Evaluation of antibody response in mice against avian influenza A (H5N1) strain neuraminidase expressed in yeast *Pichia pastoris*

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Avian influenza has raised many apprehension in the recent years because of its potential transmitability to humans. With the increasing emergence of drug-resistant avian influenza strains, development of potential vaccines are imperative to manage this disease. Two structural antigens, haemagglutinin and neuraminidase, have been the target candidates for the development of subunit vaccine against influenza. In an effort to develop a faster and economically beneficial vaccine, the neuraminidase gene of a highly pathogenic avian influenza isolate was cloned and expressed in the methylotrophic yeast *Pichia pastoris*. The recombinant neuraminidase (rNA) antigen was purified, and its bioactivity was analysed. The rNA was found to be functional, as determined by the neuraminidase assay. Four groups of mice were immunized with different concentrations of purified rNA antigen, which were adjuvanted with aluminium hydroxide. The immune response against rNA was analysed by enzyme-linked immunosorbent assay (ELISA) and neuraminidase inhibition assay. The mice groups immunized with 25 μg and 10 μg of antigen had a significant immune response against rNA. This method can be utilized for faster and cost-effective development of vaccines for a circulating and newer strain of avian influenza, and would aid in combating the disease in a pandemic situation, in which production time matters greatly.

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1. Introduction

The highly pathogenic H5N1 avian influenza virus (AIV) causes one of the most devastating diseases of the avian species, and recently of humans. The AIV is an enveloped virus of the orthomyxovirus family, with eight segmented negative sense RNA. Due to the high level of antigenic variation and frequent, and often fatal, transmission of the disease from avian species to humans (Chotpitayasunondh *et al*. 2005; Subbarao *et al*. 1998; Tran *et al*. 2004), the disease not only results in huge economic loss but also poses major health risk, as demonstrated by the number of fatalities in the last decade. According to the World Health Organization, there were 648 confirmed human cases including 384 deaths in 2003–2013 (WHO 2013). Previous studies have reported that strong pre-existing immune response plays a crucial role in protecting the animals from virus infection. Therefore, it is evident that an efficient vaccine that induces protective immune response against the highly pathogenic avian influenza is necessary to control disease transmission from infected to susceptible healthy populations.

Keywords. Enzyme-linked immunosorbent assay; highly pathogenic avian influenza; immunogenicity; neuraminidase inhibition; neuraminidase

Abbreviations used: AIV, avian influenza virus; ELISA, enzyme-linked immunosorbent assay; HA, haemagglutinin; NA, neuraminidase; NI, neuraminidase inhibition; rNA, recombinant neuraminidase; YPD, yeast peptone dextrose

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The AIV virus consists of two major surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), of which HA facilitates virus entry while NA helps virus release. The HA initiates virus entry by binding to the sialic-acid receptors on target cells (Weis *et al*. 1988), while NA facilitates virus release from infected cells by cleaving the terminal sialic-acid residue, allowing the virus to spread (Tsvetkova and Lipkind 1968). Neutralizing antibodies to HA protects animals from infection while antibodies to NA helps controlling the virus spread, making the two antigens target molecules for vaccine strategies against avian influenza. Although neutralizing antibodies to HA (Bommakanti *et al*. 2010; Steel *et al*. 2010; Weaver *et al*. 2011) alone can protect the animal from infection, inclusion of NA in vaccine is reported to enhance the level of protection in animals (Fang *et al*. 2008; Rockman *et al*. 2013), since HA together with NA is more efficient than HA alone (Fang *et al*. 2008). NA has lower antigenic diversity compared to HA, which is indicated by the limited number of variants (Fouchier *et al*. 2005; Shi *et al*. 2010). NA could be a more suitable choice for an effective vaccine against AIV. The current avian influenza vaccine is an inactive avian influenza antigen consisting both HA and NA antigens, which are prepared in embryonated eggs (Ehrlich *et al*. 2012). However, these methods results in the production of fewer vaccines (Fedson 2008; Stephenson *et al*. 2004; Wood and Robertson 2004) and demands stringent biosafety requirements. With a high degree of variability among HA and NA subtypes in avian influenza, these vaccine manufacturing methods may not be sufficient in the case of a pandemic. Therefore, it is necessary to develop a quicker method for producing large quantities of avian influenza vaccines. Although neuraminidase gene expressed in baculovirus (Brett and Johansson 2005; Mather *et al*. 1992) and DNA vaccines expressing neuraminidase (Chen *et al*. 2000) have already been reported to provide partial protection in animals, the methylotrophic yeast *Pichia pastoris* is an excellent alternative to baculovirus for producing such large quantities of vaccines, especially because of its simple scalability and higher cell mass (Cereghino and Cregg 2000). Hence, in an effort to develop a faster and cost-effective potential vaccine against avian influenza during a pandemic situation, we cloned, expressed and purified a recombinant NA and studied its immunogenicity in mice.

2. Materials and methods

2.1 *Cells and genes*

Escherichia coli (One Shot® TOP10) (Life technologies, USA) cells were used for all bacterial cloning and propagation of plasmids. The cells were grown in Luria-Bertani medium supplemented with 25 μg/mL of Zeocin (Life technologies, USA). *Pichia pastoris* GS115 was used as an expression host together with the *Pichia* expression vector pPICZC (Invitrogen, USA) and were cultured in yeast peptone dextrose (YPD) agar/broth supplemented with 100 μg/mL of Zeocin. cDNA of Avian Influenza virus NA structural gene NA of H5N1 strain (A/Hatay/2004/H5N1; GenBank Accession number: AJ867075) was prepared in virology lab at International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India, and was sent to us after cloning into the pET20b+ vector.

2.2 *Animals*

Female Swiss albino mice (6–8 weeks old, 22–25 g) were used for immunization experiments. The animals were procured from a holding farm, Indian Immunologicals, Hyderabad, India. The animals were maintained in a pathogen-free condition. All the procedures for animal experiments follow the approved protocols and are in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

2.3 *Cloning of NA into Pichia expression vector pPICZC*

The NA gene was re-amplified from pET20b+NA following standard Polymerase chain reaction (PCR) with a forward primer (ATCGGAATTCATGAATCCAAATCAGAAGA-TAA TAACCAT) consisting *EcoRI* restriction site (underlined) at its 5′ end and reverse primer (GCTGGCGGCCGC GT*ACCTTGGAAATACAAATTTTC*CTTGTCAATGGTGA-ATGGCAA) consisting *NotI* restriction site (underlined) and a TEV protease cleavage site (ENLYFQG – italicized) at its 5′ end. The resulting amplicon (figure 1A) was cloned into a yeast expression vector pPICZC under the control of an alcohol oxidase promoter in-frame with the c-terminus His-tag. The clones containing the NA gene were verified by restriction digestion and DNA sequencing. The positive clone was linearized with restriction enzyme *PmeI* and was transformed into *P. pastoris* by electroporation at 25 μF, 200 Ω, 2500 V (Biorad, USA) and plated onto YPD agar plates containing Zeocin (100 μg/mL) (Invitrogen, USA). The colonies were subcultured on YPD broth and gene integration was confirmed as described at *ww.invitrogen.com/content/sfs/manuals/pich_man.pdf*.

2.4 *Expression of rNA in Pichia pastoris*

The positive transformants were inoculated into 2 mL of Buffered Glycerol-complex Medium (BMGY) media and grown at 30°C for 48 h at 1000*g*. After 48 h, the cells were harvested by centrifugation at 3250*g* and washed with distilled water twice prior to resuspension in BMY broth. The cultures were induced with 1% methanol (v/v) for every 24 h till 120 h.

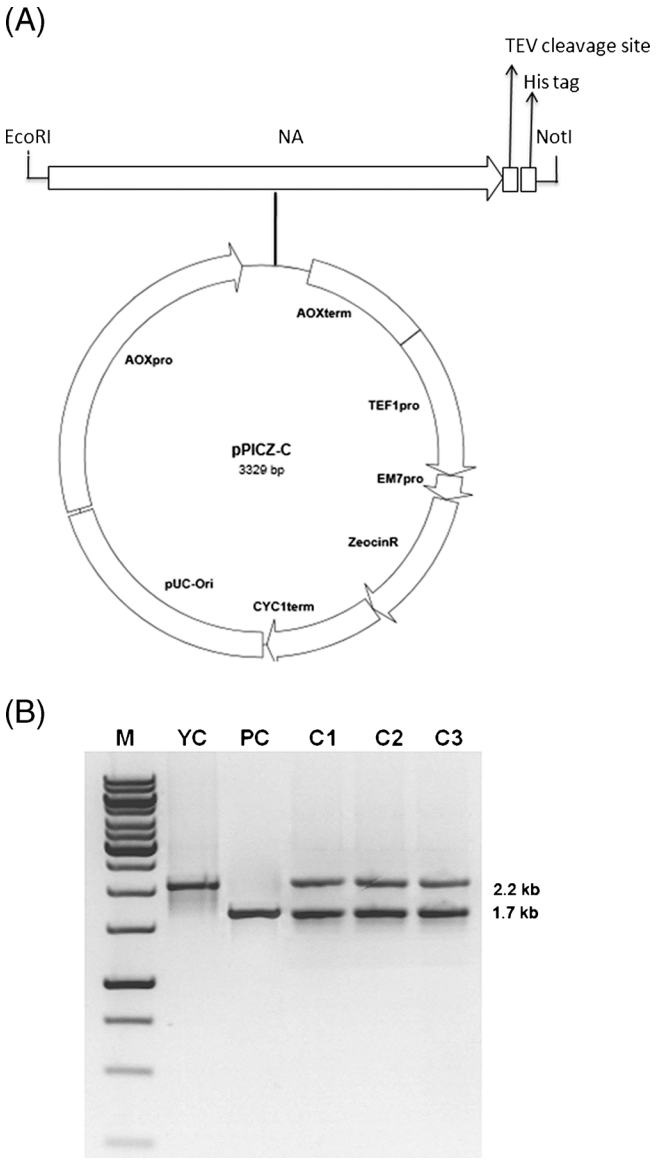

Figure 1. Cloning of NA gene into *Pichia* expression vector and confirmation of genetic integration. (**A**) Schematic representation of NA open reading frame with the in-frame TEV protease cleavage site and a 6xHis tag. (**B**) Determination of target gene (NA) integration. The genomic DNA from the positive transformants were isolated and analysed by PCR with AOX forward and reverse primers. The resulted amplicons were analysed in 1% agarose gel. The positive clones yielded two amplicon, one corresponding to the 2.2 kb AOX gene cassette and the ~1.7 kb corresponding to the NA gene. YC – yeast control, PC – plasmids control.

After 120 h, the cells were harvested and lysed using a glass bead beater. For one gram of cell wet weight 3 g of chilled glass beads were added and the suspension was subjected to vortex for 30 s and immediately transferred to ice. After 1 min, the sample was vortexed again and the cycle was repeated for 10 times. The glass beads were removed by brief centrifugation at 200*g*. The resulting supernatant was clarified by centrifuging at 15000*g* at 4°C. The clarified lysate was analysed for the expression of NA gene on SDS-PAGE and Western blot using HisProbe-HRP (Thermo scientific, USA). The positive transformants expressing NA gene were scaled up to 500 ml in shaker culture flasks and induced as above. After induction, the cells were harvested; the pellet was washed and re-suspended in 50 mM sodium phosphate buffer (pH 8). The cells were lysed in a French press cell disrupter (Constant systems, UK) at 40,000 psi, 3 cycles. The cell lysate was centrifuged at 15,000*g* at 4°C. The supernatant was filtered through 0.2 µm filter and analysed on a 12% SDS-PAGE. An immunoblot was performed using HisProbe-HRP (Thermo scientific, USA) following standard procedures to confirm the expression of rNA.

2.5 *Purification of rNA*

The purification of rNA under native condition using Ni-NTA resulted in co-purification of rNA and a non-specific host cell protein. Hence, the NA protein was purified using Ni-NTA chromatography under denaturing condition. The lysate was mixed with equal volume of 8 M urea and NaCl was added to a final concentration of 300 mM. The lysate was filtered through a 0.2 µM syringe filter and passed through a Ni-NTA Superflow resin (Qiagen, USA), washed with 50 mM sodium phosphate buffer containing 4 M urea, 300 mM NaCl and 30 mM imidazole (Sigma, USA) and the bound NA protein was eluted using 50 mM sodium phosphate buffer containing 4 M urea, 300 mM NaCl and 300 mM imidazole. The fractions were analysed on 12% SDS-PAGE and the fractions containing purified rNA protein were pooled. The protein was refolded by dialysing against 50 mM sodium phosphate buffer containing 2M urea, 50 mM glycine, 0.5 mM EDTA and 1 mM DTT (pH 8) at +4°C. The concentration of urea in the dialysis buffer was progressively reduced for every 6 h through 2, 1, 0.5 and 0.25 M till no urea. Purified, His-tagged TEV protease at a concentration of 0.5 mg/mL was added to 2 mg/mL of purified, refolded NA protein and incubated at 20°C for 4 h. The mixture containing purified rNA and TEV protease was passed through a Ni-NTA Superflow resin, to enable the his-tagged TEV protease to bind the Ni-NTA and the flow through containing cleaved rNA was collected, concentrated through a vivaspin20 (10,000 MWCO) (Vivaproducts, USA) and analysed on 12% SDS-PAGE. The protein was dialysed with 50 mM sodium phosphate (pH-8.0) and the concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL). An immunoblot was also performed to confirm the reactivity of the purified rNA using Influenza A H1N1 Neuraminidase (NA) specific rabbit polyclonal antibody (Sino Biologicals, China).

2.6 *Neuraminidase assay*

The purified rNA at 1mg/ml concentration was evaluated for its function by neuraminidase assay using EnzyChrom™ Neuraminidase Assay Kit (BioAssay Systems, USA) by following the manufacturer's guidelines. Briefly, to 20 µL of different concentrations (400, 200, 100, 50 and 25 µM) of sialic acid standard (provided with the kit), 30 µL assay buffer, 55 µL substrate, 1 µL cofactors, 1 µL enzyme and 0.5 µL dye reagent were added. Appropriate controls (blank, H_2O) were also included in the assay. The reaction mixture was incubated at 37°C. After 20 min, the OD (OD20 min) was measured at 570 nm. The reaction mixture was incubated for a further 30 min at 37°C and the OD was measured again (OD50 min) at 570 nm. The OD of sample, blank and H_2O at 20 min were subtracted from OD of sample, blank and H_2O at 50 min respectively to get ΔRsample, ΔRblank and ΔRH_2O respectively. The OD of standard was plotted against standard concentration and the neuraminidase activity of the sample was calculated as Neuraminidase Activity (Units/L) = $(\Delta RSAMPLE–\Delta RBLANK–\Delta RH_2O)/Slope \times 1/t$ where slope is the slope of the standard curve in μM^{-1} and *t* is the time of reaction between readings (30 min).

2.7 *Animal experiment*

2% aluminium hydroxide was used to adsorb the rNA protein and the vaccine preparation was stored at 4°C until further use. Four groups, each consisting 10 mice were immunized subcutaneously with different concentrations (25 µg, 10 µg, 5 µg and 2.5 µg/0.1 mL) of purified rNA and labelled as Group1, Group2, Group3 and Group4 respectively. All the mice were boosted on 28th day days post immunization (dpi) and 42nd dpi with 5 µg of purified rNA. The animals were bled on 14, 21, 35 and 49 dpi and the sera were analysed for anti-NA immune response. An unimmunized control (Group 5=10 mice) group was also included in the experiment. The immune response was analysed by indirect enzyme linked immunosorbent assay (ELISA) and neuraminidase inhibition assay using rNA.

2.8 *Indirect ELISA*

An indirect ELISA was performed for analysing the immune response against rNA as described earlier (Biesova *et al.* 2009) with minor modifications. Briefly, Maxisorp™ ELISA plates (Nunc, Denmark) were coated with 200 ng of rNA and incubated overnight at 4°C. The plate was washed four times with 15 mM Phosphate buffered saline (PBS) supplemented with 0.1% Tween20 (PBST) and blocked with blocking buffer (PBST supplemented with 5% normal bovine serum and 2% normal rabbit serum). The serum samples diluted two fold from 1:2 till 1:1024 in blocking buffer were added and incubated at 37°C for an hour. Following incubation, the plates were washed four times with PBST and anti-mouse-horseradish peroxidase (HRPO) conjugate at 1:8000 dilutions was added to all the wells and incubated for 1 h. After washing four times with PBST, the plates were developed with 0.02% ortho-phenylene diamine (OPD), 0.015% H_2O_2 in 15 mM sodium citrate buffer (pH-5.6) and the reaction was stopped after 15 minutes with 1.25 M H_2SO_4. The absorbance was read at 492 nm. The OD value > 0.150 was taken as positive. The ELISA titer was represented as the reciprocal of highest dilution at which the OD value was > 0.150.

2.9 *Neuraminidase inhibition assay*

The anti-NA immune response was evaluated by neuraminidase inhibition (NI) assay. To perform this, 50 µL of mice sera from each group was taken at 1/2 dilutions which were half diluted further till 1/1024 in a 96-well micro-titer plate. 50 µL of purified rNA (0.25 mg/ml) was added to each well and incubated at 37°C for 2 h. The non-neutralized (leftover) NA activity was evaluated as described above. The neuraminidase inhibition titer was represented as the highest dilution until there was no neuraminidase activity observed.

3. Results

3.1 *Cloning and expression of rNA in Pichia pastoris*

The NA gene was digested with *EcoRI* and *NotI* and cloned into similarly digested pPICZC vector (figure 1A). The clones were confirmed by restriction digestion with *EcoRI* and *NotI,* which released a 1359 bp NA and 3.3 kb pPICZC vector, and the cloned gene was then confirmed by DNA sequencing. The positive clones were transformed into *Pichia pastoris* after linearization with *PmeI*. The positive transformants were analysed for the NA gene integration by PCR, using AOX forward and AOX reverse primers. The PCR yielded a 2.2 kb amplicon (figure 1B) corresponding to the AOX gene, and a ~1.7 kb amplicon corresponding to the NA with its promoter and terminator. The 1.7 kb amplicon was then confirmed by DNA sequencing, and was found to be 100% homologous with the cloned NA. The rNA expression in a 2 mL culture was considerably lower. It could not be visibly seen in SDS-PAGE but the immunoblot using the His-probe showed a ~53 kDa NA protein. The expression of rNA did not increase after induction with increasing concentrations of methanol (data not shown). The clone expressing rNA cultures was scaled up and the expression of rNA was re-confirmed by SDS-PAGE and Western blot.

3.2 *Purification and characterization of rNA*

The clone expressing rNA was scaled up and induced as described above. The cells were harvested and lysed. The unlysed cells and cell debris were removed by centrifugation and the clarified supernatant was analysed for the presence of rNA. In the Western Blot with Hisprobe-HRP, it was evident that the rNA protein was present both in the cytosol and the membrane fraction (data not shown). When membrane fractions were used, multiple protein species were getting co-purified. However, the rNA protein from the cytosolic fractions were singularly purified, using Ni-NTA chromatography. The elution fractions containing rNA protein were analysed on SDS-PAGE (figure 2A) and Western Blot using HisProbe (figure 2B) and NA-specific polyclonal antibody (figure 2C), which showed a ~53 kDa rNA protein. To remove the urea and allow proper refolding, the purified protein was subjected to dialysis by gradual reduction of the urea concentration for every 6 h under reduced buffer conditions. This process left the protein completely in the soluble form. The His-tag from the purified rNA was then removed using TEV protease before analysing immunogenicity in the mice. The purified rNA protein was adjusted to a final concentration of 1 mg/mL and the activity was analysed by neuraminidase assay using EnzyChrom™ Neuraminidase Assay Kit. The purified rNA protein was found to have 274 units/mg.

3.3 *Immunogenicity of rNA in mice*

The immunogenicity of purified rNA was evaluated in mice. Four groups (Group 1– 4) of mice were immunized with different concentrations of rNA, and the immune response against rNA was analysed by ELISA and neuraminidase inhibition assay. Groups 1 and 2, which were immunized with 25 µg and 10 µg, respectively, were able to develop NA specific immune response after the first booster, as determined by ELISA. The second booster on 42nd days post immunization (dpi) induced an anamnestic immune response. Group 3 showed very less antibody response after the second booster. However, Group 4, immunized with 2.5 µg, was unable to induce higher immune response even after the second booster. Group 2 gave a maximum ELISA OD on 49th dpi, followed by Group 1 on 35 dpi and Group 3 on 49 dpi. The unimmunized control was negative throughout the experiment (figure 3). The immunogenicity was then evaluated by neuraminidase inhibition assay. The titer in NI assay was considered an indirect measure of neutralizing antibody response against rNA antigen. Group 2 showed higher antibody response than Group 1, whereas Group 3 and Group 4 gave very low neutralizing response. Group1, Group2, Group3 and Group4 showed 89, 284, 28 and 8 NI titers, respectively. Group 5 was negative throughout the experiment (figure 4).

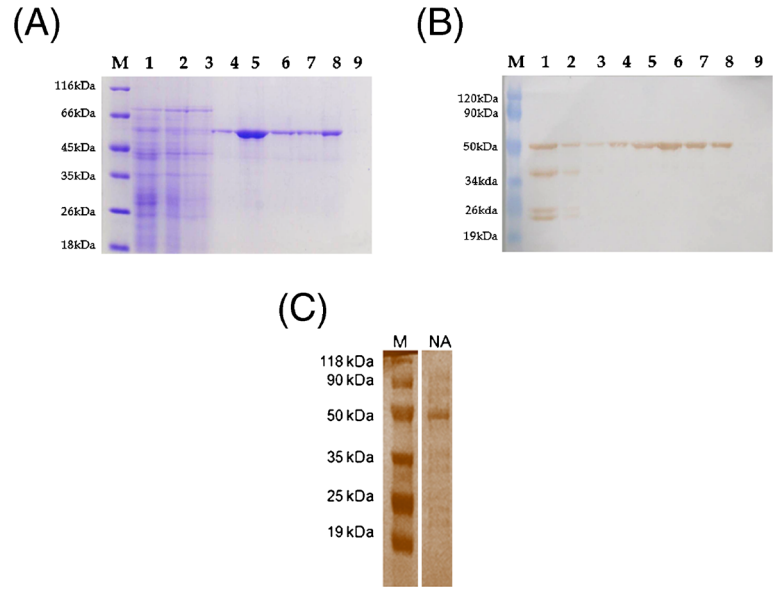

Figure 2. Purification of rNA expressed in *Pichia pastoris*. The positive transformants induced with methanol for 5 days were harvested and lysed using a French press under 40000 psi. The lysate was clarified by hi-speed centrifugation and the rNA was purified using Ni-NTA chromatography. The fractions were analysed by SDS-PAGE (**A**) and Western blot (**B**) using His-Probe which showed the ~53 kDa NA antigen. The fractions containing rNA was pooled and dialysed against 50 mM sodium phosphate buffer and stored at −80°C until further use. Lane1 – Input; Lane2 – Flow through; Lane3 – Wash; Lane 4 to 10 – elution fractions. The reactivity of purified rNA was also confirmed by western blot using NA specific rabbit polyclonal antibody (C). M – Prestained protein marker; NA – pooled fraction of purified rNA protein.

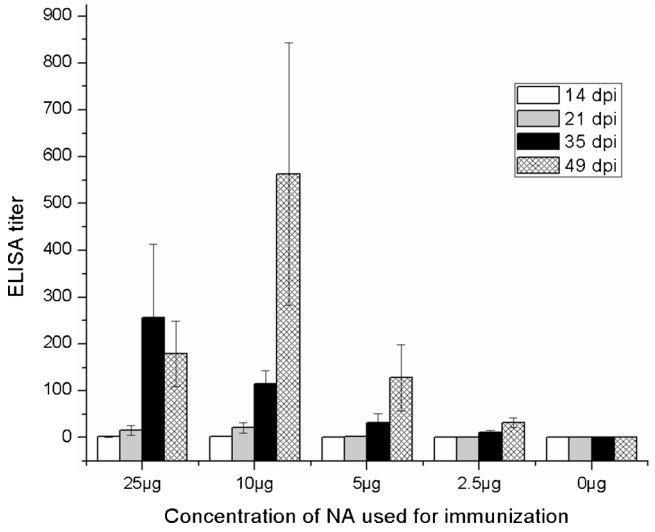

Figure 3. Determination of anti-NA immune response by indirect ELISA in mice. Four groups of mice were immunized with purified rNA antigen at different concentrations such as 25 µg, 10 µg, 5 µg and 2.5 µg/dose and the immune response was analysed by an indirect ELISA. The antibody response was high in group immunized with 10 µg, followed by 25 µg and 5 µg. group immunized with 2.5 µg failed to show NA specific antibody response even after the second booster. The mean antibody titer of each group is represented against each dpi.

4. Discussion

The HA and NA genes of AIV are highly variable, with 17 subtypes of HA and 9 subtypes of NA described so far (Fouchier *et al*. 2005; Shi *et al*. 2010). With the high possibility of an avian influenza pandemic, and the highly unpredictable combination of HA and NA in new strains, it is apparent that only a multivalent vaccine would aid animals in complete protection against the viral infection. Development of vaccines for virus strains already characterized, or at least reported, may not be the best defence in facing a future pandemic because these vaccines may not promise complete protection against a new strain. However, the development of vaccines against circulating strains of influenza viruses within a very short time span will help control the disease to a certain extent. Hence, the aim of this study was to establish a faster and economical vaccine development method, especially for developing countries where more amount of vaccine would be needed in a limited period of time if a pandemic situation arises.

It is evident from numerous reports that the two major structural antigens, HA and NA, either alone (Deroo *et al*. 1996; Johansson 1999) or in combination (Chen *et al*. 1999) with other influenza antigens (Chen *et al*. 1999; Keitel *et al*. 1996), are the backbone of current influenza vaccines, and may be for the future as well. Even though HA alone can provide maximum protection in animals from the disease (Robinson *et al*. 1993; Webster *et al*. 1994; Kodihalli *et al*. 1997, 1999), few studies have already reported that addition of NA to HA can improve the vaccine efficiency (Fang *et al*. 2008; Johansson 1999). Hence, a detailed study on the combination of these two antigens as a vaccine candidate has become necessary. Baculovirus expressing the NA antigen of highly pathogenic AIV has already been reported (Brett and Johansson 2005; Mather *et al*. 1992; Johansson *et al*. 1995, Johansson 1999) but the extent to which this will help address a possible pandemic is still a topic for debate. *Pichia,* as a platform for expressing these antigens, is an excellent choice since huge cell mass could be reached in less time. Although the *Pichia* expression system is at a disadvantage of having glycosylation patterns different than mammalian glycosylation pattern, many antigens that are expressed in *Pichia* are shown to protect against infection (Martinet *et al*. 1997; Schelleken *et al*. 1987; Saelens *et al*. 1999; Lin *et al*. 2012) which proves *Pichia* to be an efficient platform for manufacturing such vaccine candidates.

In this study, the NA of highly pathogenic avian influenza was cloned into *P. pastoris* and the recombinant protein was purified. Although the amount of rNA protein present in the cytosol was considerably lower, the rNA was also observed

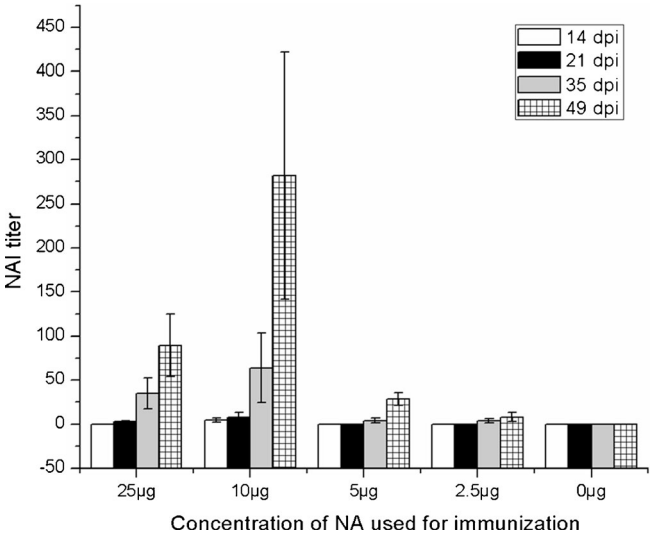

Figure 4. Determination of NI response in mice. Four groups of mice were immunized with purified rNA antigen at different concentrations such as 25 µg, 10 µg, 5 µg and 2.5 µg/dose. The immune response was analysed by an indirect neuraminidase inhibition assay. The NI titer was high in group immunized with 10 µg, followed by 25 µg, 5 µg and 2.5 µg. The mean NA inhibition titer of each group is represented against each dpi.

in the membrane fractions and culture supernatant (data not shown). The majority of these rNA can be extracted through different purification methods. The rNA purified from the cytosolic fractions was purified under denaturing conditions and latter refolded to its active state. The expression studies were performed in 2 L shake flasks and the total yield of the purified rNA was ~2 mg/L, which was comparable with the levels reported by Martinet *et al*. (1997). However, this can be increased substantially if large scale fermentor is used. The activity of purified rNA was also determined by neuraminidase assay. The activity of NA per mg of purified rNA was found to be 274 units, which is closer to what has been previously reported (Yongkiettrakul *et al*. 2009). This clearly indicates that the development of such vaccine in the *Pichia* expression system could be a better and viable option to face a future pandemic. The immunogenicity experiments showed that the purified rNA was able to elicit confirmation dependent antibody response in mice, as demonstrated by neuraminidase inhibition assay. The animals of Group 2 showed maximum antibody response followed by Group 1 as determined by both ELISA and neuraminidase inhibition assay. One or two animal from Group 1 and Group 2 produced either too high or too low antibody response, which contributed to significant level of variation between the animals of the same group. However, maximum number of animals from these groups produced higher antibody level against rNA. Group 3 and Group 4 did not elicit higher immune response even after the second booster. On the other hand, Martinet *et al.* (1997) reported that three immunizations with 2 μg of rNA offered 50% protection in animals. But, in this study, similar immune response was achieved only with 10 μg of rNA. This low immunity may be attributed to the glycosylation pattern, as reported by Martinet *et al.* (1997), that removal of glycosylation at the top of neuraminidase resulted in improved protection in mice. But, interestingly, the immune response induced with 10 μg of rNA in this study was higher than that of what is reported by Kilbourne *et al.* (2004) with 9 μg of rNA. Hence, going by the antibody titers, the immune response induced with 10 μg of rNA may be good enough to offer partial protection in animals. The use of α-1,6-mannosyltransferase (och1)-defective *P.pastoris* (Yang *et al*. 2012) strain can be used to overcome these glycosylation issues as it may further improve the antigen-specific immune response in animals.

This method of vaccine development in *P. pastoris* will take at least 2 to 3 weeks, even if the sequence information of the circulating influenza strain is available. Nonetheless, such vaccines, in combination with HA, may assist in improving (Fang *et al*. 2008; Johansson 1999) the protective immune response against highly pathogenic AIV infections. A potent subunit vaccine against avian influenza could be made if the effect of other structural antigens in eliciting anti-influenza immune response could be established. In an effort to analyse and understand the effect of other structural antigens in producing an active immune response, we also have expressed and purified the HA and M1 antigen of highly pathogenic avian influenza in *P. pastoris* (data not shown) and the evaluation of immune response both as single component subunit vaccine and as a multi-component subunit vaccine is being studied.

In conclusion, this method of avian influenza vaccine development will be safer and cost-effective because the gene encoding HA and NA can be engineered or modified using only the sequence information, which will eliminate the need of handling of live viruses and thus avoid the necessity of stringent biosafety measures. Because of the ease of vaccine development, this method may assist in addressing a future highly pathogenic AIV pandemic.

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References

Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY, Robbins JB and Keith JM 2009 Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. *Vaccine* **27** 6234–6238

Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, Lu X, Joyce JG, Shiver JW, Casimiro DR, ter Meulen J, Liang X and Varadarajan R 2010 Design of an HA2-based *Escherichia coli* expressed influenza immunogen that protects mice from pathogenic challenge. *Proc. Natl. Acad. Sci. USA* **107** 13701–13706

Brett IC and Johansson BE 2005 Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. *Virology* **339** 273–280

Cereghino JL and Cregg JM 2000 Heterologous protein expression in the methylotrophic yeast *Pichia* pastoris. *FEMS Microbiol. Rev.* **24** 45–66

Chen Z, Kadowaki S, Hagiwara Y, Yoshikawa TK, Matsuo KT, Kurata T and Tamura S 2000 Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase. *Vaccine* **18** 3214–3222

Chen Z, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T and Tamura S 1999 Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein. *Vaccine* **17** 653–659

Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, Chunsuthiwat S, Sawanpanyalert P, Kijphati R, Lochindarat S, Srisan P, *et al*. 2005 Human disease from influenza A (H5N1), Thailand. *Emerg. Infect. Dis.* **11** 201–209

Deroo T, Min Jou W and Fiers W 1996 Recombinant neuraminidase vaccine protects against lethal influenza. *Vaccine* **14** 561–569

Ehrlich HJ, Singer J, Berezuk G, Fritsch S, Aichinger G, Hart MK, El-Amin W, Portsmouth D, Kistner O and Barrett PN 2012 A Cell culture–derived influenza vaccine provides consistent protection against infection and reduces the duration and severity of disease in infected individuals. *Clin. Infect. Dis.* **54** 946–954

Fang F, Cai XQ, Chang HY, Wang HD, Yang ZD and Chen Z 2008 Protection abilities of influenza B virus DNA vaccines expressing hemagglutinin, neuraminidase, or both in mice. *Acta Virologica* **52** 107–112

Fedson DS 2008 NEW technologies for meeting the global demand for pandemic influenza vaccines. Biologicals: *J. Int. Assn. Biol. Standard.* **36** 346–349

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B and Osterhaus AD 2005 Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79** 2814–2822

Johansson BE 1999 Immunization with influenza A virus hemagglutinin and neuraminidase produced in recombinant baculovirus results in a balanced and broadened immune response superior to conventional vaccine. *Vaccine* **17** 2073–2080

Johansson BE, Price PM and Kilbourne ED 1995 Immunogenicity of influenza A virus N2 neuraminidase produced in insect larvae by baculovirus recombinants. *Vaccine* **13** 841–845

Keitel WA, Cate TR, Atmar RL, Turner CS, Nino D, Dukes CM, Six HR and Couch RB, 1996 Increasing doses of purified influenza virus hemagglutinin and subvirion vaccines enhance antibody responses in the elderly. *Clin. Diagn. Lab. Immunol.* **3** 507–510

Kilbourne ED, Pokorny BA, Johansson B, Brett I, Milev Y and Matthews JT 2004 Protection of mice with recombinant influenza virus neuraminidase *J. Infect. Dis.* **189** 459–461

Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y and Webster RG 1999 DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. *J. Virol.* **73** 2094–2098

Kodihalli S, Haynes JR, Robinson HL and Webster RG 1997 Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. *J. Virol.* **71** 3391–3396

Lin GJ, Deng MC, Chen ZW, Liu TY, Wu CW, Cheng CY, Chien MS and Huang C 2012 Yeast expressed classical swine fever E2 subunit vaccine candidate provides complete protection against lethal challenge infection and prevents horizontal virus transmission. *Vaccine* **30** 2336–2341

Martinet W, Saelens X, Deroo T, Neirynck S, Contreras R, Min Jou W and Fiers W 1997 Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase. *Eur. J. Biochem.* **247** 332–338

Mather KA, White JF, Hudson PJ and McKimm-Breschkin JL 1992 Expression of influenza neuraminidase in baculovirus-infected cells. *Virus Res.* **26** 127–139

Robinson HL, Hunt LA and Webster RG 1993 Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* **11** 957–960

Rockman S, Brown LE, Barr IG, Gilbertson B, Lowther S, Kachurin A, Kachurina O, Klippel J, *et al.* 2013 Neuraminidase-inhibiting antibody is a correlate of cross-protection against lethal H5N1 influenza virus in ferrets immunized with seasonal influenza vaccine. *J. Virol.* **87** 3053–3061.

Saelens X, Vanlandschoot P, Martinet W, Maras M, Neirynck S, Contreras R, Fiers W and Jou WM 1999 Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. *Eur. J. Biochem.* **260** 166–175

Schelleken, H, de Reus A, Peetermans JH and van Eerd PA 1987 The protection of chimpanzees against hepatitis B viral infection using a recombinant yeast-derived hepatitis B surface antigen. *Postgrad. Med. J.***63** 93–96

Shi W, Lei F, Zhu C, Sievers F and Higgins DG 2010 A Complete Analysis of HA and NA Genes of Influenza A Viruses. *PLoS One* **5** e14454

Steel J, Lowen AC Wang TT, Yondola M, Gao Q, Haye K, Garcia-Sastre A and Palese P 2010 Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **1** e00018–10

Stephenson I, Nicholson KG, Wood JM, Zambon MC and Katz JM 2004 Confronting the avian influenza threat: vaccine development for a potential pandemic. *Lancet Infect. Dis.* **4** 499–509

Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, *et al*. 1998 Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279** 393–396

Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, Le HN, Nguyen KT, *et al.* 2004 Avian influenza A (H5N1) in 10 patients in Vietnam. *New Engl. J. Med.* **350** 1179–1188

Tsvetkova IV and Lipkind MA 1968 Studies on the role of myxovirus neuraminidase in virus-cell receptors interaction by means of direct determination of sialic acid split from cells. I. Experiments on influenza virus-RBC cell system. *Archiv fur dies Gesamte Virusforschung* **23** 299–312

Weaver EA, Rubrum AM, Webby RJ and Barry MA 2011 Protection against divergent influenza H1N1 virus by a centralized influenza hemagglutinin. *PLoS one* **6** e18314

Webster RG, Fynan EF, Santoro JC and Robinson H 1994 Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* **12** 1495–1498

Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ and Wiley DC 1988 Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333** 426–431

Wood JM and Robertson JS 2004 From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. *Nat. Rev. Microbiol.* **2** 842–827

World Health Organization 2013 *Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003–2013* (Geneva: WHO) (*http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html*)

Yang YL, Chang SH, Gong X, Wu J and Liu B 2012 Expression, purification and characterization of low-glycosylation influenza neuraminidase in α-1,6-mannosyltransferase defective Pichia pastoris. *Mol. Bio. Rep.* **39** 857–864

Yongkiettrakul S, Boonyapakron K, Jongkaewwattana A, Wanitchang A, Leartsakulpanich U, Chitnumsub P, Eurwilaichitr L and Yuthavong Y 2009 Avian influenza A/H5N1 neuraminidase expressed in yeast with a functional head domain. *J. Virol. Methods* **156** 44–51

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