have been found to be particularly suitable for use in adjuvanting influenza virus vaccines. Various such emulsions are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5μm in diameter, and may even have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220 nm are preferred as they can be subjected to filter sterilization.

The invention can be used with oils such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used e.g. obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and

esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, which is particularly preferred herein. Squalene is also found in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast. Squalene is metabolisable oil by virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no. 8619). Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other preferred oils are the tocopherols. Mixtures of oils can be used.

Suitable surfactants for use in the emulsion of the invention may be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij **30**); polyoxyethylene-9-lauryl ether; and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate) and Span 85 (sorbitan trioleate).

Mixtures of surfactants may also be used e.g. Tween 80/Span 85 mixtures, or Tween80/Triton-X100 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as Tween 80) 0.01 to 1%, in particular about 0.1%; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1%, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20%, preferably 0.1 to 10% and in particular 0.1 to 1% or about 0.5%.

In some embodiments the composition of the invention includes a tocopherol, any of α, β, γ, ε or ξ tocopherols can be used, but α-tocopherols are preferred. The tocopherol can take several forms e.g. different salts and/or isomers. Salts include organic salts, such as succinate, acetate, nicotinate, etc. D-α-tocopherol and DL-α-tocopherol can both be used.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' [5-7], see WO90/14387. The MF59 emulsion advantageously includes citrate ions e.g. 10 mM sodium citrate buffer.
- An emulsion of squalene, a tocopherol, and Tween 80 like AS03 (squalene - 10.68 mg DL-alpha-tocopherol - 11.86 mg , polysorbate 80 - 4.86 mg)
- AS02 which comprises MPL and QS-21 in oil in water emulsions. The latter is an immunomodulator extracted from the bark of a South American tree, *Quillaria saponaria*.
- The AS01 Adjuvant System based on the combination of liposomes, MPL and QS21.
- An emulsion of squalene, a tocopherol, and a Triton detergent (e.g. Triton X-100). The emulsion may also include a 3d-MPL.

- An emulsion comprising a polysorbate (e.g. polysorbate 80), a Triton detergent (e.g. Triton X-100) and a tocopherol (e.g. a α-tocopherol succinate). The emulsion may also include squalene. The emulsion may also include a 3d-MPL
- An emulsion of squalane, polysorbate 80 and poloxamer 401 ("Pluronic™ L121").
- A submicron oil-in-water emulsion of non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate, dimethyldioctadecylammonium bromide and/or N, N-dioctadecyl-N, N-bis (2-hydroxyethyl) propanediamine.
- An emulsion in which a saponin (e.g. QuilA or QS21) and a sterol (e.g. a cholesterol) are associated as helical micelles
- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (e.g. an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer)
- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty alcohol, and a non-ionic lipophilic surfactant (e.g. an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer)
- Other oil-in-water emulsions include Montanide (Seppic), Adjuvant 65, and Lipovant.

The emulsions may further contain ISCOMS (immune stimulating complexes), CpG dinucleotides etc.

The most preferred emulsion of the invention is made of squalene, Tween 80, and Span 85.

The emulsions are premixed with antigen at the time of manufacture. Thus the adjuvant and antigen are formulated in a same or single unit.

Compositions of the invention are made of pharmaceutically acceptable ingredients and are typically in aqueous or fully liquid form. They may include components in addition to the antigen and adjuvant e.g. they may include one or more pharmaceutical carrier(s) and/or excipient(s) and/or diluents and /or further adjuvants.

The composition may include preservatives such as thiomersal and other equivalents.

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 30 mg/ml.

Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, etc.

Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer.

The pH of a composition may be generally between 5.0 and 8.1. Most preferably the pH of the composition is adjusted to between 6.5 -7.5 with 0.1N NaOH/0.1N HCl solution.The composition is preferably sterile.

Influenza vaccines are typically administered in a dosage volume of about 0.5 ml; although a half dose (i.e. about 0.25 ml) may be administered to children (e.g. up to 36 months of age).

Compositions and kits are preferably stored at between 2° C to 8° C. They should not be frozen. They should ideally be kept out of direct light.

In a preferred arrangement the antigen and adjuvant fully liquid components are held together in the same syringe in a single chamber.

In another preferred arrangement, the two fully liquid components of a vaccine are held together but separately in the same syringe e.g. a dual-chamber syringe. When the syringe is actuated (e.g. during administration to a patient) then the contents of the two chambers are mixed. This arrangement avoids the need for a separate mixing step at the time of use.

Suitable containers for compositions of the invention (or kit components) include vials, syringes (e.g. disposable syringes) etc. These containers should be sterile.

Where a composition/component is located in a vial, the vial is preferably made of a glass or plastic material. The vial is preferably sterilized before the composition is added to it. To avoid problems with latex-sensitive patients, vials are preferably sealed with a latex-free stopper, and the absence of latex in all packaging material is preferred. The vial may include a single dose of vaccine, or it may include more than one dose (a 'multidose' vial) e.g. 10 doses. Preferred vials are made of colorless glass.

A vial can have a cap (e.g. a Luer lock) adapted such that a pre-filled syringe can be inserted into the cap, the contents of the syringe can be expelled into the vial (e.g. to reconstitute lyophilized material therein), and the contents of the vial can be removed back into the syringe. After removal of the syringe from the vial, a needle can then be attached and the composition can be administered to a patient. The cap is preferably located inside a seal or cover, such that the seal or cover has to be removed before the cap can be accessed. A vial may have a cap that permits aseptic removal of its contents, particularly for multidose vials.

Where a composition/component is packaged into a syringe, the syringe may have a needle attached to it. If a needle is not attached, a separate needle may be supplied with the syringe for assembly and use. Such a needle may be sheathed. Safety needles are preferred. 1-inch 23-gauge, 1-inch 25-gauge and ⅝-inch 25-gauge needles are typical. Syringes may be provided with peel-off labels on which the lot number, influenza season and expiration date of the contents may be printed, to facilitate record keeping. The plunger in the syringe preferably has a stopper to prevent the plunger from being accidentally removed during aspiration. The syringes may have a latex rubber cap and/or plunger. Disposable syringes contain a single dose of vaccine. The syringe will generally have a tip cap to seal the tip prior to attachment of a needle, and the tip cap is preferably made of a butyl rubber. If the syringe and needle are packaged separately then the needle is preferably fitted with a butyl rubber shield. Preferred syringes are those marketed under the trade name "Tip-Lok"™.

Containers may be marked to show a half-dose volume e.g. to facilitate delivery to children. For instance, a syringe containing a 0.5 ml dose may have a mark showing a 0.25 ml volume.

Where a glass container (e.g. a syringe or a vial) is used, then it is preferred to use a container made from a borosilicate glass.

A kit or composition may be packaged (e.g. in the same box) with a leaflet including details of the vaccine e.g. instructions for administration, details of the antigens within the vaccine, etc. The instructions may also contain warnings e.g. to keep a solution of adrenaline readily available in case of anaphylactic reaction following vaccination, etc.

Compositions of the invention are suitable for administration to human patients. The immune response raised according to the invention will generally include an antibody response,

preferably a protective antibody response. Methods for assessing antibody responses, neutralizing capability and protection after influenza virus vaccination are well known in the art.

Compositions of the invention can be administered in various ways. The most preferred immunization route is by intramuscular injection (e.g. into the aim or leg), but other available routes include subcutaneous injection, intravenous injection, intranasal, oral, intradermal transcutaneous, transdermal etc.

Vaccines of the invention may be used to treat both children and adults. Influenza vaccines are currently recommended for use in pediatric and adult immunization, from the age of 6 months.

Treatment may be by a single dose or multiple dose schedules. The multiple doses will typically be administered at least 1 week apart (e.g. at least about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks apart, about 12 weeks, about 16 weeks apart, etc.).

Preferred dosing regimens of the invention are 2-dose regimens. Further doses may be administered in subsequent influenza seasons, typically in the usual 1-dose format, but the standard immunization in a single season (e.g. within a single 6 month period or 12 month period) according to the invention will involve 2 doses.

Vaccines produced by the invention may be administered to patients at substantially the same time as (e.g. during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines e.g. at substantially the same time as a measles vaccine, a mumps vaccine, a rubella vaccine, a MMR vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H. influenzae* type b vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A-C-W135-Y vaccine), a respiratory syncytial virus vaccine, a pneumococcal conjugate vaccine, etc. Administration at substantially the same time as a pneumococcal vaccine or a meningococcal vaccine is particularly useful in elderly patients.

Similarly, vaccines of the invention may be administered to patients at substantially the same time as (e.g. during the same medical consultation or visit to a healthcare professional) an antiviral compound, and in particular an antiviral compound active against influenza virus (e.g. oseltamivir and/or zanamivir). These antivirals include neuraminidase inhibitors, such as a (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid or 5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D -glycero-D-galactonon-2-enonic acid, including esters thereof (e.g. the ethyl esters) and salts thereof (e.g. the phosphate salts). A preferred antiviral is (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1), also known as oseltamivir phosphate (TAMIFLU™).

Examples:

The invention is explained in detail in the following examples which are given solely for the purpose of illustration only and therefore should not be construed to limit the scope of the invention.

Example 1:

<u>This example gives the method of propagation of the virus according to one of the embodiments of the invention</u>

9-12 day old embryonated hen's eggs were inoculated with live influenza virus. The eggs were incubated at 37°C for a period of two to three days. The eggs were then refrigerated at 5°C for 24 hours. The allantoic fluid was harvested. The harvested allantoic fluid was subjected to purification.

Example 2:

This example gives the method of purification of the influenza virus according to one of the embodiments of the invention

Step 1: Clarification of allantoic fluid after propagation in eggs:

To the allantoic fluid, phosphate buffer pH 7.4, sodium chloride and EDTA was added and stirring was done for 60 minutes. This was diluted five times with phosphate buffer. The resulting allantoic fluid was filtered through 5μ capsule filter and 3μ ± 0.8 μ capsule filters followed connected in series.

Step 2: Capturing of Influenza virus on anion exchange column:

The filtrate obtained from step 1 was subjected to Sartobind Q single step chromatography at a flow rate not more than 200 ml/min. The membrane was washed with equilibrium buffer followed by washing with wash buffer 2. The membrane was eluted with high salt in phosphate buffer.

The buffers and solvents that may be employed are as follows:

1. 50mM Phosphate Buffer, 200mM NaCl, 2mM EDTA pH 7.4; (Equilibration buffer)
2. 50mM Phosphate Buffer, 300mM NaCl, 2mM EDTA pH 7.4; (Wash buffer)
3. 50mM Phosphate Buffer, 2.0 M NaCl, 2mM EDTA pH 7.4; (Elution buffer 1)
4. 50mM Phosphate Buffer, 3.0 M NaCl, 2mM EDTA pH 7.4; (Elution buffer 2 or cleaning buffer).
5. 10 mM Phosphate Buffer, pH 7.4.
6. 30% Isopropyl alcohol

Step 3: Splitting of Influenza virus:

The HA containing fraction was diluted with autoclaved, 0.22 μ membrane filtered water and EDTA was added to it. Buffer exchange was done on the diluted virus solution with sodium phosphate buffer, EDTA, pH 7.4, using 100 kDa cut off membrane. 0.5% Triton X-100 was added to the virus pool and this was incubated at room temperature for 24 hrs. The protein sample was passed through Toyopearl Phenyl/Hexyl 600 M (HIC) column, pre-equilibrated with phosphate buffer, pH 7.4.The flow through was collected.

Step 4: Polishing

NaCl was added to the flow through. The virus was concentrated by using 10 kD membrane and buffer exchange to Phosphate buffer pH 7.4 containing NaCl. 0.125% Tween 80 was added to prevent protein aggregation due to low CMC.

Example 3:

<u>This example gives the method of purification of the influenza virus according to another embodiment of the invention</u>

Step 1: Clarification of allantoic fluid after propagation in eggs:

The allantoic fluid was pre- inactivated using 0.02% Formalin. Phosphate buffer, pH 7.4, NaCl and EDTA, to final concentration 50 mM, 1M and 2 mM, were added to it and was kept stirring. This was diluted with 50 mM Phosphate buffer, pH 7.4, 2 mM EDTA to bring the NaCl concentration to 200 mM. The conductivity was adjusted to be not more than 22 mS/cm. Filter allantoic fluid through 5μ and followed by 3μ+0.8 μ Capsule filter for clarification.

Step 2: Capturing of Influenza virus on anion exchange column:

The filtrate obtained was subjected to Sartobind Q single step (pre- equilibrated with 50 mM Phosphate buffer, pH 7.4 containing 200 mM NaCl, 2 mM EDTA) chromatography. The membrane was washed with the equilibration buffer followed by the washing buffer (50 mM Phosphate buffer, pH 7.4 containing 300 mM NaCl). Elution was affected using 2.0 M and 3.0 M NaCl in 50 mM phosphate buffer, pH 7.4.

Step 3: Splitting of Influenza virus:

The eluted HA containing fractions were diluted with sterile water to get the concentration of NaCl to 400-500 mM. 2 mM EDTA (final concentration) was added to it. The virus solution was then subjected to buffer exchange using 10 mM Sodium Phosphate buffer, 2 mM EDTA, pH 7.4, using 100 kDa cut off membrane (TFF). The conductivity was adjusted to around 2

mS/cm. The virus solution was then concentrated. Triton X-100 (0.5% final concentration) was added to the virus pool and incubated 24 hr at room temperature.

Step 4: Triton Removal and concentration:

Triton was removed by passing the protein sample through Toyopearl Phenyl 600 M (HIC) column, pre-equilibrated with 10 mM phosphate buffer, pH 7.4. 100 mM NaCl (final concentration) was added to flow through from the said column. The sample was subjected to concentration and sterile filtration.

Example 4:

<u>This example gives the method of purification of the influenza virus according to one another embodiment of the invention</u>

Step 1: Clarification of allantoic fluid after propagation in eggs:

The allantoic fluid was buffer exchanged with 1 x PBS (136.89 mM NaCl, 10.73 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4), 1% Sucrose-pH 7.4, using 300 kDa Sartorius PES membrane. The original volume of allantoic fluid was maintained. NaCl was added to it to final concentration of 1M and the solution was incubated in ice for 60 mins under stirring condition. The sample was then diluted with water to get the conductivity to 13.5±1.5 mS/cm^2. The diluted allantoic fluid was then filtered through 5μ, followed by 3μ+0.8 μ Capsule filter for clarification.

Step 2: Capturing of Influenza virus on anion exchange column:

The above obtained clarified fluid was then subjected to Sartobind Q single step (pre equilibrated with 1 x PBS (136.89 mM NaCl, 10.73 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4), 1% Sucrose, chromatography. The column was washed with 1 x PBS (136.89 mM NaCl, 10.73 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4), 1% Sucrose, followed by 1 x PBS (161.89 mM NaCl, 10.73 mM KCl,10.14 mM Na2HPO4, 1.76 mM KH2PO4), 1% Sucrose, pH 7.4. The elution was affected by 1 x PBS (136.89 mM NaCl,

10.73 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4), 1% Sucrose, 1.363 M NaCl, pH 7.4.

Step 3: Splitting of Influenza virus:

The eluted HA containing fractions were diluted with sterile water to get the concentration of NaCl to 300-350 mM. 2 mM EDTA (final concentration) was added to it. The diluted virus solution was subjected to buffer exchange using 10 mM Sodium Phosphate buffer, 2 mM EDTA, 1% sucrose, pH 7.4, using 100 kDa cut off membrane (TFF) to get the conductivity to 2 to 2.5 mS/cm. The sample was subjected to concentration. Triton X-100 was added to final concentration of 0.5% to the virus pool and the sample was incubated for 24hr at room temperature.

Step 4: Triton Removal and concentration:

The Triton X-100 was removed by passing the protein sample through Toyopearl Phenyl 600 M (HIC) column that was pre-equilibrated with 10 mM phosphate buffer + 1% sucrose, pH 7.4. 100 mM NaCl (final concentration) was added to eluted sample thus obtained. Inactivation was done using 0.02% formalin (final concentration) at 37°C for 24hr. The sample was then subjected to buffer exchange and concentration.

Example 5:

<u>This example gives the preparation of adjuvant to be used in the preparation of a vaccine formulation, according one aspect of the invention</u>

Step 1: Preparation of aqueous phase

Polysorbate 80 was dissolved in water for injection .To this solution sodium citrate & citric acid was added. This formed the aqueous phase.

Step 2: Preparation of oily phase

Squalene and sodium trioleate were dissolved to form an oily solution. This formed the oily phase.

Step 3: Preparation of oil in water emulsion

Oil phase was added to aqueous phase using high shear mixer at 10,000 rpm for 30-45 minutes .pH was adjusted at 6-7. This was then subjected to high pressure homogenization (HPH) at 100 bars. The pressure was later increased up to 1000-1100 bars using first stage pressure adjusting knob. The collected sample was added to the inlet funnel and the same process was repeated for a total of 06 times. After completion of process, the bulk was collected and filtered through 0.2 μm Polyethersulfone (PES) filter.

Example 6:

This example gives the preparation of vaccine formulation according to one aspect of the invention

Sodium chloride was added to water for injection and this was stirred till complete dissolution of the salt. To this solution, potassium chloride potassium dihydrogen phosphate, Sodium hydrogen phosphate dehydrate, and Magnesium chloride hexahydrate were added sequentially. Stirring was carried out after each addition to ensure complete dissolution. Sterilization of the above solution was carried out by passage through sterile 0.2 μ Polyethersulfone (PES) filter.To the above solution, sterile thiomersal solution was aseptically transferred, with complete mixing, followed by the aseptic addition of Adjuvant emulsion prepared in example 3, with complete mixing. To this solution, the active raw material prepared in example 2 was aseptically added. The pH of the solution is adjusted between 6.5 -7.5 with 0.1N NaOH/0.1N HCl solution. Finally water for injection was added to make up the volume, and the solution so formed was stored at 2-8^{0}C.

The composition of the formulation so formed is as follows:

Table 1

S. No.	Ingredients	Quantity per dose
1	Split influenza virus, inactivated, containing Reassortant virus strain X-179A	3.75/7.5 µg*
2	Emulsion Adjuvant	0.25 ml**
3	Sodium chloride	3.75 mg
4	Potassium chloride	0.1 mg
5	Potassium dihydrogen phosphate anhydrous	0.19 mg
6	Di Sodium hydrogen phosphate dihydrate	0.65 mg
7	Magnesium chloride hexahydrate	0.11 mg
8	Thiomersal	0.025 mg
9	Water for injection	q.s

* Haemagglutinin Antigen

** Equivalent to 9.75 mg of squalene content. Antigen and adjuvant are aseptically filled into same sterile vials and stored at 2-8°C.

Example 7:

Randomized, double blind, comparative, study of influenza A H1N1 vaccine, according to the invention, in human subjects.

Healthy individuals, males and females, aged 18 years and above, were immunized with a vaccine according to the present invention with an objective to evaluate and compare the immunogenicity, safety and any observed adverse events (commonly solicited 'local and systemic reactions'), etc. of each of the formulations used for the clinical study. Another objective of the study was to determine the optimal dose and the number of doses of the H1N1 vaccine, according to the invention, for further clinical studies based upon safety, tolerability and immunogenicity data.

0.5 ml of each of the vaccine, according to the invention was given intramuscularly, in the respective subjects, on Day 0 and Day 21.

The results of the study indicate that two doses of (15 µg without adjuvant, 7.5 µg with adjuvant and 3.75 µg with adjuvant) inactivated, split virion, egg based, monovalent pandemic influenza A (H1N1) vaccine were well tolerated, safe and immunogenic in adults.

7.1 Selection criteria:

Screening of subjects was done within 14 days prior to dosing. Healthy individuals, males and females, aged 18 years and above, were selected for the study. For female candidates, pregnancy was an exclusion criterion.

7.2 Study design:

Selected subjects were randomly divided into three groups. Each group had approximately 24 subjects. Subjects in each group received two doses of one of the three test vaccines (15 µg without adjuvant, 7.5 µg with adjuvant and 3.75 µg with adjuvant, according to the invention). Subjects were administered with the study vaccines on Day 0 & Day 21 and were followed up to Day 63. Blood samples were collected on Day 0 (Pre-dose), Day 21 (Predose; i.e., before the administration of the second dose), and Day 42 for immunogenicity parameters (HI antibodies). Blood and urine sampling for screening and safety analysis were done for all subjects at Day –14 (pre-immunization) to, day 0 (screening), Day 7, and Day 28. Urine pregnancy test at screening, Day 0 and Day 21 was done to rule out pregnancy. **7.3**

7.3 Method of administration of the vaccine:

First dose of the three test formulations were administered by deep intramuscular (IM) injection in the deltoid region of the arm. The second doses of vaccines were administered in the deltoid region of the contra lateral arm.

7.4 Formulations:

The formulations used for the study were- Inactivated, split virion, egg based, monovalent influenza A H1N1 (2009) vaccine of Panacea Biotec Ltd. Doses of the vaccine used in the study were 15 µg without adjuvant, 7.5 µg with adjuvant and 3.75 µg with adjuvant.

7.5 Statistical methods:

All p-values reported were based on two-sided tests and p-values < 0.05 were considered to be statistically significant using SAS version 9 or above.

All analysis was performed using SAS version 9.

Statistical Evaluation of Safety-

All adverse events reported were categorized into solicited (local and systemic) and unsolicited adverse events and were summarized by frequencies and percentages.

Summary statistics was computed for laboratory (hematology and biochemistry) and vital parameters during all visits.

Statistical evaluation of Immunogenicity-

Geometric Mean Titres (GMT) of all treatment Groups were calculated by taking the antilog exponential of the mean of log exponential transformed antibody titres for pre-dose (visit 1) and post vaccination (visit 3 and visit 5).

The ratio of GMT from baseline (visit 1) to post dose (visit 3 and visit 5) were calculated.

7.6 Results:

Safety Results-

The maximum incidence of local solicited reaction was pain (4.17% in 15 µg without adjuvant, 54.17% in 7.5 µg with adjuvant and 45.83% in 3.75 µg with adjuvant group). All local reactions were mild/moderate in intensity. The maximum incidence of solicited systemic adverse event reported in the study was headache (16.67% in 15 µg without adjuvant, 41.67% in 7.5 µg with adjuvant and 12.5% in 3.75 µg with adjuvant group). All unsolicited AEs were considered as mild/moderate except one (gastroenteritis with dehydration) which was severe in intensity. Ciprofloxacin was the most common concomitant medication used in the trial.

Immunogenicity Results-

Seroprotection

Pre-dose blood samples showed serum HI antibodies titers ≥1:40 for 20.83 % in 15 μg without adjuvant, 20.83% in 7.5 μg with adjuvant and 12.5% in 3.75 μg adjuvant groups.

Twenty one days after first dose, samples showed serum antibodies HI titre ≥1:40 for 50% in 15 μg without adjuvant, 75.00 % in 7.5 μg with adjuvant and 58.33% in 3.75 μg with adjuvant group. Twenty one days post second dose the proportion of subjects achieving serum HI antibody titres ≥1:40 increased to 91.67% in 3.75 μg with adjuvant and 83.33 % in 7.5 μg with adjuvant group. The second dose showed no effect on HI antibodies titers for 15 μg without adjuvant group. The percentage of subjects achieving seroprotection after second dose administration was more than 70% in both 3.75 μg with adjuvant and 7.5 μg with adjuvant groups.

Seroconversion

The proportion of subjects who achieved seroconversion 21 days after first dose were 31.82% in 15 μg without adjuvant, 54.17% in 7.5 μg with adjuvant and 37.5% in 3.75 μg with adjuvant group. Seroconversion rates 21 days after second dose increased to 75% in 3.75 μg with adjuvant and 62.5% in 7.5 μg with adjuvant group. There was no change in seroconversion after second dose in 15 μg without adjuvant group. The percentage of subjects achieving seroconversion after second dose administration was more than 40% in both 3.75 μg with adjuvant and 7.5 μg with adjuvant groups.

Geometric mean ratio (GMR) of HI antibody Geometric Mean Titres (GMTs post dose 1 and 2 were compared to baseline antibody titers) achieved cut of level of 2.5 in all the treatment groups at 21 days after first as well as second dose. These results indicated immunologically significant difference observed in pre-dose and post first and second dose GMT.

Table 2 below, gives the immunogenicity analysis of different dosages of pandemic influenza A (H1N1) vaccine according to the invention, as measured by HI assay (Immunogenicity Cohorts) [No. of subjects (%)]

	15 µg without Adjuvant (N = 24)	**7.5 µg with Adjuvant (N = 24)**	**3.75 µg with Adjuvant (N = 24)**
Day 0			
Total no. of subjects immunized	24	24	24
Seroprotection	5 (20.83%)	5 (20.83%)	3 (12.5%)
Seroconversion			
Day 21			
Total no. of subjects immunized	22	24	24
Seroprotection	11 (50%)	18 (75%)	14 (58.33%)
Seroconversion	7 (31.82%)	13 (54.17%)	9 (37.5%)
Day 42			
Total no. of subjects immunized	22	24	24
Seroprotection	11 (50%)	20 (83.33%)	22 (91.67%)
Seroconversion	7 (31.82%)	15 (62.5%)	18 (75%)

Seroprotection: titre value ≥40

Seroconversion: (0 ≤ baseline titre ≤ 9 and after dose titre value ≥ 40) or (baseline titre > 9 and after dose titre value ≥ 4* baseline titre value

Table 3 below gives the Geometric Mean Titre of different dosages of pandemic influenza A (H1N1) vaccine according to the invention, as measured by HI assay (Immunogenicity Cohorts) [No. of subjects (%)]

Table 3

	15 µg without Adjuvant (N = 24)	**7.5 µg with Adjuvant (N = 24)**	**3.75 µg with Adjuvant (N = 24)**
Day 0	12.2405	12.9684	9.4387
Day 21	31.0881	59.9323	32.6783
Day 42	30.1239	69.2429	59.9323

Table 4 below, gives the Geometric Mean Ratio of different dosages of pandemic influenza A (H1N1) vaccine according to the invention, as measured with HI assay (Immunogenicity Cohorts) [No. of subjects (%)]

Table 4

	15 µg without Adjuvant (N = 24)	**7.5 µg with Adjuvant (N = 24)**	**3.75 µg with Adjuvant (N = 24)**
Dose I	2.91896	4.62141	3.46215
Dose II	2.82843	5.33936	6.34960

7.7 Conclusion:

The results of this study indicate that two doses of (15 µg without adjuvant, 7.5 µg adjuvant and 3.75 µg with adjuvant) inactivated, split virion, egg based, monovalent pandemic influenza A (H1N1) vaccine were well tolerated, safe and immunogenic in adults. The vaccine of the invention was most immunogenic at low dose (3.75 ug) with adjuvant.

Claims:

1. A process for purification of influenza virus from allantoic fluid, comprising a step of chromatography using strong anion exchanger quaternary ammonia bound to regenerated cellulose membrane.
2. The process as claimed in claim 1, wherein the process comprises the use of Sartobind Q single step chromatography.
3. The process as claimed in claim 1, wherein the allantoic fluid is subjected to inactivation by an inactivating agent, prior to the step of chromatography.
4. The process as claimed in claim 1, wherein the process comprises inactivation step by an inactivating agent, after the step of chromatography.
5. The process as claimed in claim 3 and 4, wherein the inactivating agent is formalin.
6. The process as claimed in claim 5, wherein the concentration of formalin used is 0.005 – 0.1%.
7. The process as claimed in claim 6, wherein the concentration of formalin used is 0.01-0.05%.
8. The process as claimed in claim 7, wherein the concentration of formalin used is 0.02%.
9. The process as claimed in claim 1, wherein the conductivity of the allantoic fluid used for chromatography is not more than 22 mS/ cm.
10. A process according to claim 1, wherein the virus preparation is further treated with a splitting agent by passing through hydrophobic interaction chromatography column.
11. The process as claimed in claim 10, wherein the splitting agent is Triton X- 100.
12. The process as claimed in claim 11, wherein the concentration of Triton X- 100 used is 0.1- 1.0%.
13. The process as claimed in claim 12, wherein the concentration of Triton X- 100 used is 0.5%.
14. The process as claimed in claim 10, wherein the virus preparation to be treated with splitting agent has a conductivity not more than about 3 mS/ cm
15. The process as used in claim 10, wherein the hydrophobic interaction chromatography column used is the Toyopearl Phenyl 600 M (HIC) column.
16. A process for purification of influenza virus comprising the steps of
    a. propagating candidate influenza virus strains, in embryonated hens' eggs
    b. providing a harvested mixture of cultured influenza virus in allantoic fluid

c. optionally adding a salt to the allantoic fluid
d. optionally carrying out one or more clarification steps or filtration steps to separate whole virus from non-virus material
e. optionally carrying out one or more concentration steps
f. carrying out column chromatography using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane.
g. splitting of the whole virus using a suitable splitting agent
h. carrying out hydrophobic interaction chromatography
i. optionally carrying out one or more further purification and/or concentration steps

17. The process as claimed in claims 1 and 16, for purification of influenza virus, to provide highly pure viral antigen, for use in the preparation of a vaccine formulation for inducing immunological response in a subject, by administering immunologically active amount of the antigen.

18. A process for preparing influenza virus vaccine formulation comprising the steps of

a. propagating candidate influenza virus strains, in embryonated hens' eggs,
b. providing a harvested mixture of cultured influenza virus in allantoic fluid,
c. optionally adding a salt to the allantoic fluid,
d. optionally carrying out one or more clarification steps or filtration steps to separate whole virus from non-virus material,
e. optionally carrying out one or more concentration steps,
f. carrying out column chromatography using strong anion exchanger having quaternary ammonia bound to regenerated cellulose membrane,
g. splitting of the whole virus using a suitable splitting agent,
h. carrying out hydrophobic interaction chromatography,
i. optionally carrying out one or more further purification and /or concentration steps,
j. providing a pure fully liquid low dose antigenic component,
k. providing a fully liquid oil in water emulsion component,
l. optionally providing other carriers, excipients, adjuvants and diluents

wherein, the components of steps j) to l) are mixed during manufacture and formulated in a single unit.

19. The process as claimed in claim 18, for purification of influenza virus, to provide highly pure viral antigen, for use in the preparation of a vaccine formulation for inducing immunological response in a subject, by administering immunologically active amount of the antigen.

20. A fully liquid influenza vaccine formulation comprising

a. one or more highly pure, low dose split influenza virus antigen component(s)
b. an emulsion comprising squalene
c. Sodium chloride
d. Potassium chloride
e. Potassium dihydrogen phosphate
f. Disodium hydrogen phosphate
g. Magnesium chloride
h. Thiomersal

wherein, the dose of haemagglutinin (HA) is less than 8 micrograms or less than 4 micrograms and wherein components a) to h) are mixed during manufacture and formulated in a single unit.

21. The vaccine formulation as claimed in claim 20, for inducing immunological response in a subject, by administering immunologically active amount of the antigen.

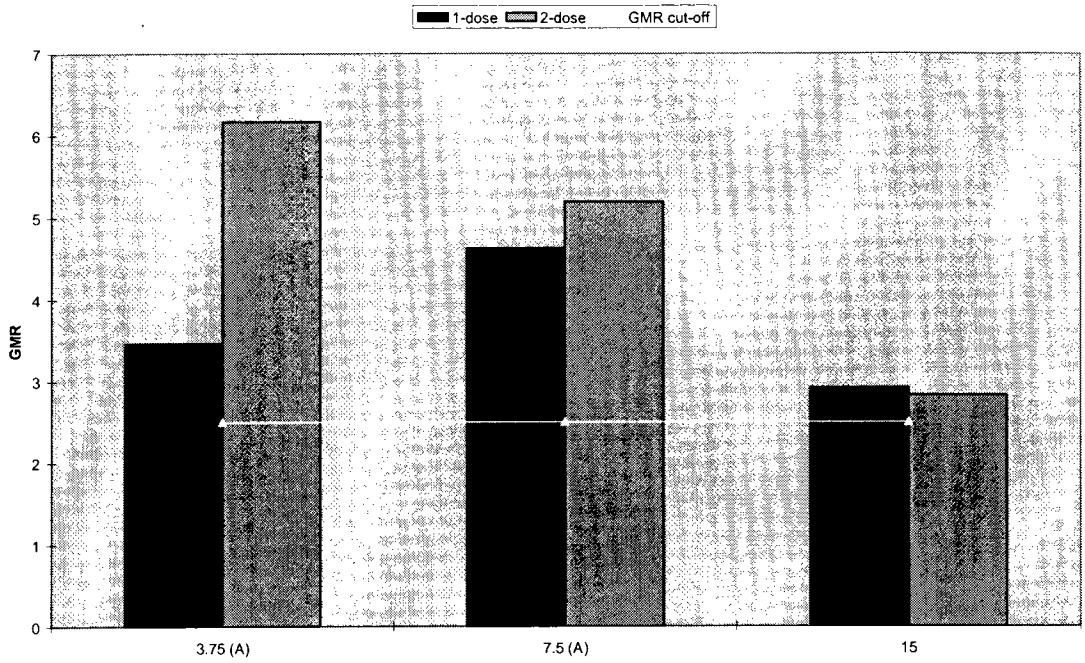

Figure 1