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Characterization and Protection in Mice of Outer Membrane Proteins from P*asteurella multocida* A:1 Incorporated in Lipid Vaccine Delivery Systems

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ABSTRACT: Fowl cholera is an infectious disease affecting poultry and is caused by *Pasteurella multocida*. To develop a subunit vaccine, outer membrane proteins (OMPs) from *P. multocida* serotype A:1 strain NIAH DU1551/97 were extracted and characterized using SDS-PAGE. The OMPs were solubilized in detergent or incorporated in lipid-based antigen delivery systems, namely virosomes made from Newcastle disease (ND) virus and immunostimulating complexes (ISCOMs). The formulations were characterized by several physicochemical methods. To evaluate the formulations as possible vaccine, their potency was tested in mice. Eleven proteins that range in size from 30 to 80 kDa were detected in the OMP fraction. The most prominent protein bands were 30, 33, and 45 kDa. Virosomes and ISCOMs showed an average diameter of 180 and 30 nm, respectively. OMPs incorporated in the virosomal membrane inhibited the capability of virosomes to agglutinate chicken red blood cells. Animals were challenged after two immunizations. All vaccines fully protected mice against a low dose challenge. In the case of a high dose challenge, micellar OMPs provided 80% protection, whereas OMPs incorporated in virosomes or ISCOMs gave 100% protection, which is comparable to that of inactivated whole cell vaccines. In conclusion, antigen loaded virosomes and ISCOMs are potential pasteurella subunit vaccines.

KEYWORDS: ISCOMs, Newcastle disease-virosomes, Outer membrane proteins, *Pasteurella multocida*, subunit vaccine.

List of abbreviations: BHI, brain heart infusion; CFU, colony forming units; Chol, cholesterol; DLS, dynamic light scattering; EPC, egg phosphatidyl choline; F protein, fusion protein; HA, hemagglutination activity; HN, hemagglutinin-neuraminidase; ISCOMs, immunostimulating complexes; MW, molecular weight; ND, Newcastle disease; OMPs, outer membrane proteins; PE, phosphatidyl ethanolamine; PBS, phosphate buffered saline; PI, protective index; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TSAB, tryptic soy agar with 5% defibrinated sheep blood; WC, whole broth culture.

INTRODUCTION

Pasteurella multocida, a gram-negative bacterium, is the causative agent of fowl cholera and other diseases of wild and production animals.[1] Based on epidemiological information collected in Thailand, strains of serogroup A are recognized as the primary cause of fowl cholera, whereas isolates of serogroups B, D and F are less frequently associated with disease.[2] Vaccination is the most attractive approach for controlling the disease. Two types of vaccine against *P. multocida* are currently being used for immunization of poultry: attenuated and inactivated vaccines. Recently, the development of subunit vaccines comprising purified subunits of this microorganism has been promoted. Outer membrane proteins (OMPs) from *P. multocida* have been isolated. They provided various degrees of protection against a challenge.[3-6]

Apart from the discovery and selection of potent immunogens, a key factor to the success of vaccine development is proper delivery of the immunogens to the immune system.[7] To date, there are no data available in the literature on the role of antigen delivery systems for OMPs of *P. multocida* on vaccine efficacy. This

prompted us to prepare several formulations of OMPs of *P. multocida* and to test their protective efficacy in mice. Membrane proteins, such as OMPs, generally can be presented as protein micelles, liposomes, or immunostimulating complexes (ISCOMs). Liposomes are phospholipid vesicles, which have been evaluated as antigen delivery systems. However, they frequently show relatively low adjuvant activity and need co-incorporation of adjuvants.[8] Almeida *et al.* produced a liposomal influenza vaccine with influenza hemagglutinin proteins intercalated in the lipid bilayer.[9] These new liposomes are termed "virosomes". Virosomes offer the opportunity to exploit the targeting and fusogenic properties of the native viral membrane proteins, which may result in effective delivery of antigens into the immuno-responsive cells.[8, 10, 11]

Newcastle disease (ND) virus is an avian paramyxovirus that also causes an economically important and highly contagious disease of poultry. Two surface membrane proteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), of the ND virus are the important immunogenic antigens. These two proteins play a role in viral infection with HN mediating the attachment of virus to the host cell receptor, followed by F facilitating the fusion between viral particles and the host cell membrane.[12] For poultry vaccination, therefore, virosomes containing HN and F proteins of ND virus might offer the potential for delivery of co-incorporated antigens analogous to influenza virosomes for human vaccines.[13]

Immunostimulating Complexes (ISCOMs) are another lipid particulate system that has been studied for the delivery of subunit antigens. ISCOMs are small colloidal (30-40 nm) structures composed of phospholipid, cholesterol, a saponin mixture (Quil A), and the antigens to be incorporated. In particular, ISCOMs are potent enhancers of the immune response against membrane proteins.[7, 8]

Since no studies reported the employment of antigen delivery systems for OMPs isolated from *P. multocida*, the present study was designed to evaluate the protective efficacy of pasteurella subunit vaccines based on OMPs, formulated as protein micelles, virosomes, and ISCOMs, against a bacterial challenge in a mouse model.

Materials and Methods

Reagents

Egg phosphatidyl choline (EPC) and phosphatidyl ethanolamine (PE) were purchased from Avanti Polar Lipids Co. (Birmingham, AL, USA). Cholesterol (Chol) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Quil A was supplied by Iscotec (Lulea, Sweden). All other chemicals were analytical grade and used as received without further purification.

Animals

Specific pathogen-free ICR mice, 4 weeks of age, were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed in cages (10 per cage) and supplied with food and water *ad libitum*.

Bacterial Strain

P. multocida strain NIAH DU1551/97 of serotype A:1 was selected as a strain representative of local isolates for extraction of outer membrane proteins (OMPs). Before use, the bacterial culture was passaged in a mouse to enhance the virulence.

Preparation of Inactivated Bacterial Vaccine

P. multocida, FC-Pakchong, a commercial strain currently used in Thailand and *P. multocida*, strain NIAH DU1551/97, were grown in 100 ml brain heart infusion (BHI) media (Difco) for 18-20 h at 37°C with gentle aerated shaking. The final concentrations were 1.15×10^{10} cfu/ml and 8.5×10^{9} cfu/ml of *P. multocida* strains FC-Pakchong and NIAH DU1551/97 respectively, as determined by standard plate count technique. Purity was checked by Gram-staining and biochemical testing.[14] The broth culture was inactivated by addition of formalin to a final concentration of 0.3% (v/v) and overnight incubation at room temperature with stirring. The inactivated whole broth culture (WC) was finally adsorbed onto aluminum hydroxide gel (25% of $Al(OH)_3$ suspension, provided by the Veterinary Biologic Products Division, Department of Livestock Development, Thailand).

Isolation of Outer Membrane Proteins (OMP's) from *P. multocida*

The OMPs were extracted following the method of Choi-Kim *et al.* with some modifications.[15] *P. multocida* strain NIAH DU1551/97 was grown on the surface of tryptic soy agar (Difco) with 5% defibrinated sheep blood (TSAB). The agar plates were incubated at 37°C for 18 h. Two colonies of the culture were inoculated into 12 ml of BHI broth and incubated without shaking at 37°C for 18 h. Next, 10 ml of broth culture was transferred into 1 liter of BHI medium and incubated in a shaker chamber at 37°C for 18 h. Bacterial cells were sedimented by centrifugation at 12,000 x g for 20 min at 4°C. The cells were then washed twice with sterile saline (0.15 M NaCl) and finally resuspended in 25 ml of Tris-EDTA (50 mM Tris; 1 mM EDTA, pH 7.2). The suspension of cells was passed three times through a French press cell at 1,160 kg/cm^2 (40K Spectronic). Cell debris was removed by centrifugation at 5,000 x g for 15 min at 4°C. The suspension containing OMPs

was further pelleted by ultracentrifugation (180,000 x g in a 50 Ti fixed angle rotor at 4°C for 1 h). The pellet was re-suspended with 1.8 ml of Tris-EDTA buffer and solubilized by adding 200 µl of 20% w/v Triton X-100 (giving a final concentration of 2% w/v). The mixture was incubated at room temperature for 1 h and kept at –70°C until use.

Preparation of ND virus

The La Sota strain of ND virus was propagated by inoculation of diluted viral stocks in phosphate buffered saline (PBS, pH 7.2) into the allantoic sac of 10-day-old embryonated chicken eggs. After 72 h of incubation at 37°C, the eggs were cooled overnight. All subsequent steps were carried out at 4°C. The allantoic fluid was harvested and clarified by centrifugation at 3000 x g for 30 min. ND virus in allantoic fluid was pelleted by ultracentrifugation at 100,000 x g for 150 min (Beckman, 50Ti fixed angle). The pelleted virus was suspended in 1/50th of the original volume in Tris-EDTA buffer, pH 7.2. The virus suspension was layered on top of a discontinuous gradient of 1.5 ml each of 50%, 40%, 30%, 20%, and 10% (w/w) sucrose.[12] After ultracentrifugation at 140,000 x g for 6 h (Beckman, SW40 Ti), virus at the interface was collected and disaggregated by passing the suspension up and down in a pipette several times. The virus was stored at –70°C until needed.

Incorporation of OMPs in ND-Virosomes

Virosomes were produced as previously described for influenza virus with some modifications.[16] The purified viral particles were ultracentrifuged at 100,000 x g for 150 min at 4°C (fixed angle rotor 50Ti), and the pellet obtained was suspended in 1 ml Tris-EDTA buffer containing 2% w/v Triton X-100. After incubation at room temperature for 1 h, the suspension was layered on top of 20% sucrose in Tris-EDTA solution. Next the mixture was ultracentrifuged at 140,000 x g for 1 h at 4°C to remove detergent-insoluble substances, which presumably contained nucleocapsid proteins. The clear supernatant containing the phospholipids and glycoproteins of the ND virus in Triton X-100 was analyzed for phospholipid, protein content, and hemagglutination activity as described below.

For the preparation of OMP-containing ND virosomes, firstly, a lipid film composed of EPC:PE:Chol at a mol ratio of 2:1:2 was prepared by evaporating the organic solvent. Then a purified viral envelope solution was added to the dried lipid film to give a mol ratio between viral lipid and external phospholipid of 3:7. Next, a volume of 100 µL solution of OMPs (1.5 mg solubilized in 2% w/v Triton X-100) was added to the viral envelopes/lipid mixture followed by incubation at room temperature for 1 h. OMP-containing ND virosomes were then formed by detergent removal using Biobeads SM-2. For each 20 mg of Triton X-100 present in the preparation, 80 mg of Biobeads SM-2 (previously washed with methanol and dried on Wattman filter paper) was added. After incubation for 2 h at room temperature with robust shaking (1400 min^{-1}; MTS-2, IKA Schüttler, Staufen, Germany), the mixture was incubated further for 30 min without shaking, as a resting period. Next, a second portion of 35 mg Biobeads SM-2 was added and incubation was continued for 1 h at room temperature with vigorous shaking, followed by 30 min without shaking. The last portion, 35 mg of Biobeads SM-2, was added and the mixture was shaken for another 30 min. After incubation, the liquid phase containing the formed OMP-containing ND virosomes was sucked out with a 1 mL tuberculin syringe, into which Biobeads SM-2 cannot penetrate. To separate the OMP-containing ND virosomes from non-incorporated components, the preparation was layered on top of a discontinuous sucrose gradient containing 1 mL each of 50% and 40%, 2 mL of 10%, 1 mL of 5% sucrose. The rest of the centrifuge tube was filled with Tris-EDTA buffer. The samples were ultracentrifuged at 140,000 x g for 2 h at 4°C (SW40 Ti). The virosomal band was then isolated and dialyzed (with 10,000 molecular weight cut-off membrane) against several changes of Tris-EDTA buffer.

Incorporation of OMPs in ISCOMs

OMP containing ISCOMs were prepared by the classical centrifugation method.[17] Briefly, a lipid film containing PE and Chol (0.5 mg each) was prepared by evaporating the organic solvent under a nitrogen stream. The lipid film was solubilized with 0.8 ml of Tris-EDTA buffer containing 2% Triton X-100 (w/v). Next, 100 µL of 10% w/v Quil A and 100 µL of OMPs (containing 1.5 mg OMPs) were added to the lipid solution. The mixture was incubated for 1 h at room temperature. The weight ratio of Quil A: PE: Chol: protein antigens was 2:1:1:3. The Triton X-100 present in the mixture was then removed by vigorously shaking the mixture with 150 mg Biobeads SM-2 at room temperature for 4 h as described in 'Incorporation of OMPs in ND virosomes' (above). To remove non-incorporated components from ISCOM particles, the mixture was then applied onto a discontinuous sucrose gradient comprising of 1.5 ml each of 10%, 20%, 30%, 40%, 50%, and 60% w/v sucrose solution in 0.05 mM Tris-1 mM EDTA. After ultracentrifugation at 140,000 x g (SW40 Ti) for 18 h at 25°C, the ISCOMs band was collected and subjected to dialysis (10,000 molecular weight cut-off membrane) for 48 h against Tris-EDTA buffer. The resulting dispersion was collected.

Characterization of OMP Formulations

The micellar OMPs and OMP-containing ND-virosomes and ISCOMs were assayed for protein composition by SDS-PAGE under non-reducing conditions. Total protein concentration was determined by the method of Peterson using bovine serum albumin as relative standard.[18] Size and morphology of virosomes and ISCOMs were verified by negative-staining electron microscopy (H-7500, Hitachi, Tokyo, Japan) using 2% uranyl acetate. Size of virosomal vesicles and ISCOMs was measured by dynamic light scattering (DLS) (Malvern 4700, Malvern Instrument Ltd., Worcestershire, UK). Phospholipid content was measured according to Rouser's procedure.[19]

Hemagglutination Activity (HA)

The hemagglutination assay was performed as described.[20] In short, 50 µL of dispersion of ND virus, empty virosomes, or virosomes containing OMPs were applied to the first well of each row in a U-shaped 96-wells plate and serial 2-fold dilutions were made until the last well of each row. A 1% suspension of freshly isolated chicken erythrocytes in PBS (pH 7.2) was prepared and 100 µL of this suspension was applied to each well. Erythrocytes were allowed to sediment for 45 min. The agglutination was monitored visually.

Determination of the LD_{50} of the Challenge Strain in Mice

ICR mice, 4 weeks of age, were used throughout. The serial tenfold dilutions of exponential phase broth cultures grown at 37°C without shaking of *P. multocida* NIAH DU1551/97 were prepared in normal saline solution. Groups, of 10 mice each, were injected with serial dilutions. Mice which were moribund and deemed incapable of survival were euthanized. The LD_{50} was determined to be 6 cfu/animal by the method of Reed and Muench.[21]

Immunization and Challenge Experiments in Mice

Each group of 10 mice was vaccinated intraperitoneally with 0.2 ml vaccine. Five formulations were tested: micellar OMPs (60 µg protein), virosomal OMPs (60 µg protein), ISCOMs containing OMPs (60 µg protein), vaccine containing 2.3×10^9 of *P. multocida* commercial strain (FC-Pakchong), and 1.7×10^9 cfu of *P. multocida* selected strain (NIAH DU1551/97) adsorbed onto $Al(OH)_3$ adjuvant, respectively. Two weeks later, a booster dose of the corresponding vaccine formulation was administered intraperitoneally as well. Mice of group 6 were the unvaccinated controls.

The challenge of vaccinated and unvaccinated control mice was done by subcutaneous administration of 100 x (low dose) and 1,000 x (high dose) LD_{50} of a 18-h culture of *P. multocida* strain NIAH DU1551/97 in BHI one week after the booster vaccination. Challenged mice were observed for a period of 5 days and mortalities recorded. Moribund mice were euthanized and counted as mortality. The protective index (PI) was calculated for each group with the following formula:

$$PI = \frac{\%\text{Mortality in controls} - \%\text{Mortality in vaccinates}}{\%\text{Mortality in controls}} \times 100$$

Table 1. Physicochemical analysis of virosomes and ISCOMs containing OMPs of *P. multocida* strain NIAH DU1551/97

Preparation	Phospholipid (nmole/ml)	Protein(µg/ml)	Particle size (nm)	PD[a]
Virosomes	0.35 ± 0.07	288 ± 11	183 ± 4	0.35
ISCOMs	0.22 ± 0.05	273 ± 22	30 ± 3	0.42

[a]Polydispersity index (PD) indicates particle size distribution, which ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.
Data represent mean ± S.D. of 3 different samples for each preparation.

Table 2. Effect of OMPs associated with the virosomal bilayer on the hemagglutination capacity of virosomes.

Preparation	Protein concentration (µg/ml)	Hemagglutination end point
ND virus[a]	656	1:4,096
Plain virosomes[b]	160	1:2,048
OMP-containing virosomes[c]	288	<1:2
No virosomes	0	<1:2

[a]Purified Newcastle disease (ND) virus
[b]Virosomes prepared from ND viral envelope bearing viral protein spikes.
[c]Virosomes prepared from ND viral envelope bearing viral protein spikes and OMPs of *P. multocida* strain NIAH DU1551/97.

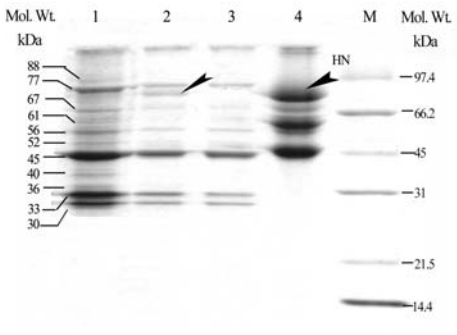

Fig 1. SDS-PAGE of micellar outer membrane proteins (OMPs) isolated from *P. multocida* (lane 1), virosomal OMPs (lane 2), ISCOMs containing OMPs (lane 3), NDV (lane 4), and molecular weight marker proteins (M).

Results

Isolation of OMPs

P. multocida strain NIAH DU1551/97 serotype A:1 was disintegrated using a French press technique. The OMPs were extracted by incubation with Triton X-100. As shown in Fig. 1 (lane 1), the protein profile of OMPs in SDS-PAGE analysis revealed up to eleven protein bands. The molecular weights (MW) of the major polypeptide bands were in the range of 30 to 80 kDa, as estimated by comparison with the MW marker. The most prominent polypeptide bands had a MW of 30, 33, and 45 kDa.

Characterization of OMP-Containing ND-Virosomes and ISCOMs

OMPs were reconstituted in the ND-virosomes and in ISCOMs. The average diameter of the OMP-containing ND-virosomes was about 180 nm with a polydispersity index of 0.35 as measured by DLS. The OMP-containing ISCOMs had an average size of about 30 nm with a relatively high polydispersity index of 0.42. The size of the virosomes and ISCOMs was confirmed by TEM, which also demonstrated the unilamellar nature of the virosomes and the cage-like structures of the ISCOMs (results not shown). Physicochemical characteristics of OMP-containing virosomes and ISCOMs are summarized in Table 1.

SDS-PAGE analysis of virosomes and ISCOMs (Fig. 1, lane 2 and 3) showed typical OMP patterns with prominent bands at MW 30, 33, and 45 kDa. An additional protein band of MW 70 kDa was also apparent in the virosome sample. This band coincided with one ND viral membrane protein, hemagglutinin-neuraminidase (HN) (of lane 2 and 4, Fig. 1). The influence of the presence of bacterial membrane proteins on the surface of the virosomes on the capacity to agglutinate chicken red blood cells was studied by an hemagglutination assay. Co-reconstitution of ND viral membrane and OMPs affected the capacity to agglutinate red blood cells. As shown in Table 2, plain ND-virosomes had high HA, but ND-virosomes bearing OMPs did not show any agglutination. Apparently, the presence of OMPs on the surface of virosomes completely inhibited HN functionality.

Vaccination and Challenge Exposure

To assess the adjuvant potential of virosomes and ISCOMs containing OMPs, mice were immunized twice by intraperitoneal immunization with various OMP formulations, followed by a challenge with 100x (low dose) or 1000x (high dose) of the LD_{50} of *P. multocida* NIAH DU1551/97. The results of this study are presented in Table 3. For the low dose challenge, mice

Table 3. Protection against low and high dose homologous challenge[a] induced by various formulations containing OMPs extracted from *P. multocida* in ICR mice.

Challenge Dose	Formulation[b]	Mortality	Protective Index (%)
100 x LD_{50}	Micellar OMPs	0/10	100
	OMPs Virosomes	0/10	100
	OMPs ISCOMs	0/10	100
	WC-FC Pakchong[c]	0/10	100
	WC-NIAH DU1551/97[c]	0/10	100
	Negative Control	8/10	
1,000 x LD_{50}	Micellar OMPs	2/10	80
	OMPs Virosomes	0/10	100
	OMPs ISCOMs	0/10	100
	WC-FC Pakchong	0/10	100
	WC-NIAH DU1551/97	0/10	100
	Negative Control	10/10	

[a]The mice were challenged by injecting 0.2 ml brain-heart infusion broth containing live *P. multocida* NIAH DU1551/97 subcutaneously.
[b]All subunit vaccine formulations contained approximately 60 mg OMPs/dose.
[c]Inactivated whole broth culture of *P. multocida* strain FC-Pakchong and NIAH DU1551/97, respectively that were adsorbed onto aluminum hydroxide gel to 25%(v/v).

immunized with any of the subunit pasteurella vaccines (micellar OMPs, virosomes, and ISCOMs) or inactivated whole cell vaccines showed 100 percent survival, whereas the survival of non-immunized mice was low (20%). For the high dose challenge, none of the control mice survived. The protective index (PI) was 80% for the group receiving micellar OMPs. OMPs associated with virosomes and ISCOMs gave a PI of 100%, as did the inactivated whole cell vaccines.

Discussion

OMPs of Pasteurella spp. have been studied as potential immunogens and antibodies against them demonstrate a strong protective activity.[5,6,22,23] Several authors demonstrated that OMPs of *P. multocida* may be used as the components of subunit vaccines.[1,3,4,22] Membrane proteins used as candidate vaccine components usually require a delivery system that preserves and stimulates the immunogenicity. To date, there is no study that investigates the role of the antigen delivery system for OMPs of *P. multocida* on vaccine efficacy. Hence, it was desirable to characterize and compare the efficacy of subunit vaccine candidates in the form of micellar OMPs, OMP-containing ND-virosomes and ISCOMs.

In this study, *P. multocida* serogroup A:1 strain NIAH DU1551/97 isolated from dying birds was disrupted by the French press technique. The disintegrated bacteria were treated with Triton X-100 to extract OMPs. The polypeptide bands from SDS-PAGE analysis of isolated OMPs in our study are similar to results obtained by Pati *et al*. They described ten major polypeptide bands of MW 25 to 88 kDa of OMPs isolated from *P. multocida* B:2.[5] The 30, 33, and 45 kDa polypeptides were the three principal protein bands present in the OMPs isolated from *P. multocida* A:1 (Fig. 1). This finding is consistent with a previous study where three major protein bands of *P. multocida* reference strain X-73 (serogroup A) with approximate MW of 29, 34.5, and 45 were observed.[15]

Inspired by the hemagglutination and fusogenic activity of ND-virosomes, which might assist delivery of foreign antigens, OMPs were co-reconstituted in virosomal membranes.[13] It was a disappointment that the capacity of the HN protein to agglutinate chicken red blood cells was totally inhibited in the presence of OMPs on the surface of ND-virosomes. A possible explanation may be that HN is masked by the OMPs, which may therefore diminish the accessibility of HN protein to sialic acid-containing receptors present on red blood cells. A similar inhibition of hemagglutination was seen after anchoring polyethylene glycol (PEG) chains on the surface of influenza virosomes.[24] Alternatively, dilution of the HN with the OMPs on the virosomal surface might have reduced its hemagglutination activity.

An established potency test for *P. multocida* vaccine is vaccination and challenge in animals. A significant finding in this study was that mice immunized with OMPs formulations were protected against a subsequent challenge infection. OMPs associated with ND-virosomes and ISCOMs yielded higher PI than micellar OMPs at a high dose challenge. Based on these data, we conclude that OMPs extracted from *P. multocida* are potent immunogens. Moreover, antigen loaded virosomes and ISCOMs are potential pasteurella subunit vaccines. Therefore, further investigation of these OMPs formulations in birds is warranted, such as studies on the level of cross-protection against heterologous Pasteurella strains, the duration of protection in target species, and the immune mechanisms underlying the observed immunological response. Additionally, the combination of Newcastle disease viral envelope and OMPs from *Pasteurella multocida* might serve as a multi-disease combination vaccine, which should be a subject of further study as well.

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