Vaccine 27 (2009) 4161–4168

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Sialivac: An intranasal homologous inactivated split virus vaccine containing bacterial sialidase for the control of avian influenza in poultry

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ARTICLE INFO

Article history:
Received 27 February 2009
Received in revised form 17 April 2009
Accepted 20 April 2009
Available online 9 May 2009

Keywords:
Intranasal vaccine
Chitosan
Sialidase
Homologous split virus
HP H5N1

ABSTRACT

A simple, effective inactivated avian flu vaccine composed of three homologous highly pathogenic (HP) H5N1 strains combined with *Clostridium perfringens type A 107* sialidase/neuramindase and chitosan as a trans epithelial carrier adjuvant applied intranasally to poultry is described. Poultry were vaccinated with an inactivated, solvent split, chitosan adjuvanted intranasal (IN) vaccine with and without *C. perfringens* sialidase and the resulting serum IgG antibody measured by haemagglutination inhibition (HI) and mucosal IgA by ELISA. The clinical effectiveness was demonstrated by disease intervention field trials, where the ability of an intranasal vaccine containing three homologous inactivated solvent split HP H5N1 strains, *C. perfringens* sialidase and chitosan was successful in controlling the disease in intensively reared commercial chickens. Evidence is presented by demonstrating effective intervention with IN vaccine during outbreaks in poultry previously vaccinated with commercial heterologous H5N2 intramuscular (IM) vaccine and reassorted H5N1 Re-1 vaccine which had failed to protect intensively reared birds. Intervention with the IN vaccine in such flocks completely halted the infection within 2–5 days. Survivors ceased to excrete live virus. Stimulation of the common mucosal immune system (CMIS) and the early production of secretory IgA and subsequently humoral IgG demonstrated by laboratory controlled experiments and field studies revealed the ability of intranasally vaccinated birds to resist lethal virus challenge. A strategy of mucosal immunisation is recommended to reduce the incidence of disease in intensively reared poultry and thus minimise the generation and transfer of mutated highly pathogenic subtypes to humans and other animals.

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1. Introduction

More than 10 years have elapsed since the first appearance of avian influenza H5N1. The subtype highly pathogenic avian influenza type A (HPAI) has proven notoriously difficult to control in endemic regions of SE Asia, where the density of people poultry and pigs provide the ideal conditions for virus proliferation and mutation. The socio-economic toll is huge, for every human infected a disproportionate and intolerable number of animals are dying from this disease. Attempts to control it with heterologous vaccines are not working. It is now recognised that the intensification of poultry production has created a "Trojan Horse" where low pathogenic strains of virus circulating in water fowl for millennia as subclinical commensals without serious mortality, have been presented with the opportunity to jump into terrestrial avian species such as poultry favouring the evolution of high pathogenic subtypes capable of causing high mortality in poultry, and mammalian species including man [1]. Despite the danger presented by the continued production of poultry by intensive means, the demand for a cheap source of animal protein encourages intensive methods of production. Current national control measures are compromised by the use of heterologous vaccines of low efficacy which are failing to completely control the disease in endemic regions.

A preventive strategy by mass vaccination with a bacterial sialidase potentiated, inactivated homologous vaccine, which can block the entry of the virus at the vulnerable surface epithelia and give time to allow a cascade of immune responses to support cross-reactive heterosubtype (HetI) immunity is a viable option for the immunisation of poultry [2–4]. Influenza vaccines in present use are effective at producing IgG, evident in the high HI titres however they are poor at stimulating mucosal secretory IgA. Trans epithelial vaccines produce early mucosal secretory IgA and exhibit both HetI and substantial immunological memory [5,6] which can protect against mutant subtypes which have drifted antigenically from vaccine strains in current use. Using a trans epithelial immunisation strategy, a method is described in which the inactivated main antigenic components of the H5N1 virion haemagglutinin, neuraminidase, and the solvent split virus contents including the

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doi:10.1016/j.vaccine.2009.04.058

M2 ion channel proteins, are combined with a bacterial sialidase prepared from *Clostridium perfringens type A 107*, adsorbed onto chitosan producing a polyvalent antigenic cocktail with adjuvant properties for presentation as an intranasal vaccine for the immunisation of poultry.

Many respiratory and intestinal pathogens gain entry via the mucosal epithelial tissues. Most produce sialidase examples include: *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Streptococcus pneumonia*, *Vibrio cholera*, *Salmonella typhi* [7], *C. perfringens* [8], and avian *orthomyxoviruses*. Sialidases have been identified as enzymes that selectively remove sialic acid from sialated membrane glycolipids present on the surfaces of animal tissues. In the case of influenza the mode of action within the influenza infected host is the recognition of host cell receptors enabling virus attachment to the specific α 2–3 and α 2–6 receptor sites on the mucous epithelial cells and at a later stage in the infection, enabling the detachment of newly formed virus from infected cells facilitating the invasion of fresh tissue.

It was reported in 1947 by McCrea [9] that *C. perfringens* culture filtrates contained an enzyme that destroyed erythrocyte receptor sites (receptor destroying enzyme RDE) which inhibited the agglutination of red cells by *orthomyxoviruses*. Invasion by infectious agents through mucosal surfaces can be prevented by immunological stimulation of the common mucosal immune system (CMIS). Stimulation of the CMIS can be triggered by the presentation of inactivated pathogen associated antigens combined with a mucosal adjuvant to the nasopharyngeal associated lymphoreticular tissue (NALT).

We demonstrate that a bacterial sialidase included in the vaccine composition assisted the early production of secretory IgA which can effectively block entry of the avian flu virus. In addition to the initial active blocking of virus by IgA, the bacterial sialidase possibly competes for host cell receptors and the adjuvant chitosan enables the intra- and para-cellular transport of the virus vaccine antigens across and into the mucosal epithelial linings stimulating a delayed systemic immune response a combination which appears to assist in clearance of HP H5N1 virus.

Other workers [10] have shown that dendritic cell (DC) replication occurs and amplification results with antigen being transferred to progeny DCs. This action stimulates the production of T lymphocytes, and B lymphocytes with subsequent presentation of the antigens to dendritic cells in the underlying epithelium. Once activated by antigens the DCs migrate to the lymphoid tissues where they interact with T cells and B cells.

It was observed during field experiments that birds in a flock which were vaccinated with intranasal *Sialivac* vaccine at 9 days of age survived a natural accidental challenge 6 months later by HPAI whereas the remainder of the flock which had not received *Sialivac* died, this appears to confirm immunological memory.

Similar incidents illustrate the high degree of efficacy of this vaccine and the conclusion that not only prophylactic but also therapeutic capability is being expressed.

It has been reported that the poor human to human transmission of type A HP H5N1 has been attributed to the fact that there are few α 2–3 receptors in the human upper respiratory tract, however recent reports in Nature Medicine by Nicholls et al. [11], showed that human nasopharyngeal, adenoid and tonsillar tissues can be infected with H5N1 despite the apparent lack of these receptors in these tissues [12].

Control strategies using antiviral drug therapy is expensive and inappropriate for use in poultry. Uncontrolled use of earlier drugs rimantadine and amantadine has produced 90% drug resistance [13–17]. There is an important evidence [18] that the neuraminidase activity of *P. aeruginosa* (PA) can be blocked by the action of Peramivir and Oseltamivir two of the neuraminidase inhibiting drugs used in the treatment of influenza. It was shown by these workers that bacterial and viral neuramindases share the same amino acid positions (ASPs), which interact with sialic acid. They further observed that *V. cholera* neuraminidase was also blocked by Oseltamivir. This suggests a common affinity in the active sites of both *P. aeruginosa* and *V. cholera* and possibly indicates an evolutionary conserved feature shared by *C. perfringens* and by many other pathogens whose mode of entry is via the mucosal epithelium.

This knowledge indicated a possible way of preventing the access of the *Orthomyxoviridae* family to the mucosal upper respiratory tract receptors in poultry by receptor site competition by occupation with *C. perfringens* sialidase.

Pinto and Lamb [19] describe the M2 ion channel protein and its function in clinical influenza. This proton pump is vital for virus replication thus neutralisation of this channel by specific antibody could disable its function. The M2 ion channel protein is a more stable target which is less prone to mutational changes than H and N antigens. The third feature of the *Sialivac* vaccine is the release and exposure by splitting the virus envelope with chloroform and the incorporation of the split virus contents including the M2 proton channel protein in the vaccine and its conjugation to the chitosan adjuvant together with the H and N antigens for delivery by the intranasal route. It was shown [20] that a formalinised inactivated whole cell culture vaccine of *P. aeruginosa* is non-toxic and antigenic when applied to mucosal epithelia by the oral route, producing IgG, IgM and IgA with the highest levels being IgA. Although the influence of neuraminidase was not specifically investigated serum samples from the vaccinated subjects enabled opsonophagocytosis of live *P. aeroginosa* by a human macrophage cell line indicating stimulation of the innate and acquired immune response and possible stimulation of the microRNA (miRNA) regulation of immunity [21].

The neuraminidase inhibitors Oseltamivir and Zanamivir were developed using crystal structures of N9 and N2 from the B genus of the influenza viruses [22]. The significance of their action was that these compounds recognised the three-dimensional structure on the enzyme site and that it was the same for all of these subtypes and that the inhibitors bind to the active sites in the same way. They were also found to have a similar activity against N1 neuraminidase and are effective against flu viruses that contain an N1 neuraminidase [23].

As described in this work a more effective form of control was investigated which might prevent access of the virus to host cell receptors on the sero mucosal epithelium by competitive degrading of these sites by a bacterial sialidase and the mopping up of virus by early IgA and possibly IgM thus reducing localised viral load. Ineffectiveness of heterologous vaccines is frequently revealed by increased mortality in both laying birds and broilers with deaths appearing within the first 14 days and gradually increasing to 20–30% at 4–5 weeks culminating in mortalities in excess of 90%. Following close scrutiny of a number of diseased field flocks it appeared that the viruses were possibly mutating under vaccine and management practices into more resistant subtypes (Fig. 1).

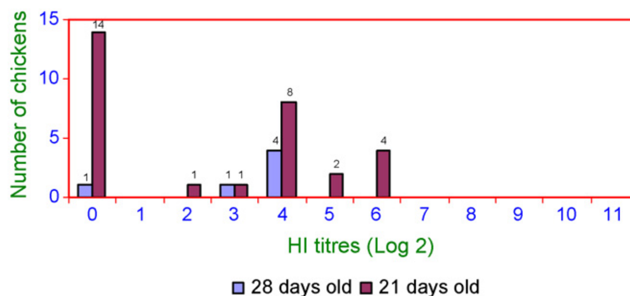

Fig. 1. HI test on serum samples taken from layer chickens at 21 days and 28 days of age which were vaccinated with *Sialivac* at 12 days of age.

This situation has been reported on numerous occasions in endemic regions where control with heterologous vaccines was the norm. This might also indicate batch-to-batch variation in the vaccine potency. The authors lacked the expertise and facilities to identify unequivocally the precise and detailed nature of this immunological event except to report that after application of the *Sialivac* intranasal vaccine, full recovery proved to be a constant and repeatable result.

2. Materials and methods

2.1. Observation and selection of three field HPAI virus isolates from disease outbreaks

Disease outbreaks in large commercial flocks were reported and strains of high pathogenic virus were isolated and positively indentified as HP H5N1. Three virus isolates originating from three separate regions of West Java indentified as HPAI by (Anigen Rapid H5 Ag test) which differed sufficiently in their HI titres and clinical pathology to suggest subtypical differences. All the birds in these outbreaks had received parenteral heterologous vaccine which had failed to control the disease. The viruses were propagated in 10-day-old embryonated eggs and the haemagglutination titre of the harvested allantoic fluid estimated by the HA test using chicken RBCs. HA antigen from three virus isolates was used in haemagglutination inhibition (HI) tests [25] and tested against serum taken from vaccinated chickens from the affected areas. Equal volumes from each strain were mixed to form a common pool. The vaccine was potentiated by the incorporation of a bacterial sialidase from a strain of *C. perfringens Type A 107*. The potency of the sialidase was estimated by measuring the activity of the receptor destroying enzyme RDE using chicken RBCs and 4HA units of virus haemagglutinin in a modified HI test where the resulting titre was expressed as the reciprocal of the highest dilution blocking the haemagglutination of the RBCs. A suitable concentration, found by experiment was added to the vaccine to stimulate the hetero subtypical immunity (HetI). It is interesting to note that the formalin inactivated sialidase had the same RDE titre as the untreated raw material. The *Sialivac* vaccine as described previously was applied to the birds by intranasal drop of 30 μl to one nostril. Immunologically naïve chicks destined for broilers were vaccinated once at 12 days old. Poultry destined to become laying birds were given an additional booster dose of 0.03 ml IN at 5 weeks of age.

2.2. Virus isolation and virus identification and confirmation

Heads were removed from dead chickens which had been identified as HPAI positive by rapid test (“ANIGEN” Rapid H5 AIV Ag Test Kit, subtype H5 Animal Genetics, Inc. 404-5, Woncheon-dong, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea). Isolation of influenza viruses was done using embryonated eggs according to the procedure (WHO Manual on AI Diagnosis and Surveillance) [25].

The virus isolates were obtained from dead birds originating from the following: strain 1 from a breeding chicken farm, strains 2 and 3 from commercial layer farms. Confirmation of virus identification by PCR and gene sequencing was done at the Tropical Diseases Laboratory East Java, Indonesia. Homogeneity tests confirmed all three isolates to be HP H5N1 in reference to A/Goose/Guangdong/1/96. The viruses were purified [24] and stored at 4 °C for 24 h to await purity and identity checks, thereafter stored at −20 °C.

2.3. Virus master seed and virus working seed

Before storage, samples were tested for sterility by standard approved methods (OIE Manual of Standards for Diagnostic Tests and Vaccines). The confirmed virus allantoic fluid was mixed 1:1 with an excipient composed of aqueous 10% (w/v) sucrose and 5% (w/v) lactalbumin hydrolysate, and stored in liquid nitrogen at −196 °C.

The working seed was adjusted to a titre of 128 HA units/25 μl in the same excipient as the master seed, sterility tested (OIE Manual of Standards for Diagnostic tests and Vaccines) and stored at −196 °C. The identity and origin of all virus seed was recorded and documented as strains F1, F2, and F3.

2.4. Preparation and purification of C. perfringens sialidase/neuraminidase

In order to avoid genetic drift and deterioration in sialase/neuraminidase production by repeated subculture of stock strains, master and working seed banks were prepared. Samples from a production batch of centrifuged cells were resuspended in an equal volume of an aqueous excipient containing 10% (w/v) sucrose and 5% (w/v) lactalbumin hydrolysate aliquots of 0.2 ml were placed in glass tubes, freeze-dried and sealed under vacuum. The master seed was stored at −196 °C in liquid nitrogen and a sample tested every 12 months to check for viability and neuraminidase production. The working seed bank was stored under the same conditions containing a greater number of vials or ampoules sufficient to prepare a starter inoculum for each production batch. One ampoule was checked for viability and sialidase activity every 6 months.

The medium for the cultivation of *C. perfringens* type A consisted of peptone 2%, lactalbumin hydrolysate 1%, yeast extract 0.5%, NaCl 1.0%, pH 7.4, autoclaved at 121 °C for 30 min. Glucose was added from a 50% (w/v) sterile aqueous solution to give 1.0% in final medium. The medium was cooled rapidly to 37 °C to maintain reducing conditions.

The main batch consisting of 4 l of the above medium was eventually inoculated by a progressive multiplication of bacteria at 37 °C commencing with the reconstituted freeze-dried working seed into Robertson’s Cooked Meat culture in test tubes culminating in 400 ml of a 3 h starter in the logarithmic phase into the final growth medium where growth was allowed to continue for 3.5 h with the pH maintained at 7.0 by the addition of 5N NaOH. The final culture was cooled and centrifuged to remove cells. 0.6% (v/v) formalin was added to the supernatant and incubated for 18 h at 37 °C. The purity was checked and stored at 4 °C to await estimation of sialidase receptor destroying enzyme (RDE).

Batches of *C. perfringens* toxoid were concentrated by salting out with $(NH_4)_2SO_4$ as described below. It was found that the increase in purity by fractionation with ammonium sulphate did not increase the concentration of sialidase by a significant amount, this was abandoned as the extra cost and time compromised the aim to keep the vaccine as cheap as possible.

Ammonium sulphate 50% (w/v) was added to the toxoid. The precipitate was discarded and retained the supernatant. Fully saturated by adding a further 35% (w/v) ammonium sulphate and allowed to stand for 12 h. The resulting brown solid which floated on the surface was skimmed off and dialysed against distilled water. The dialysate was concentrated further by dialysis using PEG MW 20,000 and stored at −20 °C.

The sialidase potency test was a modification of the HI test, and was carried out to determine the titre of the RDE (sialidase) in the *C. perfringens* toxoid. This was done by mixing twofold serial dilutions of the toxoid with 1% washed chicken RBCs allowing an adsorption time of 1.5 h at 28 °C. Following adsorption, 4HA units of formalin inactivated virus haemagglutinin was added to each well and the result was read after 3 h at 28 °C. The degree of inhibition of agglutination was recorded as the reciprocal of highest dilution of toxoid which inhibited agglutination. A titre of 1024 RDE units/25 μl

(40,960 U/ml) was normally obtained. It is interesting to note that the RDE titre of the formal toxoid sialidase was exactly the same as the non-treated parent enzyme.

2.5. Preparation of the stock chitosan solution

Batches of chitosan from numerous sources were tested. Although the pharmaceutical grades of chitosan chloride were effective it was decided that the cost was too high making the vaccine too expensive for rural farmers. A commercial grade of chitosan was selected which was cheap and functional (Biotech Surindo). Preparations from many batches were tested and a 0.5% (w/v) solution of 85% deacetylated chitosan dissolved in 1.0% (v/v) aqueous glacial acetic acid, sodium actetate buffer pH 5.0 sterilised by autoclaving at 121 °C for 20 min proved suitable free from any observed clinically adverse reactions following intranasal and parenteral administration in poultry of all ages.

2.6. Intranasal inactivated split virus vaccine (Sialivac) preparation

The F1, F2 and F3, highly pathogenic HP H5N1 homologous strains were propagated in 11-day-old specific pathogen-free (SPF) embryonated eggs. Using a working seed virus containing 128 HA units/25 μl from each of the three isolates. The eggs were inoculated with 0.2 ml of a 1/1000 dilution in phosphate buffered saline pH 7.4 (PBS) + Kanamycin 20 mg/ml incubated for 72 h and observed for the dead embryos. The dead embryos were chilled at 4 °C and the allantoic fluid harvested and clarified by centrifugation at 3000 rpm and tested for potency by the HA test. Equal volumes of the three viruses were pooled and a sample tested for HA titre.

The virus pool was inactivated by the addition of 0.1% formalin incubated at 37 °C for 18 h, split with 0.5% (v/v) chloroform for 18 h with constant stirring at 4 °C, the residual chloroform removed under a vacuum of 100 mbar for 2 h at 28 °C and retested by HA (512–1024 HA units being normal). Loss of virulence was determined by inoculating 11-day-old embryonated eggs with 0.2 ml of the split inactivated virus suspension. All embryos were alive after 5 days incubation at 37 °C. The virus fluid was further clarified by filtration, to remove accumulated debris from the chloroform treatment and stored at −20 °C awaiting conjugation with chitosan. Commercial grade 85% deacetylated chitosan of crab origin was prepared as a 0.5% (w/v) solution in 1% (v/v) pH 5.0 aqueous acetic acid, sodium acetate buffer and sterilised by autoclaving at 121 °C for 20 min in a tightly sealed bottle.

To every 100,000 HA units of inactivated virus were added 3.0 ml (122,880 RDE units) of *C. perfringens* sialidase made up to a total volume of 30 ml with 0.5% (w/v) of pH 5.0 acetic acid/acetate buffered sterile chitosan. Inactivated virus and sialidase were mixed and then added to the 0.5% (w/v) chitosan and stirred for 1 h at 4 °C.

The final vaccine composed as above was aliquoted in 30 ml volumes in a sterile 30 ml polypropylene bottle incorporating a dropper nozzle delivering 0.03 ml per drop. A final sterility and freedom from abnormal toxicity test were carried out before use.

2.7. Haemagglutination inhibition test

All measurement of antibody titres in vaccinated chickens was done by following WHO Manual on Avian Influenza Diagnosis and Surveillance [25].

2.8. Humoral IgG response of Sialivac vaccinated birds as measured by HI test

HI tests were done on serum samples taken from layer chickens at increasing ages which were vaccinated solely with *Sialivac* at 12 days of age. This was undertaken to reveal how soon the humoral IgG response appeared in birds vaccinated at an early age.

Thirty birds were vaccinated at 12 days of age with 0.03 ml of *Sialivac* vaccine intranasally into one nostril.

At 21 days and 28 days of age serum samples were taken from all birds. The recorded HI titres are shown in Fig. 1.

2.9. Mucosal IgA response

2.9.1. Chicken IgA response using plain vaccine without bacterial sialidase

The IgA serological response was measured in a group of chickens which had been vaccinated intranasally at 12 days old with 0.03 ml chitosan adjuvanted inactivated homologous vaccine prepared in embryonated eggs. The plain vaccine dose consisted of 100 HA units of inactivated virus in a solution of 0.4% (w/v) 85% deacetylated commercial chitosan in pH 5.0 acetic acid/actetate buffer.

Thirty 9 weeks old layer chickens were selected from the group which had previously been vaccinated at day 12. A second vaccination with the same vaccine was carried out at 9 weeks of age at which time the first tracheal swabs were taken. The chickens were divided into 6 groups consisting of 5 chickens per group. Sampling by tracheal swabs was carried out weekly as follows: pre-vaccination control samples taken designated group I. Samples from groups II, III, IV, V and VI were taken at 1, 2, 3, 4 and 5 weeks post-vaccination, respectively.

ELISA for measuring IgA in the samples was done by using ELISA microplates Maxisorp (Nunc), U bottom 96 wells. ELISA was done by using inactivated AI (H5N1) split virus: 1/200 of 4HA, diluted in carbonate buffer pH 9.6 as the coating antigen, stored at 4 °C overnight. Blocking was carried out using TEN (Tris Base, EDTA, NaCl) + 0.2% casein overnight. Tracheal swab samples were diluted in 300 μl PBS, shaken for 1 h at room temperature. Conjugate used was 1:1500 (Affinity purified HRP conjugated goat anti-chicken IgA–HRP (Bethyl Lab, Inc. www.bethyl.com) and ABTS was used as substrate. The results were read using ELISA reader (Titertek EX) at 415 nm.

2.9.2. Chicken IgA response using intranasal solvent split virus vaccine (Sialivac)

Another group of 20 20-week-old birds originating from SPF stock were immunised intranasally as in Section 2.9.1 with a single 0.03 ml dose of chloroform split virus vaccine prepared from the same base components as for experiment in Section 2.9.1. This vaccine contained in each 0.03 ml dose 100 split virus HA units, 122 *C. perfringens* RDE units suspended in 0.5% (w/v) chitosan pH 5.0 acetic acid/sodium acetate buffer. The chickens were divided into six groups, each consisting of 4 chickens per group. Tracheal mucosal swab samples were taken at weekly intervals post-vaccination. The contents of each swab was eluted in 0.4 ml PBS + antibiotic (Kanamycin 10 mg/ml) and stored at 4 °C.

Pre-vaccination control samples designated group I. Samples from groups II, III, IV, V and VI were taken at 1, 2, 3, 4 and 5 weeks post-vaccination, respectively. The ELISA test was performed according to the previous test procedure (Section 2.9.1) for the detection of avian IgA.

The resulting titres were expressed as the mean values of each group. The results from both groups were superimposed, Fig. 2, to enable a clearer comparison.

2.10. Intervention with an inactivated homologous HP H5N1 intranasal vaccine (Sialivac) during field outbreaks of HP H5N1 AI

Visits were made to four commercial breeding farms in West Java which had reported and identified HP H5N1 outbreaks of avian

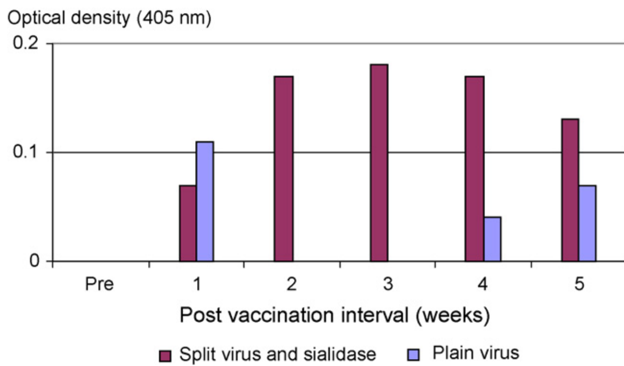

Fig. 2. Detection of mucosal IgA response of chickens vaccinated intranasally with plain inactivated AI vaccine and split virus + sialidase (*Sialivac*) by ELISA.

influenza. In all cases mortality had reached alarming levels and characterised by rapid spread, which from previous experience led to 100% mortality. All the birds in every farm were vaccinated intranasally with 0.03 ml using the vaccine formulation described in Section 2.6 *Sialivac* preparation.

2.11. Serological responses in broiler breeder chickens vaccinated with plain intranasal and without plain intranasal vaccine

Two groups of broiler breeder chickens each consisting of 5000 birds located in two geographically separate farms under the same ownership and using the same management practices were vaccinated as follows.

Farm A: Birds on this farm were vaccinated intranasally with 0.03 ml of the plain vaccine into one nostril at 12 days of age followed by further intramuscular oil adjuvant Re-1 H5N1 reassorted vaccine at weeks 5, 14, and 20. Twenty random blood samples were taken at intervals indicated in Fig. 3 and the HI titre represented as the mean of the individual titres.

Farm B: Birds on this farm did not receive the plain intranasal vaccine but were vaccinated intramuscularly with oil adjuvant Re-1 H5N1 reassorted vaccine at weeks 5, 14, and 20. Twenty random blood samples were taken at the intervals indicated in Fig. 3 and the HI titre represented as the mean of the individual titres.

2.12. Control of infection by intervention with intranasal Sialivac vaccine

This was a field experiment to determine the efficacy of *Sialivac* vaccine by the addition of *Clostridial* sialidase and split virus as described in Section 2.6, in controlling a disease outbreak.

In a flock of 6700 laying birds (breed Hi sex) being a separate part of a total flock of 60,000 birds, abnormal mortality of chickens started at 28 weeks of age on April 16th, 2008 when 17 chickens were found dead. This was confirmed to be HP H5N1 by rapid diagnostic test. Egg production started to drop as mortality increased. On April 22nd, 2008 mortality peaked and 611 chickens died. At this point chickens were vaccinated with 0.03 ml inactivated *Sialivac* intranasal AI vaccine. Five days after vaccination, on April 27th, 2008, high mortality ceased with only three recorded deaths. Egg production increased and the haemagglutination inhibition titre of serum increased to a peak 20 days post-vaccination. To prevent accidental spread to the main flock of 60,000 birds all were vaccinated with the intranasal *Sialivac* vaccine.

2.13. Possible genetic drift observed in virus antigen of three field isolates

Serum samples obtained from a farm where the birds had been vaccinated with intramuscular oil adjuvant Re-1 reassorted vaccine in the years 2003–2005. Each year the farm had suffered serious losses from HPAI during these years and where the AI vaccine in use Re-1 appeared to be losing efficacy. Serum samples taken from birds in each year were tested for haemagglutinating antibody against the HA from each respective years strains in order to gain some idea if this was the appearance of subtypical mutation or was a possible reflection of batch to batch variation in vaccine potency.

2.14. Plain intranasal vaccine

The formulation of the plain vaccine which differs from the *Sialivac* vaccine was composed as described and used in experiment in Sections 2.9.1 and 2.11 does not contain split virus or *Clostridial* sialidase.

3. Results

3.1. Serological results of chickens vaccinated with Re-1 vaccines detected by HI against three HPAI virus isolates

The results displayed indicate that HI antibodies produced by Re-1 vaccine induced a protective level of antibody against strains isolated in 2003 and 2004 (titres 6.22 and 6.11, respectively) but produced insufficient protective antibody against the 2007 virus isolate. Re-1 vaccine was developed by reverse genetic re-assortment using wild type H5N1 virus isolated from goose in Guangdong, China in 1996 as HA and NA donors to PR8 virus. This observation might indicate mutational changes in the virus and that AI vaccination by the parenteral route using RE-1 and pos-

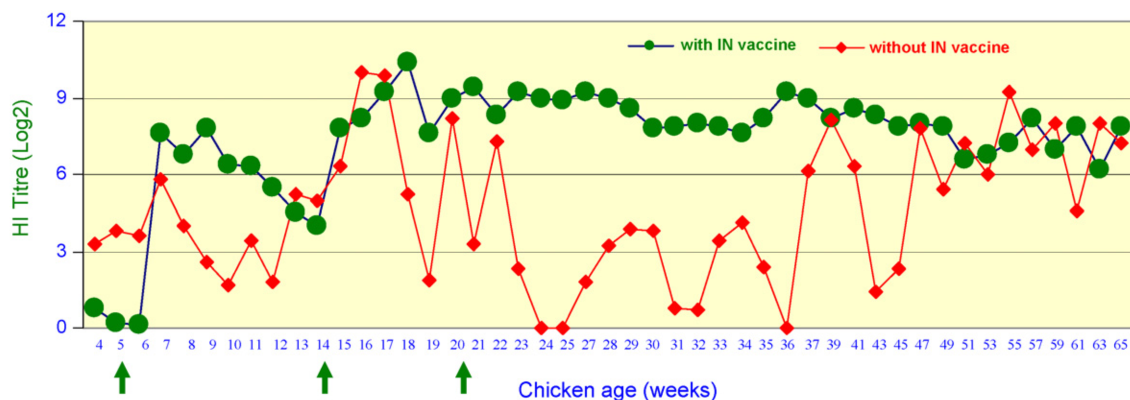

Fig. 3. The HI serological profiles of chickens with and without intranasal vaccination.

Table 1
Serological results of chickens vaccinated with Re-1 vaccines detected by HI against different AI virus isolates.

AI virus isolates/antigen	HI titres	
	Mean	% Coeff. of variation
2003	6.22	29.8
2004	6.11	32.15
2007	3.09	67.0

sibly other heterologous vaccines whilst stimulating the humoral immune system are not sufficient to establish complete protection from mutational changes in avian influenza subtypes encountered in the field. This observation could be supported by HA and NA gene sequences of each virus isolate, it might also reflect changes or reduced efficacy in the vaccine composition (Table 1).

This observation supported by more recent evidence of apparent vaccine breakdown or presumed subtype changes encouraged the authors to seek a better method of control by actively blocking virus entry which ultimately led to the development of the *Sialivac* intranasal vaccine.

3.2. Humoral IgG response of Sialivac vaccinated birds as measured by HI test

The results indicate the humoral IgG antibody response as measured by the HI test. The antibody titres of the sero converted birds are within the accepted limits for what is considered to be a protective level of antibody by the OIE (4 log 2). Birds vaccinated at 12 days of age with intranasal vaccine produced as seen in Fig. 2 produced early IgA, the humoral response noted here was delayed and full seroconversion was not seen until around 10 weeks post-initial vaccination. Although 100% IgG sero conversion was not achieved after one vaccination a second vaccination at 5 weeks stimulated the anamnestic response with greater levels of HI antibody produced.

3.3. Detection of mucosal IgA response of chickens vaccinated intranasally with plain inactivated AI vaccine and split virus + sialidase (Sialivac) by ELISA

The immune response to a single 30 µl dose of *Sialivac* in this experiment produced higher levels of IgA than was observed in the double dose plain vaccine which was without split virus and sialidase. The immune response observed by the raised levels of mucosal IgA as measured by ELISA supports the evidence that the same vaccine applied intranasally to both immunologically naïve and diseased poultry provides a recognised level of protection from naturally acquired infection which is borne out by the observed results of field trials (Table 2 and Fig. 4). No explanation can be given for the apparent disappearance of IgA in weeks 2 and 3 and reappearance in weeks 4 and 5 for the plain virus vaccine other than it lacked the potentiating effect of the sialidase split virus formulation.

3.4. Intervention with an inactivated homologous HP H5N1 intranasal vaccine (Sialivac) during field outbreaks of HP H5N1 AI

Birds in every farm with the exception of farm 1 were vaccinated with heterologous intramuscular inactivated AI vaccines, which were failing to control the outbreak.

The results show that during existing avian influenza outbreaks intervention with *Sialivac* vaccine applied intranasally can dramatically influence the course of the disease in poultry of all ages housed in intensive breeding systems. It can be seen from the above interventions that the sole use of intranasal *Sialivac* vaccine in farms 1 and 4 effectively controlled the disease within 3–5 days, whereas in

Table 2
Influence of *Sialivac* during field outbreaks of HP H5N1 avian influenza.

	Farm 1	Farm 2	Farm 3	Farm 4
Age of birds	20 days	>5 months	6 months	<4 months
AI vaccination	Nil	H5N1, Re-1/IM, twice (5 and 15 weeks)	H5N2/IM twice (6 and 16 weeks)	H5N2/IM at 5 weeks
Approx. mortality (day)	500	500	1000	100
Total population	25,000	40,000	50,000	30,000
Total losses at intervention	Approx. 5000	4730	Approx. 35,000	Data not available
Vaccine applied at the outbreak	*Sialivac* only	*Sialivac* + H5N1 Re-1/IM	*Sialivac* + H5N2/IM	*Sialivac* only
Responses	Mortality ceased in 3 days	20% survived	No survivors	Mortality ceased in 5 days

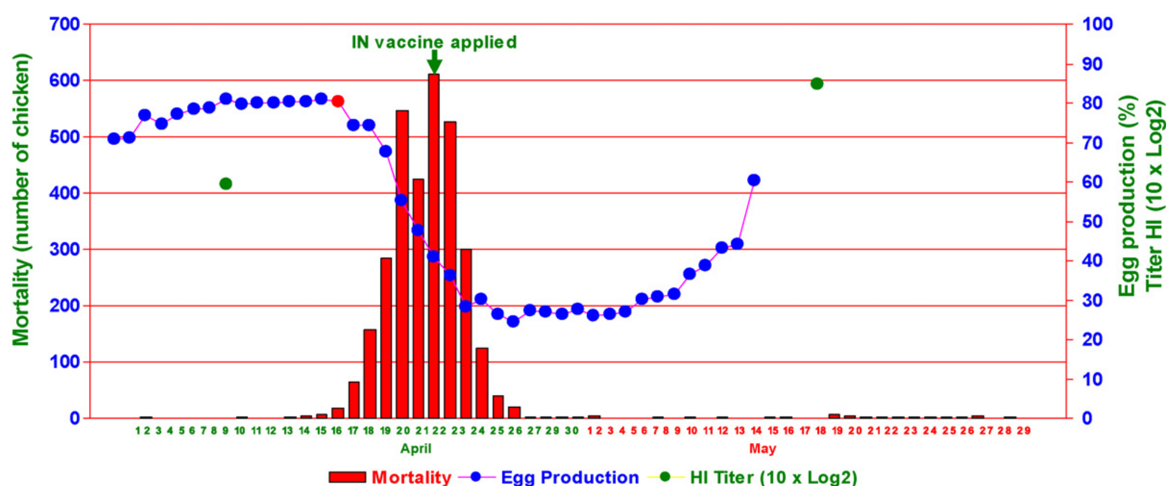

Fig. 4. Control of infection following intervention with *Sialivac* intranasal vaccine.

farms 2 and 3 where revaccination took place with Re-1 and H5N2 vaccine IM, respectively, plus *Sialivac*, failure to control ensued possibly due to mechanical transmission of virus from subclinically infected birds to the whole flock by multidose syringe thus bypassing the early protective influence of the *Sialivac* vaccine.

In cases where both intranasal and commercial intramuscular or subcutaneous vaccines were applied simultaneously control failed and the disease continued.

In cases where the application of intranasal vaccine alone was carried out complete control was observed and fatalities ceased within 2–5 days.

Birds in farm A which had been vaccinated with intranasal vaccine once at 12 days of age with the plain vaccine without bacterial sialidase or split virus presented a sustained higher level of HI antibody than the birds in farm B which had not received IN vaccine but only the intramuscular Re-1 vaccine. During and up to the 6 weeks period the level of HI antibody in the farm A birds remained low but then continued to rise possibly in response to the introduction of further antigen from the Re-1 vaccine at 5-, 14-, and 20-week intervals, indicated by the arrows on the *x*-axis. The sustained higher level of antibody seen in the intranasally vaccinated birds with plain intranasal vaccine may indicate not only the anamnestic response but some degree of synergism with the Re-1 antigen. The combination of mucosal immunity from the IN vaccine combined with the humoral immunity from the Re-1 IM vaccine could be the basis for a more comprehensive disease prophylaxis.

Intervention with *Sialivac* showed a dramatic fall in mortality within 5 days post-vaccination, followed by the recovery of egg production and a rise in HI antibody. Faecal swabs taken monthly from recovered birds, examined by egg inoculation and PCR failed to isolate or reveal AI virus indicating post-vaccination systemic clearance of virus. At the dosage level of 0.03 ml no clinically adverse reaction was observed in the vaccinated birds and 0.2 ml of the vaccine injected into the amniotic sac of 12-day-old embryonated eggs demonstrated complete freedom from abnormal toxicity. The birds remained free from further HPAI infection.

4. Discussion

Evidence of the failure to control HPAI with current heterologous parenteral vaccines in the endemic region of West Java Indonesia is described. This situation accentuated by the desperation of farmers and commercial poultry producers facing crippling losses presented a unique opportunity in an endemic environment to prepare and test under laboratory, and most importantly field conditions, a more effective vaccine.

Highly pathogenic avian influenza disease in poultry is peracute, and in an infected flock a dramatic progressive mortality is apparent. In most outbreaks a proportion are subclinically infected and showing no apparent clinical disease. Reference is made to farms 2 and 3 (Table 2) here the farmers had reservations about the effectiveness of the intranasal vaccine and decided to vaccinate all birds at the same time with a commercial intramuscular vaccine using a multidose syringe with the result that this operation possibly transmitted the virus from subclinically infected birds throughout the whole flock.

The findings support the strategy of prophylactic immunisation with homologous vaccine as the most cost effective means of reducing the high mortality caused by the avian influenza virus by the incorporation into the inactivated homologous influenza vaccine a bacterial sialidase from *C. perfringens type A*.

There is growing awareness of the value of mucosal immunity induced by needle free application of efficacious, adjuvanted and enzyme potentiated vaccines an example of which we report in this paper.

We have described that intervention with *Sialivac* vaccination in commercial farms experiencing progressive mortality stopped the deaths within 2–5 days and successfully protected the survivors which subsequently ceased to excrete live virus in the faeces. This form of intervention using the sialidase/chitovac intranasal vaccine was carried out on numerous occasions at the request of large scale poultry farmers who experienced serious losses in flocks which had been vaccinated with heterologous H5N2 intramuscular oil adjuvant vaccines and in some cases intramuscular reverse genetic Re-1 vaccines which were failing under the existing management conditions to control the disease.

The benefit of an immunisation strategy using intranasal rather than parenteral vaccination favours ease of application in the field by unskilled operators and by the increased levels of protective mucosal IgA as a first line of defence against highly invasive HPAI. This report also highlights the danger of revaccination in infected flocks with heterologous IM vaccines which have failed to protect the birds and where such application favours the mechanical spread from symptomless subclinically infected birds to the whole flock causing increased mortality in some cases up to 100%.

We showed that an intranasal vaccine *Sialivac* applied to birds at 12 days of age is capable of stimulating the common mucosal immune system and can provide protection to subsequent field exposure to HPAI. To achieve a solid immunity it is recommended to vaccinate at 12 days of age followed by a second application at 5 weeks.

An important consistent feature of this vaccine is its ability to produce a protective immune response as observed by the early production of IgA and later IgG but also what appears to be a profound therapeutic action revealed by the arrest of infection in diseased flocks possibly due to the blocking action of the host cell receptors by the bacterial sialidase and the action of mucosal IgA. This combination of both prophylactic and therapeutic action is a novel feature of the *Sialivac* vaccine.

An important question brought to the attention of the authors that is not addressed in the current study is, what is the definitive action of the *Sialivac* antigenic components when chickens are immunised in parallel with *Sialivac*, intranasal, with the same vaccine without sialidase and a commercial oil adjuvanted parenteral vaccine based on the same isolates and then subjected to experimental challenge with defined viruses from the family *orthomyxoviridiae*.

The authors envisage the possibility of inducing a comprehensive broad spectrum cross-protection against both human and avian strains by the use of this vaccine, which will need further experimentation and subsequent challenge experiments with a range of specific viruses. This will require the use of a BSL 3 bio-secure facility.

A further important requirement is to consider the thermostabilisation of the present "wet vaccine" formulation to improve efficacy for use in tropical and subtropical environments. Dehydration of the vaccine will be a future consideration, a cost–benefit appraisal will be needed to justify this improvement; the present "wet vaccine" is offered at 1.2 cents U.S. per dose. At present the long term thermostability of "wet vaccine" *Sialivac* is unknown.

For any vaccine to be adopted for general use in developing countries, despite its obvious benefit, it must be affordable to all farmers. This was the underlying guiding principle but also keeping in mind the maintenance of potency safety and product efficacy.

The inclusion of the crude *C. perfringens type A* toxoid which contains in addition to the sialidase, formalin toxoided lethal alpha toxin plus minor antigens, theta haemolytic, kappa proteinase, Mu hyaluronidase, and v antigen a deoxyribonuclease [26]. It is unknown what influence these antigens may have if any, or what immunological effect they might have. We have shown however

that the finished *Sialivac* vaccine is completely innocuous and free from abnormal toxicity.

Anecdotal evidence from farmers using *Sialivac* report that there is a marked absence of the condition known as "wet litter" caused by the disease *C. perfringens* infectious necrotic enteritis, this might be due to the production of anti-alpha toxin antibody from the IN vaccine and will need further investigation.

The results described in this paper pose as many questions as answers and require further investigation to unequivocally establish *Sialivac's* full immunological profile, for example the duration of immunity, its action on ducks and turkeys, does the bacterial sialidase produce humoral antibodies sufficiently competent to neutralise virus neuraminidase, and is the production of *C. perfingens* anti-alpha antibody sufficient to control infectious necrotic enteritis.

Acknowledgements

The authors would like to acknowledge the help of Dr. C.A. Nidom of The Tropical Diseases Laboratory, East Java, Indonesia, for the typing of the virus isolates, drh. L.N. Darmawan, drh. Teguh Sunaryo, drh. I. Karyahadi, technical assistance of Firmansyah, A. Mulyadi and M. Syafarudin. The cooperation of LDCC Bogor, poultry farmers: Mr. Dharmawan Moelyadi and Mr. F.X. Supardinata, P.T. Peternakan Ayam Manggis, Indonesia.

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