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Anne Prel, Ghislaine Le Gall-Recule, Véronique Jestin. Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low pathogenic avian influenza strains in ducks. *Avian Pathology*, 2008, 37 (05), pp.513-520. 10.1080/03079450802357001 . hal-00540132

HAL Id: hal-00540132

<https://hal.science/hal-00540132>

Submitted on 26 Nov 2010

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Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2008-0085
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	03-Jun-2008
Complete List of Authors:	Prel, Anne; AFSSA, UVIPAC Le Gall-Reculé, Ghislaine; AFSSA, UVIPAC Jestin, Véronique; AFSSA
Keywords:	Avian influenza, virus-like particles, duck, baculovirus expressed proteins

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Cavp-2008-0085

Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low pathogenic avian influenza strains in ducks

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Running title: Avian influenza virus-like particles

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Received 3 June 2008:

Abstract

Infections with H5/H7 low pathogenic avian influenza (LPAI) viruses are now notifiable because such viruses can mutate into highly pathogenic avian influenza (HPAI) viruses, leading to serious problems for both animal and public health. Domestic ducks can play a crucial role in the transmission of H5 LPAI viruses to other poultry. Although prime boost vaccination using, respectively, a recombinant and an inactivated vaccine was shown to be protective in ducks against H5N1 HPAI, vaccination of domestic ducks against H5 LPAIV is poorly documented. However, substituting inactivated vaccines with subunit vaccines might be more advantageous. In this context, we generated a triple recombinant baculovirus composed of HA and NA proteins derived from a French H5N3 LPAI virus strain and the M protein derived from an Italian H7N1 LPAI virus strain. We describe a molecular construction strategy that enabled the development of virus-like particles (VLPs). Western blot analyses and neuraminidase inhibition assay of cell supernatants purified by sucrose density gradient ultracentrifugation showed that HA, NA and M1 proteins were expressed and co-released. Electron microscopy examination revealed VLPs that were morphologically identical to wild-type virus. Immunogold electron microscopy demonstrated that H5 and N3 proteins were present on the VLP surface, and haemagglutination and neuraminidase assays showed that the H and N proteins, respectively, were biologically active. In addition, VLP immunogenicity (induction of haemagglutination-inhibiting antibodies) was demonstrated in SPF Muscovy ducks. According to our successful previous experimental results of protection in ducks following vaccination with the three baculovirus-expressed proteins, the present results make feasible the reliable use of H5N3 VLPs as a subunit vaccine in this species.

Introduction

Avian influenza (AI) remains an economic threat to commercial poultry worldwide. Since 1997, direct avian-to-human transmission of lethal AI viruses of the H5 subtype has elevated the need to control AI beyond economic considerations. Domestic ducks can play crucial roles in the transmission of H5 low pathogenic (LP) (Cherbonnel *et al.*, 2007) and highly pathogenic (HP) avian influenza viruses (Sturm-Ramirez *et al.*, 2005), and a few inactivated and recombinant commercial or experimental vaccines have been assessed experimentally in these species essentially for the prevention of H5N1 HP (Middleton *et al.*, 2007; Steensels *et al.*, 2007). However, in the field the protection of vaccinated ducks remains questionable as suggested by the reoccurrence of H5N1 HP in Vietnam despite massive vaccination (OIE). Few data have been reported concerning the protection afforded by inactivated vaccines against H5 LPAI infection in ducks (Prel *et al.*, 2007). However, alternative vaccines may increase this protection. Recombinant avian influenza vaccines such as fowlpox virus-based AI vaccines cannot be used in prime-boost because a strong immunity may be directed against the vector (Swayne *et al.*, 2000). This is the reason why, in a recent study, a boost with an inactivated AI vaccine was performed after a prime with a fowlpox virus-based AI vaccine (van den Berg *et al.*, 2008). Although the latter schedule was shown to be efficient in a HPAI challenge model, it might be more advantageous to substitute inactivated vaccines with subunit vaccines in order to better differentiate vaccinated birds from infected ones by testing antibodies against other influenza proteins that would not be included in the subunit vaccine (Differentiating Infected from Vaccinated Animals, DIVA, strategy). Vaccines composed of virus-like particles (VLPs) have been generated for a variety of viruses (Noad & Roy, 2003) and have demonstrated a great potential for the prevention of infectious diseases. Indeed VLPs

were shown to be very immunogenic. In addition, humoral, cell-mediated, and mucosal immune responses have been reported in sheep following immunization with bluetongue virus VLPs (Roy, 1996). The strategy using the immunogenic properties of avian influenza virus surface glycoproteins seems accessible since Latham & Galarza (2001) showed the possibility of producing VLPs using a quadruple recombinant baculovirus encoding hemagglutinin and neuraminidase surface glycoproteins and M1 and M2 matrix proteins of H3N2 human influenza virus. Pushko *et al.* (2005) confirmed this approach by producing VLPs using a triple recombinant baculovirus encoding HA, NA, and M1 matrix of an avian influenza virus H9N2 isolated from children. They also showed that immunization of mice with these VLPs reduced challenge virus replication and conferred a protection against a homologous influenza virus challenge. Although recombinant HA protein has been shown to be immunogenic in poultry (Crawford *et al.*, 1999), no data are available regarding VLPs in this species. If avian influenza virus VLP immunogenicity were to be shown in ducks, VLPs could provide a solution to the drawbacks of inactivated vaccines.

In this context, we previously attempted to develop a H5N3 avian influenza VLP vaccine expressed from insect cells, comprising the two structural proteins HA and NA derived from a recent French LPAI virus strain A/Duck/France/02166/2002 (H5N3) and the M matrix protein derived from an Italian LPAI virus strain A/Chicken/Italy/1067/99 (H7N1) (Prel *et al.*, 2007). We generated a triple recombinant baculovirus and we demonstrated that the three proteins were successfully expressed in infected Sf9 insect cells and displayed the expected biological activity. Although we showed that the three proteins were co-released in cell media, our results in electron microscopy did not display the formation of VLPs. One of the proposed explanations was that one protein might have been expressed in a too limiting quantity that could decrease the yield of VLP released from Sf9 cells. Indeed, the achievement of appropriate expression levels of each protein

represents one of the difficulties in making complex VLPs (van Oers, 2006). In this previous work, the HA and NA proteins were both expressed under the control of the polyhedrin promoter. After several amplifications, the HA gene could be excluded by recombination between the two promoters.

In this study, we have changed the molecular construction strategy and have achieved a novel triple recombinant plasmid that allowed us to optimise and finalize the development of an H5N3 avian influenza VLP vaccine for ducks.

Materials and Methods

Generation of a triple recombinant baculovirus. We used the double recombinant baculovirus plasmid pMH5 previously generated by our team (Prel *et al.*, 2007). In this construction, each gene was located within its own expression cassette that included either the polyhedrin or the p10 promoter and transcription termination sequences. The HA protein (GenBank accession number: AJ632268) was produced under the control of the polyhedrin promoter whereas the M protein (GenBank accession number: AJ416630) was produced under the control of the promoter p10. A full-length cDNA copy of the NA gene of A/Duck/France/02166/2002 (H5N3) strain (GenBank accession number: AJ849934) was cloned downstream of the p10 promoter into pFastBac Dual vector (Invitrogen) at the *XhoI* and *NheI* sites to generate a pN3-2 recombinant plasmid. Then, using a subcloning strategy, the “p10 promoter, N3 gene and HSV *TK* termination sequence” was inserted into a pMH5 recombinant plasmid at the *BstZ17I* and *AvrII* sites to generate the pMH5N3-2 recombinant plasmid (Figure 1). The NA gene and the junctions were sequenced to check that no mutation had been introduced during cloning.

Competent *E. coli* DH10Bac cells (Invitrogen) containing bacmid (baculovirus shuttle vector) and helper plasmid were used to generate recombinant bacmid according to the manufacturer's instructions. Confluent *Spodoptera frugiperda* (Sf9) insect cells seeded in a 25 cm² flask at 2×10^6 cells were transfected with a mixture of 1 µg of bMH5N3-2 bacmid (15 µl) and 15 µl of Cellfectin reagent (Invitrogen). The supernatant was harvested 5 days after transfection. An amplification of virus stock was performed to obtain a higher titre. The infectious titre of the recombinant baculovirus stock was determined by a plaque assay using Sf9 cells.

Indirect immunofluorescence examination of infected Sf9 cells. Sf9 cells were seeded in 96 microwell plates at a cell density of 1×10^5 cells/well, and were infected with the triple recombinant baculovirus at an MOI of 5. At 72 h post infection, Sf9 cells were fixed in methanol-acetone. Fifty µl of SPF chicken serum (1:10 dilution) were dispensed in two wells as negative controls. To examine the expression of H5, N3, and M1 proteins respectively, 50 µl of either chicken-specific H5 or N3 serum (1:10 dilution) or 50 µl of M1 murine monoclonal antibody (ATCC HB 64) (1:100 dilution) were dispensed in duplicate in the other wells. Chicken-specific anti-H5 serum and anti-N3 serum were prepared from SPF chickens that had received three inoculations of either 100 µg pcDNA-H5 plasmid (Prel *et al.*, 2007) or 100 µg pcDNA-N3 plasmid (Prel *et al.*, 2007) in 100 µl with the Medijector device (Antares pharma, Inc, Minneapolis, MN), respectively. The plate was incubated at 37 °C for 45 min. At the end of incubation, the wells were washed three times for 5 min in 200 µl PBS. Then, 50 µl of either rabbit anti-chicken FITC-conjugated antibody (Nordic, 1:36 dilution) or goat anti-mouse FITC-conjugated antibody (Rockland, 1:500 dilution) and Evans Blue (1:75 working dilution) were added in each well and incubated at 37 °C for 45 min. After incubation, wells were washed three times

for 5 min in PBS. Wells were overlaid with 200 µl of PBS/glycerol (v/v) and read under an UV microscope (Olympus, IMT 2). Uninfected cells were used as negative controls and were treated with the different sera as infected cells.

Purification of influenza VLPs. To examine whether the recombinant proteins could self-assemble into VLPs, Sf9 insect cells (35×10^6 cells/flask of 175 cm²) were infected with triple recombinant baculoviruses at an MOI of 5. Three days post infection, infected cells and culture supernatants were harvested and clarified by centrifugation for 30 min at 2,000 g at 4 °C to remove cell debris. VLPs in supernatants were pelleted by ultracentrifugation at 130,000 g at 4 °C for 3 h.

To assess protein expression, the sedimented particles were resuspended in 2 ml of phosphate-buffered saline (PBS) at 4 °C overnight, then loaded onto a 15-55% (w/w) continuous sucrose density gradient and sedimented by ultracentrifugation at 130,000 g at 4°C for 16 h. Fractions of 1 ml were collected from the top of the sucrose gradient, washed by dilution in PBS and sedimented by ultracentrifugation at 195,000 g at 4 °C for 1 h. The pellets were resuspended in 200 µl of PBS and stored at -70 °C.

For electron microscopy and haemagglutination activity, the sedimented particles were purified through a 20-30-50% sucrose density cushion at 130,000 g at 4 °C for 1.75 h. The VLP band was collected at the 30-50% interface and washed with PBS. VLPs were pelleted by ultracentrifugation and resuspended in 60 µl of PBS.

Protein expression. HA and M1 protein expression in each sucrose fraction was analysed by western blotting. The proteins (20 µl) from sucrose-gradient purified VLPs were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10-20% gradient polyacrylamide gels (Biorad), and transferred onto PVDF

membranes (Polyvinylidene fluoride, Amersham). HA protein was identified using specific-pathogen free (SPF) chicken specific serum H5N3/02166 and a peroxidase-labelled rabbit anti-chicken IgG conjugate (Sigma). M1 protein was identified using M1-murine monoclonal antibody (ATCC HB 64) and a peroxidase-labeled goat anti-mouse IgG conjugate (Sigma). NA protein expression and its activity were analysed by neuraminidase inhibition assay using fetuin as substrate for the cleavage of sialic acid. In this test, the NA antibodies present in the serum inhibited the action of the viral enzyme on the fetuin substrate. An aliquot of each sucrose fraction was incubated with chicken H2N3 antiserum for 1 h at room temperature. Then, the neuraminidase inhibition assay was performed as described by van Deusen *et al.* (1983).

To estimate the efficacy of the purification method and the ratio of the three proteins in the VLPs, 10 µl of the 30-50% sucrose interface purified VLPs was analysed by Coomassie blue-stained 10% SDS-PAGE. Otherwise, haemagglutination activity of VLPs was tested using a haemagglutination assay according to international standards (O.I.E., 2004). Two-fold serial dilutions of 25 µl of 30-50% sucrose interface purified VLPs in phosphate-buffered saline were prepared and incubated at room temperature for 30 min with 50 µl of chicken blood cells. The haemagglutination was inspected visually.

Electron microscopy. For negative staining of VLPs, 20 µl of 30-50% sucrose interface purified VLPs were incubated with chicken-specific serum H5N3/02166 (1:10 dilution) at room temperature for 30 min. Then, the sample was adsorbed onto a carbon- and formvar-coated copper grid for 30 min. The excess VLP suspension was removed by blotting with filter paper. The grid was negatively stained with 2% phosphotungstic acid (pH 6.45) for 2 min, air-dried, and examined with a transmission electron microscope (JEOL).

175 **Immunogold electron microscopy.** Twenty μ l of 30-50% sucrose interface purified VLPs
176 were incubated with chicken-specific anti-H5 or anti-N3 serum (1:10 dilution) for 30 min
177 at room temperature. The sample was adsorbed onto a carbon- and formvar-coated grid for
178 30 min and washed three times in PBS. The grids were exposed to 5 nm gold particle-
179 labelled rabbit anti-chicken IgG (Sigma) diluted in PBS (1:30) for 30 min at room
180 temperature, and washed three times in PBS, 5 min each. Finally, the grids were negatively
181 stained with 2% phosphotungstic acid (pH 6.45) for 2 min, air dried, and examined with a
182 transmission electron microscope (JEOL).

183

184 **Immunisation of ducks and haemagglutinin inhibition (HI) test.** For large scale
185 production of VLPs for inoculation of ducks, Sf9 cells were grown in suspension in two
186 250 ml spinner flasks at a density of about 1.0×10^6 cells/ml. They were infected with
187 triple recombinant baculovirus at an MOI of 4 and harvested 90 h post infection. Cell
188 supernatants were purified through a 20-30-50% sucrose density as previously described.
189 VLPs were resuspended in 180 μ l of PBS. Protein concentration of the purified fraction
190 was determined by using a colorimetric assay (MicroBC Assay Protein Quantification Kit,
191 Uptima) with bovine serum albumin as the standard. Fifteen 3.5-week-old specific-
192 pathogen-free (SPF) Muscovy ducks (*Cairina moschata domestica*) from our institute,
193 split into 3 groups of 5 ducks each, were housed in a room in our BSL2+ experimental
194 facilities with filtered air. The first group was used as an SPF control and ducks remained
195 unvaccinated. Ducks of the second and the third groups were inoculated subcutaneously
196 three weeks apart with 20 μ g and 2 μ g (total protein per dose), respectively, of purified
197 VLPs formulated with an oily adjuvant (kindly provided by Merial), in a final volume of 1
198 ml/duck. Blood samples were collected from 5 ducks at random before immunisation, then

in all ducks just before boost, and when the ducks were killed three 3 weeks after the boost.

To assess HA responses, every serum sample was analysed by HI test. HI tests were performed according to international standards (OIE, 2004) using 4 haemagglutinin units of inactivated A/duck/France/02166/2002(H5N3) AI as antigen. Antibody titres were expressed as $\log_2$ of the highest dilution giving 100% inhibition of the haemagglutination. The threshold was defined as $\geq 4 \log_2$.

Results

Expression of recombinant proteins in insect cells. In order to generate H5N3 VLPs in Sf9 insect cells, HA and NA genes derived from a recent French low-pathogenic avian influenza A/Duck/France/02166/2002 (H5N3) virus strain and the M gene derived from an Italian A/Chicken/Italy/1067/1999 (H7N1) virus strain were introduced into a baculovirus shuttle vector to obtain a triple recombinant plasmid pMH5N3-2. In our previous study (Prel *et al.*, 2007), HA and NA proteins were both expressed under the transcriptional control of the polyhedrin promoter. In the present study, the NA gene was inserted in the same orientation under the control of the p10 promoter in order to avoid eventual competition between HA and NA genes. The M gene was inserted in the opposite orientation under the control of the p10 promoter (Figure 1).

To evaluate the expression of HA, NA, and M1 proteins in Sf9 insect cells, the pellets of insect cells infected with this triple recombinant baculovirus were analysed by western blot and neuraminidase inhibition assay. As previously reported using the first baculovirus construct (Prel *et al.*, 2007), we could check that the three proteins were efficiently

expressed in Sf9 cells following infection with the new triple recombinant baculovirus construct (data not shown). To localize the subcellular distribution of the expressed proteins, we performed indirect immunofluorescence analyses of Sf9 cells infected with bMH5N3-2. Observations confirmed the expression of all three proteins. Staining experiments with anti-H5 and anti-N3 antibodies showed that HA and NA expressed proteins were localized at the level of the cell membrane (Figure 2), as previously shown with recombinant baculoviruses expressing human influenza HA and NA proteins (Latham & Galarza, 2001). Staining experiments with anti-M1 antibodies showed that the M1 expressed protein was localized at the level of the cell membrane, although the fluorescence intensity was poor (Figure 2).

Influenza VLPs were purified from culture media 72 h post-infection by 15-55% continuous sucrose gradient centrifugation. Seven fractions (sucrose densities: 26.9% to 52.7%) were analysed by western blotting for HA and M1 expressions. The same fractions were assessed by a neuraminidase inhibition assay for NA expression and activity.

As shown in Figure 3A, the majority of HA protein (66 to 68 kD, approximately) was detected in fractions 7 to 11 corresponding to sucrose densities of 33.1% to 49.2%. The HA electrophoretic migration pattern differed from native HA (75 kD) synthesized during virus multiplication on embryonated-eggs, presumably reflecting glycosylation differences between avian and insect cells. We observed the same HA electrophoretic migration pattern for the HA protein expressed in Sf9 mimic insect cells (Invitrogen) which have been modified to greatly enhance the production of complex sialylated N-linked glycans to express recombinant proteins like those found in mammalian systems (data not shown). Regarding the M1 protein (29 kD), the majority was detected in fractions 8 to 11 corresponding to sucrose densities of 36.5 to 49.2% (Figure 3B). Western blot analyses revealed that HA and M1 protein expressions were lower in fraction 9 (Figure 3, A and B)

but this observation was correlated with a lower protein concentration in this fraction. The NA protein and NA activity were detected in the 7 to 11 fractions (Figure 3C). The yield of VLPs production in the whole of the 7 to 11 sucrose fractions was estimated to be about 1.4 µg per ml of Sf9 cell culture by using a colorimetric assay.

All these results demonstrated that insect cells infected with triple recombinant baculoviruses co-expressed the three proteins. These were co-released in the culture medium and were all present at the same sucrose densities of 36.5% to 49.2%, indicating that HA, NA, and M1 proteins could self-assemble into VLPs.

Evaluation of influenza VLPs by electron microscopy and by Coomassie blue-stained SDS-PAGE. In order to check whether these proteins were released from cells into culture medium as VLPs that budded from the cell surface, concentrated supernatant was purified on 20-30-50% sucrose density cushions. Influenza VLPs were stopped by 50% sucrose cushion while baculovirus passed through (Pushko *et al.*, 2005). The 30-50% interface band was analysed by electronic microscopy and Coomassie blue-stained SDS-PAGE.

Immuno-electron microscopy examination of negatively stained samples revealed the presence of a great number of VLPs. These particles were morphologically identical to the wild-type virus. Indeed, there were spherical-shaped particles with a diameter of approximately 80 to 120 nm, with surface spikes (Figure 4). In the 20-30 % sucrose interface, we observed membrane parts that confirmed western blotting results for which, only HA and NA proteins gave a positive signal at sucrose density fractions of 33.1 %.

Coomassie blue-stained SDS-PAGE of 30-50% sucrose interface purified VLPs showed the good purity of the fraction containing VLPs (Figure 5). The 3 proteins were visualized and we estimated the HA and NA protein ratio in particles to be one NA for at

least four HA, which is very close to the theoretical ratio of 5 to 1, whereas the relative amount of the M1 protein was low.

Immunogold electron microscopy and haemagglutination activity of VLPs. We investigated the protein composition of the spikes that protruded from the surface of the VLPs using immunogold electron microscopy. VLPs incubated first with an anti-HA serum, then with a gold-labelled anti-chicken conjugate, were displayed by transmission electron microscopy. The examination revealed gold particles on the surface of VLPs and demonstrated the presence of HA proteins (Figure 6A). Similarly, immunogold labelling with the anti-NA antibody and electron microscopy revealed the presence of NA glycoproteins on the surface of VLPs (Figure 6B).

Regarding haemagglutination activity of VLPs, the assay revealed a high activity (1:256 dilution) and demonstrated that the HA proteins present on the surface of VLPs were biologically active.

Immunogenicity in ducks. The immunity induced by the VLPs was checked in SPF Muscovy ducks. As expected, no H5-specific serum antibody was detected in ducks before vaccination or in unvaccinated animals during the assay. After the prime inoculation all ducks developed HI titres that increased after the boost. After the boost and regardless of the VLP quantity inoculated, all the ducks exhibited positive HI titres ($\geq 4 \log_2$) (Table 1). Geometric means of HI titres of both inoculated groups were almost similar, indicating that a 10-fold quantity of VLP did not enhance the serum antibody response. In addition, the standard deviations were very low whatever the tested dose, underlying no significant individual variation in the antibody response.

Discussion

Since H5 LP avian influenza viruses can mutate into HPAI viruses, it is necessary to prevent their introduction and spread in poultry because such infections can be the source of serious epizootics that could have public health consequences. Recombinant vaccines have been generated and evaluated for ducks. They were used in combination with inactivated vaccines (van den Berg *et al.*, 2008). Since influenza VLPs produced by recombinant baculoviruses emerged as promising candidates for influenza vaccines (Latham & Galarza, 2001), we thought that substituting inactivated vaccines by subunit vaccines as VLPs might be more advantageous, notably regarding the ability to easily differentiate vaccinated birds from infected ones. In addition, Bright *et al.* (2007) confirmed that human influenza VLPs elicited broader reactive immune responses than baculovirus-produced recombinant HA. Regarding the strategy used to produce influenza VLPs, when we initiated this work, several models were described that differed on the type of viral proteins required. Latham & Galarza (2001) demonstrated that the expression of four structural proteins (HA, NA, M1, and M2) was sufficient for the formation and the release of VLPs, whereas one other team (Pushko *et al.*, 2005; Pushko *et al.*, 2007; Bright *et al.*, 2007) described the development of H9N2 or H3N2 influenza VLPs comprised of only three proteins (HA, NA, and M1). Another study reported the assembly and the release into cell culture supernatant of VLPs containing only HA and M1 proteins following co-infection with two individual recombinant baculoviruses (Galarza *et al.*, 2005). This approach was recently used by Quan *et al.* (2007) but not by Galarza's team that used again quadruple recombinant baculovirus proteins to prepare a VLP vaccine aimed to protect against infection with the H1N1 1918 influenza virus (Matassov *et al.*,

2007). The various models of avian influenza VLPs that have been developed to date have always included the matrix protein M1. This protein, which is the most abundant in the virus particle, was shown to be critically important for viral morphogenesis and budding (Lohmeyer *et al.*, 1979; Roberts *et al.*, 1998). Studies of VLP formation have demonstrated that the assembly, budding, and release process was driven solely by the expression of the M1 protein (Gomez-Puertas *et al.*, 2000). However, a recent study seems to refute this. Chen *et al.* (2007) developed a noncytotoxic plasmid-based VLP system and showed that M1 was not required to produce VLPs morphologically similar to authentic virions. They revealed that influenza particle budding is not driven by M1 but is rather dependent on the expression of cytoplasmic tails of the two proteins HA and NA. However, they confirmed that M1 is required to produce an infectious VLP. Currently, no data are available to confirm that VLPs comprised of only HA and NA proteins could be developed.

Taking these results into account and considering the fact that the different studies did not experimentally demonstrate the role of the M2 protein in VLPs (Latham & Galarza, 2001), we chose to construct a triple recombinant baculovirus plasmid encoding HA, NA, and M1 proteins. In the context of production of an avian subunit vaccine, it was interesting to exploit the immunogenicity of the NA protein besides the HA one. Indeed, Brett & Johansson (2005) showed that immunization of mice with both baculovirus-expressed HA and NA proteins resulted in a broader immune response and induced a greater reduction in viral replication following a viral challenge by a distantly related heterologous influenza strain, in contrast to the protection afforded by immunization based on the HA protein alone. These results suggest that a vaccine containing the NA protein tolerates more antigenic drift (van Oers, 2006).

We have followed the strategy used by Latham & Galarza (2001) and Pushko *et al.* (2005) that consists of expressing several proteins simultaneously using one recombinant

348 baculovirus instead of several individual ones. Indeed, the achievement of VLPs following
349 either co-infection of cells with three single recombinant baculoviruses HA, NA and M1,
350 or co-infection of cells with a double recombinant baculovirus HA-M1 and a single
351 recombinant baculovirus NA, was shown to be inconclusive (Pushko *et al.*, 2005). In a first
352 approach, we constructed a triple recombinant plasmid by subcloning the expression
353 cassette “polyhedrin promoter, N3 gene and HSV *tk* termination sequence” of the
354 recombinant baculovirus plasmid pNA downstream the HA gene into the double
355 recombinant plasmid pMH5 previously generated, resulting in a pMH5N3 plasmid (Prel *et al.*, 2007). Thus in this approach, HA and NA proteins were both expressed in the same
356 direction under the control of the polyhedrin promoter, according to the strategy followed
357 by Pushko *et al.* (2005). Although Prel *et al.* (2007) showed that the three proteins were
358 co-released into the cell supernatant, electron microscopy did not reveal the presence of
359 VLPs, suggesting that either the number of VLPs generated might have been too low to be
360 detected by electron microscopy or no VLPs had produced.

362 Compared to the strategy followed by Pushko *et al.* (2005), we have kept HA and NA
363 genes in the same orientation but have changed the molecular construction by expressing
364 each protein under the transcriptional control of two different promoters to avoid eventual
365 competition between the two genes. Western blots and neuraminidase inhibition assay
366 indicated that all three proteins were expressed in infected Sf9 insect cells and were
367 localized at the level of the cell membrane, as expected (Latham & Galarza, 2001).
368 Electron microscopy results showed that these proteins were assembled and budded from
369 insect cells to be released as virus-like particles in the culture medium and resembled
370 influenza virus particles in morphology and size. VLPs bore surface spikes that were
371 anchored in the membrane of the particles and were projected outward as HA and NA

proteins, as shown by immunogold labeling and electron microscopy. In addition, VLPs exhibited haemagglutination and neuraminidase activities.

The experimental assay performed in SPF Muscovy ducks showed that inoculation of VLPs induced a positive HA antibody response for all the VLP-inoculated animals that confirm the biological activity of the VLPs and their immunogenicity in ducks. The levels of H5 antibodies elicited by the 2 tested VLP doses were similar suggesting that vaccination with as little as 2 µg of total protein is sufficient to induce a significant immune response. In our previous study, we immunized specific pathogen free Muscovy ducks with lysates of Sf9 cells infected with a triple recombinant baculovirus (bMH5N3) to assess protein immunogenicity and protection afforded following a homologous A/Duck/France/02166/2002 (H5N3) virus challenge (Prel *et al.*, 2007). We showed that the three baculovirus-expressed proteins were immunogenic. The ducks of the vaccinated group exhibited a geometric mean of 5.9 log₂ titres using HI test and after challenge, the vaccination afforded a significant decrease of cloacal shedding and a delayed peak of tracheal shedding. In the present study, we demonstrate that VLPs induce HI titres similar to those shown as being protective in ducks. Although further experiments in ducks will be necessary to definitely demonstrate the protective immune response induced by the VLPs, given our previous results of protection, the functional properties of the HA and NA proteins presently expressed as VLPs, and the immunogenicity of VLPs in ducks, we are optimistic. A trial is scheduled as soon as is available the recombinant vaccine intended for ducks and currently in preparation through the European project NOVADUCK. Indeed, we target the use of our VLPs for boost after priming with the recombinant vaccine.

In conclusion, the achievement for the first time of low pathogenic H5 avian influenza VLPs should allow the development of safe and effective subunit vaccines to control the spread of LPAI in ducks. VLPs are more easily purified than recombinant proteins and

often give strong immune reaction (van Oers, 2006). Moreover, according to the epidemiological context, it would be easier to replace H5 or/and N3 gene from the triple-recombinant plasmid by more adapted subtype genes, and to rapidly produce a new vaccine. All these results make feasible the reliable use of avian influenza VLPs as subunit vaccines in ducks, notably as a boost immunisation by either the parenteral or mucosal route, after a prime immunisation with a recombinant vaccine. In addition, very recently, Bright *et al.* (2008) confirmed the potential of such a VLP vaccine as an effective immunogen and delivery system for influenza antigens to protect mice against lethal human H5N1 influenza isolates.

Acknowledgement

The authors are grateful to Anne-Cécile Nignol, from the Virus Genetics and Biosecurity unit at AFSSA, for insect cell cultures. They thank Eric Niqueux, Carole Guillemoto, and Marie-Odile Le Bras from the unit for their assistance in purification of VLPs and serological analyses, as well as the personal of the Service of Breeding and Experimentation in Avian Pathology of AFSSA. They wish to acknowledge Elisabeth Reperant for the review of English expression. This work was partially funded by the European REX Epizone.

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Figure legends

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Table 1. *Immunogenicity of VLPs for Muscovy ducks.*

Group (5 ducks)	HI titre (log ₂) ^a	
	3 weeks post-prime (before boost)	3 weeks post-boost
1 (unvaccinated)	<2	<2
2 (20 µg VLPs)	3.2 (0.4)	5.4 (0.9)
3 (2 µg VLPs)	2.8 (0.4)	5.2 (0.4)

^a Geometric mean (standard deviation in parenthesis). The HI test was performed using 4 HA units of the inactivated A/duck/France/02166/2002(H5N3) AI strain. Positive results are defined as $\geq 4 \log_2$.

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