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Author(s)	Poolperm, Pichayanut; Apinda, Nisachon; Kataoka, Yasushi; Suriyasathaporn, Wittaya; Tragoolpua, Khajohnsak; Sawada, Takuo; Sthitmatee, Nattawooti
Citation	Japanese Journal of Veterinary Research, 66(4), 239-250
Issue Date	2018-11
DOI	10.14943/jjvr.66.4.239
Doc URL	http://hdl.handle.net/2115/72016
Type	bulletin (article)
File Information	p239-250 Nattawooti Sthitmatee.pdf



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Protection against *Pasteurella multocida* conferred by an intranasal fowl cholera vaccine in Khaki Campbell ducks

Pichayanut Poolperm¹⁾, Nisachon Apinda¹⁾, Yasushi Kataoka²⁾, Wittaya Suriyasathaporn¹⁾, Khajohnsak Tragoolpua³⁾, Takuo Sawada^{1, 2)} and Nattawooti Sthitmatee^{1, 4, *}

¹⁾ Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand

²⁾ Laboratory of Veterinary Microbiology, Nippon Veterinary and Life Science University, Tokyo 180–8602, Japan

³⁾ Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai 50200, Thailand

⁴⁾ Excellence Center in Veterinary Bioscience, Chiang Mai University, Chiang Mai, 50100 Thailand

Received for publication, November 9, 2017; accepted, April 6, 2018

Abstract

Fowl cholera affects the poultry farming including ducks. The commercial fowl cholera vaccines using parenteral administration are available. Recently, an intranasal fowl cholera vaccines have been developed and tested in layers. This study, we analyzed the biological function of recombinant outer membrane protein H (rOmpH) of *Pasteurella multocida* strain X-73 and its antiserum. In addition, we also evaluated the protective efficacy in Khaki Campbell ducks. An adhesion inhibition assay on duck embryo fibroblast (DEF) cells was performed demonstrating that rOmpH-immunized duck sera had a potential inhibitory effect on adhesion ability of bacterial strain. An intranasal fowl cholera vaccine was formulated containing 100 µg rOmpH and 3 µg *E. coli* enterotoxin B (LTB) as an adjuvant. Ducks were intranasally immunized three times at three-week intervals. Challenge exposure was conducted by inoculation at 3.5×10^3 CFU/ml of a strain of X-73 at four weeks after the last immunization. Sera IgY and secretory IgA antibody titers were significantly increased ($P < 0.05$) post immunization. Lymphocytes from ducks immunized with the rOmpH-LTB-based intranasal vaccine showed higher proliferative response to *P. multocida* antigens than those from ducks immunized with only rOmpH or LTB ($P < 0.05$). Protection conferred by immunization with an intranasal or bacterin vaccine in ducks against challenge-exposure were 90% and 80%, respectively. We conclude that the intranasal fowl cholera vaccine protected ducks from artificial *P. multocida* infection. However, the rOmpH will be formulated with the commercial adjuvant and will be conducted against more *P. multocida* field strains in the duck flocks.

Key Words: Intranasal fowl cholera vaccine, Khaki Campbell ducks, *Pasteurella multocida*, Protection, Recombinant outer membrane protein H

*Corresponding author: Nattawooti Sthitmatee, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
Phone: +66-53-948-002. Fax: +66-53-948-065. E-mail: drneaw@gmail.com (N. Sthitmatee)
doi: 10.14943/jjvr.66.4.239

Introduction

Fowl cholera affects the poultry farming includes chickens, turkeys and ducks very severely because of their high morbidity and high mortality, resulting in large economic losses. The major causative pathogen of this disease is capsulated strains of *Pasteuralla multocida* serovar A and somatic serotypes 1, 3 and 4²²⁾. The duration of infection may range from peracute or acute to chronic form.

The outer membrane proteins (Omp) H of avian *P. multocida* is the major outer membrane protein that is highly immunogenic, and is found in the envelope and also in bacterial capsule^{11,16,27-28)}. It plays an important role in bacterial adhesion to epithelial cells of the host at early stages of infection^{3,10)}. Previous study demonstrated that a capsular 39 kDa protein of *P. multocida* serovar A:1 and A:3 acts as an adhesion factor¹⁴⁾. In addition, the adhesion ability of *P. multocida* type A to CEF cells was inhibited significantly by antisera against 39 kDa protein indicating that antigenic 39 kDa protein in the capsule may be responsible for adhesion of avian *P. multocida* type A¹⁾. Not only a 39 kDa protein is a capsule-associated adhesin but also a cross-protective antigen among *P. multocida* capsular serotypes A strains²⁾.

Commercial live attenuated vaccines and bacterin vaccines are available⁸⁾. The live attenuated vaccines provide protective immunity but the residue of virulence can affect the laying rate and the outbreaks can occur. Inactivated or killed vaccines are less effective but can not cause disease as live attenuated vaccines; furthermore, the vaccines can provide a high level of protection against disease but require regular booster. Inactivated vaccines do not effectively stimulate the local or mucosal immunity, therefore the mucosal vaccine needs to be developed¹⁷⁾.

Parenteral administration, such as an intramuscular or subcutaneous injection, is generally practiced but may cause injury or induce stress in the animals. Injection in ducks,

particular in laying ducks, will affect their egg production due to the stress during the vaccination procedure. Thus, a non-invasive administration of the vaccine such as mucosal administration is desirable. Mucosal vaccination is a non-invasive method with several advantages over traditional systemic vaccines, such as less risk of needle injury or cross-contamination^{12,21,32)}. Moreover, mucosal vaccination is widely considered to be more acceptable and simpler to administer orally or nasally as compared to administration via injection. The mucosal vaccine is able to induce secretion of surface immunoglobulin A (IgA), the first in the line of the host defense mechanism against adhesion and colonization at the mucosal surface¹²⁾.

At present, a variety of modern vaccines (e.g., recombinant vaccines or DNA vaccines) have been recognized as novel veterinary vaccine candidates²⁵⁾. Recombinant vaccines rely on the capacity of one or multiple epitopes of the pathogen and are capable of inducing immunity when administered in the presence of appropriate adjuvants or with bacterial or viral vectors²⁰⁾. Past decade, OmpH is considered to be a potential fowl cholera vaccine candidate antigen^{11,16,26)}, as it could confer sufficient protectivity against fowl cholera in chickens by intranasal administration²⁹⁾. Unfortunately, to date, an intranasal fowl cholera vaccine has not been developed for laying ducks. Thus, in the present study, we investigated the antibody responses in ducks against recombinant outer membrane protein H (rOmpH) derived from *P. multocida* strain X-73 and developed an intranasal rOmpH-LTB-based fowl cholera vaccine and evaluated the protective efficacy of the vaccine in Khaki Campbell ducks.

Materials and Methods

Preparation and purification of recombinant OmpH (rOmpH) protein

Bacterial strains, plasmids, media, and growth conditions for recombinant OmpH (rOmpH) protein

expression: *P. multocida* strain X-73 (serovar A:1, ATCC 11039) was kindly provided by Professor Dr. Takuo Sawada, Nippon Veterinary and Life Science University, Tokyo, Japan. It was grown aerobically in tryptose broth (TB; Difco Laboratories, MD, USA) at 37°C for 6 h and then subcultured on dextrose starch agar (DSA; Difco Laboratories) at 37°C for 18 h. *E. coli* strain PQE-ompH²⁶ was grown aerobically at 37°C in Luria–Bertani (LB; Difco Laboratories) broth or on LB agar supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin (Sigma Aldrich, St. Louis, MO, USA).

Preparation and purification of recombinant OmpH: The rOmpH was expressed according to the previous study by Thanasarakulpong *et al.*³⁰. Briefly, *E. coli* strain PQE-ompH glycerol stock was streaked on LB agar containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated at 37°C for 18 h. After the incubation, a single colony was chosen and inoculated into 20 ml LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The culture was grown at 37°C for 18 h, with horizontal shaking at 210 rpm. One liter of the LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin was inoculated in the ratio 1:50 with the overnight culture and allowed to continue growing under the same conditions until an OD₆₀₀ of 0.5–0.7 (mid-log phase) was reached. The recombinant protein expression was subsequently induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Amresco, Solon, OH, USA) to a final concentration of 1 mM, and the culture was incubated for an additional 4–5 h. Finally, the cells were harvested by centrifugation at 4,000 × g for 20 min at 4°C and kept at –20°C for purification.

The purification process of the recombinant protein in this study was conducted by an electroelution method as described previously³⁰. Briefly, the cell pellets were lysed in native lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea; pH 8.0), with gentle shaking at 4°C for

1 h. Then, the suspension was centrifuged at 10,000 × g at 4°C for 30 min. The supernatant was collected and placed into the chamber of an electroelutor (Nativen; ATTO, Tokyo, Japan). Approximately 1,500 µl of the total protein solution was run on a preparative 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel column (10 mm stacking gel and 30 mm separating gel) in the sample buffer (4% SDS, 50 mM Tris, 20% glycerol, 0.005% Bromophenol blue). The conditions for the protein collection were calculated according to the manufacturer's instructions (delay time: 320 min; eluting time: 6 min; filling time: 100 sec; collecting time: 120 sec; 15 mA). The rOmpH fractions were collected in the collection buffer (371 mM Tris, 5% sucrose; pH 8.8) and kept at –20°C for further analysis.

Electrophoresis and immunoblotting: Samples were subsequently analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the Laemmli method¹³ in order to detect the expressed target recombinant protein. The samples were prepared in the sample buffer (50 mM Tris, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, 4% SDS) and boiled for 5 minutes. Then the samples were analyzed on a 12.5% SDS-PAGE slab gel in a mini-slab apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The SDS-PAGE slab gels were then subjected to staining with Coomassie blue R-250 (Sigma-Aldrich) for protein band detection. For the immunoblotting procedure, the proteins were transferred from the SDS-PAGE slab gel to a nitrocellulose membrane (Bio-Rad). The membranes were incubated with a dilution of 1:5,000 Anti-6-His-tag horseradish peroxidase conjugated antibody (Anti-HisG-HRP Antibody, Invitrogen, Carlsbad, CA, USA) in blocking buffer (1% BSA, 0.05% Tween20 in PBS) for 1 h at room temperature to detect the 6×His-tag rOmpH, or incubated with a dilution of 1:1,000 duck serum against rOmpH from our sera bank (Sthitmatee N., personal sera bank) in blocking buffer for 1 h at room temperature followed by

incubation with a 1:1,500 dilution of HRP-conjugated rabbit anti-duck IgY (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. The proteins were visualized via incubation with 3,3'-diaminobenzidine (DAB; Invitrogen).

Adhesion of avian P. multocida strain to duck embryo fibroblast (DEF) cells

Preparations of polyclonal antibodies: Duck antisera were prepared against the rOmpH protein. Briefly, six ducks were divided into 3 groups consisting of immunization with rOmpH (100 µg rOmpH with Montanide (SEPPIC, Paris, France; N1 = 2), immunization with fowl cholera bacterin [*P. multocida* serotype 8:A (Namioka : Carter) or A:1 (Carter : Heddleston)], Bureau of Veterinary Biologics, Department of Livestock Development, Ministry of Agriculture and Cooperative, Thailand (N2 = 2), and non-immunized control groups (N3 = 2). Ducks in Immunized groups were intramuscularly immunized 3 times at 2-week intervals. The blood samples were taken one week after the last immunization. The sera were separated and stored at -20°C until use for adhesion inhibition assay.

DEF cell culture: Duck embryo fibroblast cells (ATCC® CCL-141™) were cultured in Eagle's minimum essential medium (MEM; Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 1% L-glutamine (Invitrogen), and 100 U/ml of penicillin and streptomycin solution (Invitrogen). The numbers of cells were adjusted to 1.25×10^5 cells/ml and seeded into 35 mm Corning® culture dishes (Corning) containing 22 × 22 mm cover slips in the bottom of the well and incubated at 37°C with 5% CO₂ for 24 h. Then the dishes were washed 3 times with 2 ml sterile PBS (pH 7.4) and used for the adhesion inhibition assay.

Adhesion inhibition assay: The adhesion inhibition assay was performed according to a modified

method described previously¹⁾. Briefly, *P. multocida* strains grown on blood agar plates at 37°C for 18 h were suspended in sterile PBS solution. The bacterial suspensions were adjusted to an optical density of 0.1 at 600 nm (approximately 1.5×10^8 CFU/ml). To evaluate the ability of immunized duck sera to inhibit bacterial adherence to DEF cells, 0.5 ml of the bacterial suspension was added to 0.75 ml of pooled duck serum which were derived from experiment above, and then incubated at 37°C for 1 h. As negative control cultures, PBS solution was used instead of duck sera. Then the suspension was added to the monolayer of DEF cells and incubated at 37°C with 5% CO₂ for 1 h. Non-adherent bacteria were removed by washing with 2 ml of sterile PBS solution and by centrifugation at 11,000 g for 10 min. The washing step was done twice. After washing the coverslips were fixed in 4% formaldehyde solution and stained with Wright-Giemsa solution (Sigma-Aldrich). The cover slips were observed under a light microscope with 1000× magnification. A total of 100 DEF cells were observed by randomly scanning the magnification field from left to right and from top to bottom coverslips, and bacterial adhering to the cells were enumerated. The selected DEF cells were counted in triplicates and used for statistical calculations.

Protection against P. multocida infection conferred by an intranasal fowl cholera vaccine in Khaki Campbell ducks

Ducks and sample collection: Seventy-eight Khaki Campbell ducks (*Anas platyrhynchos*) at the age of three to four weeks were used in this study (APA Farm Co. Ltd., Chiang Mai, Thailand) (Table 1.). Briefly, groups 1 and 2 had 22 ducks per group; there were 12 ducks chosen for tracheal lavage and 10 ducks for challenge-exposure. Groups 3 and 4 each had 17 ducks per group; there were 12 ducks for tracheal lavage and 5 ducks for challenge-exposure. Among ducks for tracheal lavage in each group, four ducks were sampled for tracheal lavage and euthanized

Table 1. Experimental design, vaccine types, route of vaccine administration and number of ducks in each group

Group	Vaccine formulation	Route	Total ducks/ group	Number of ducks	
				Tracheal lavage ^a	challenge- exposure
1	Fowl cholera bacterin ^b	IM	22	12	10
2	100 µg rOmpH + 3 µg LTB ^c	IN	22	12	10
3	3 µg LTB ^{c,d,e}	IN	17	12	5
4	100 µg rOmpH ^{b,e}	IM	17	12	5

^a Four ducks were sampled from each group for preparing the tracheal lavage and euthanized on day 0, 21 and 42 of the experiments

^b Ducks were immunized at day 0 and 28 via an intramuscular administration at 1 ml per dose.

^c Ducks were immunized at day 0, 21 and 42 via an intranasal administration at 100 µl per dose.

^d *E. coli* heat-labile enterotoxin, B subunit.

^e Control groups

on day 0, 21 and 42 of the experiments. Moreover, blood samples were collected on day 0, 14, 28, 42 and 56 of the experiments from challenge-exposure group.

The project underwent ethical review and was given approval by an institutional animal care and use committee or by appropriately qualified scientific and lay colleagues. The care and use of experimental animals complied with local animal welfare laws, guidelines and policies. The animal welfare committee of the Faculty of Veterinary Medicine, Chiang Mai University, controlled the use of the laboratory animals, in accordance with the laboratory animal ethics protocols (license number R15/2558). The experiments followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (the Ag Guide, FASS 2010).

Duck immunization: The ducks were divided into four groups based on the vaccine formulations (Table 1). The eluted recombinant protein was passed through a detergent-removing minicolumn (Ampure DT, Amersham Biosciences, Little Chalfont, UK) in order to remove SDS from the protein solution. The rOmpH concentration was measured with the bicinchoninic acid method using the bicinchoninic acid (BCA) protein assay kit following the manufacturer's instructions (Pierce®, Rockford, IL, USA). The rOmpH-LTB-based intranasal vaccine was prepared with 3 µg

E. coli Heat-Labile Enterotoxin, B subunit (LTB, Sigma-Aldrich) as a mucosal adjuvant in a total volume of 100 µl per dosage. The vaccine was freshly prepared and stored at 4°C until use. Ducks were intranasally immunized three times at three-week intervals. Additionally, a commercial fowl cholera bacterin was used to immunize twice at one-month intervals by the intramuscular administration in order to compare the protectivity effects. Immunization with only LTB or rOmpH were used as a control group in order to examine the antibody response that does not result from either one. All ducks were also observed for clinical signs and behavioral changes pre and post-immunization.

Challenge-exposure: According to Table 1, all of the immunized ducks were challenge-exposed with 3.5×10^3 CFU/ml of strain X-73²⁴ via intranasal administration at four weeks after the final vaccination. The ducks were observed for their mortality rates and clinical signs for 7 days.

Necropsies and bacterial isolation were taken for any dead ducks. Gross lesions were recorded and lungs, livers, spleens, kidneys and hock joints were collected for the bacterial isolation. Moreover, nasal discharge and rectal swabs were also collected from all ducks in each group. The bacterial isolation was performed by direct culture using DSA plates (Difco) and species was identified by biochemical tests as described

previously¹⁸⁾.

Determination of antibody response: Duck serum immunoglobulin Y (IgY) and secretory IgA were determined by ELISA. Ninety-six-well plates were coated with 100 μ l of 0.3% formalinized whole cells of *P. multocida* strain X-73 (approximately 10^4 CFU/well) or 1 μ g of rOmpH and incubated overnight at 4°C. After washing 3 times with washing buffer (PBS plus 0.05% Tween: PBST), the plates were blocked with 1% bovine serum albumin (BSA)-PBS for 1 h at 37°C. The plates were then washed, and 100 μ l of 1:100 dilution of duck sera or 1:10 of tracheal lavage were added in triplicate. All wells were incubated for 1 h at 37°C. Horseradish peroxidase (HRP)-conjugated rabbit anti-duck IgY or IgA antibody (Sigma-Aldrich) was used as the secondary antibody, at a 1:4,000 or 1:1,000 dilution, respectively, and incubated for 1 h at 37°C. After washing three times, 100 μ l tetramethylbenzidine (TMB; Pierce[®]) were added. After incubation for 15 min, the reaction was terminated by addition of 100 μ l 2 M sulfuric acid. The optical density at 450 nm (OD_{450}) was measured in each well. The average log titer and the standard error of the mean of each group were calculated according to the previous report²⁹⁾.

Determination of cellular immune response: An *in vitro* lymphocyte proliferation assay (LPA) was adapted and performed as described previously^{9,28)}. Peripheral blood mononuclear cells (PBMCs) were prepared from three milliliters of blood by a gradient centrifugation technique using a Ficoll[®] gradient (Amersham Biosciences) and centrifuged at $400 \times g$ for 30 min. The PBMC fraction was collected and washed twice with sterile Rothwell Park Memorial Institute (RPMI) tissue culture medium (RPMI1640, 31800-022, Invitrogen) supplemented with 100 IU/ml streptomycin, and 100 IU/ml penicillin (RPMI). Subsequently, pellets were resuspended with 4 ml R10 culture medium [RPMI tissue culture medium supplemented with 100 IU/ml streptomycin, 100 IU/ml penicillin, 10%

fetal calf serum (FCS, 10270-098, Gibco) and 2.5×10^{-5} M 2-mercaptoethanol], before enumerating the number of cells. PBMCs at 1×10^6 cells/well were stimulated in triplicate with 5.0 μ g/ml (final concentration) of *P. multocida* strain X-73 and rOmpH in a 96-well U-bottom microtiter plate. R10 culture medium and 10 μ g/ml of Concanavalin A (Con A, C-2010, Sigma) were used as a cell control and mitogen control, respectively. The microtiter plate was incubated at 42°C for 48 h in a humidified atmosphere with 5% CO₂. In the last 16 h before harvesting, cultures were pulsed with 0.25 μ Ci of methyl-[³H]-thymidine. Thymidine uptake was determined with a liquid scintillation counter (MicroBeta TriLux, Wallac, Finland). Results were expressed as stimulation indices (SI), calculated as SI = mean counts per minute in stimulated wells/mean counts per minute in media wells.

Statistical analyses: The results of bacterial adhesion inhibition assay were examined for statistically significant differences using general linear mixed model analysis ($P < 0.05$). In addition, the antibody response data were analyzed using Stata SE 13.1 software (StataCorp LP, College station, TX, USA) and IBM SPSS Statistics version 22. The means between different groups were compared using simple contrasts. The survival of ducks was compared between different treatment and *in vivo* infection challenge groups using binary logistic regression. Exact estimation was used given the small sample sizes in this study. The log ELISA titers were calculated from the highest dilution of the number that exceeded the cut-off value as described in the instruction manuals. Antibody titers and SI among each group were analyzed by ANOVA.

Results

Expression and purification of recombinant proteins

The whole cell lysates of *E. coli* strain

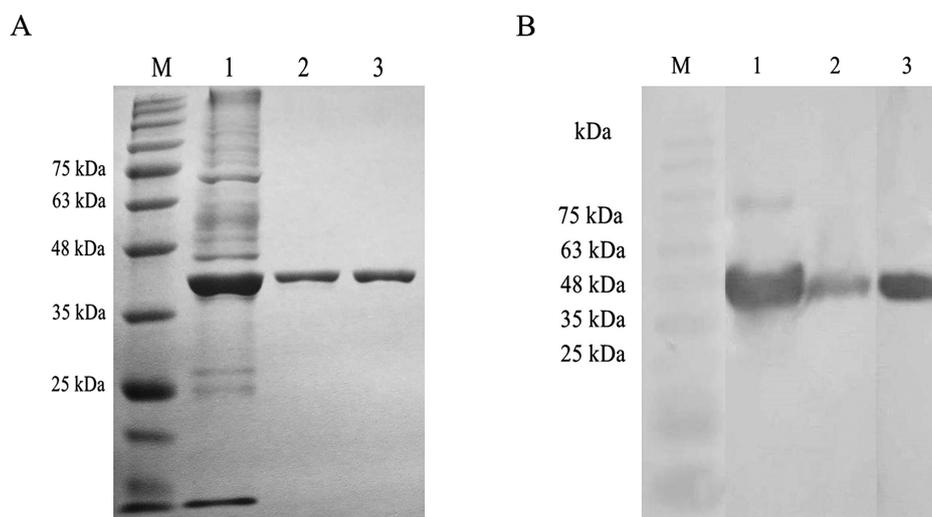


Fig. 1. The rOmpH fractions purified were analyzed using (a) SDS-PAGE stained with Coomassie brilliant blue and (b) western blotting on nitrocellulose membrane (M: molecular mass standards; 1: unpurified rOmpH; 2: rOmpH fractions purified by affinity chromatography; 3: rOmpH fractions purified by electroelution).

PQE-ompH showed an over-expressed band at approximately 39 kDa (6×Histidine tag included), while purified rOmpH fractions using affinity chromatography and electroelution showed specific bands on SDS-PAGE (Fig. 1A). The anti-HisG-HRP antibody was employed in order to confirm that the over-expressed band was the recombinant 6×Histidine-tagged rOmpH protein, as illustrated in Fig. 1B.

The ability of rOmpH-immunized duck sera to inhibit P. multocida adhesion to DEF cells

The mean number of adherent bacteria on 100 DEF cells were 100.41 ± 1.60 , 110.08 ± 3.37 , 280.50 ± 5.23 and 247.08 ± 5.94 for fowl cholera bacterin immunized sera, rOmpH immunized sera, PBS and non-immunized sera, respectively. As shown in Fig. 2., the rOmpH-immunized group was significantly decreased in bacterial adhesion compared to the PBS and non-immunized groups ($P < 0.001$), but no significant difference with bacterin vaccine group.

Protection against P. multocida infection conferred by an intranasal fowl cholera vaccine in Khaki Campbell ducks

Serum IgY profile: Determination of serum

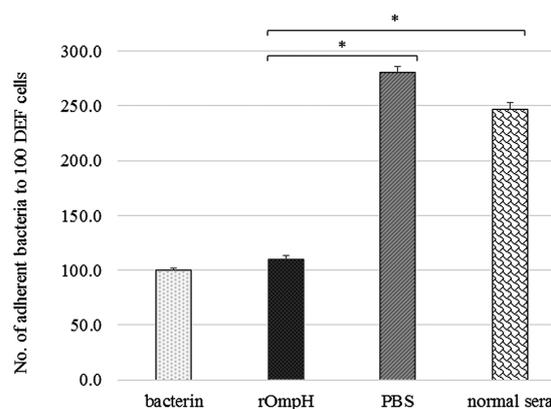


Fig. 2. Mean numbers of adherent of *P. multocida* strains to 100 DEF cells. The results are represented as mean \pm SE; General linear mixed model analysis; * $P < 0.001$.

IgY titers using an indirect ELISA was shown in Fig. 3. The serum IgY titer profile against *P. multocida* strain X-73 (Fig. 3A) was similar to rOmpH (Fig. 3B). There were no differences in pre-immunized antibody titers between groups at day 0. The anamnestic antibody responses to fowl cholera bacterin or rOmpH-LTB-based intranasal vaccine were observed at day 14 after first immunization and antibody titers were continued to increase throughout the experiment. In contrast, no response was observed in antibody titers to both *P. multocida* strain X-73 and

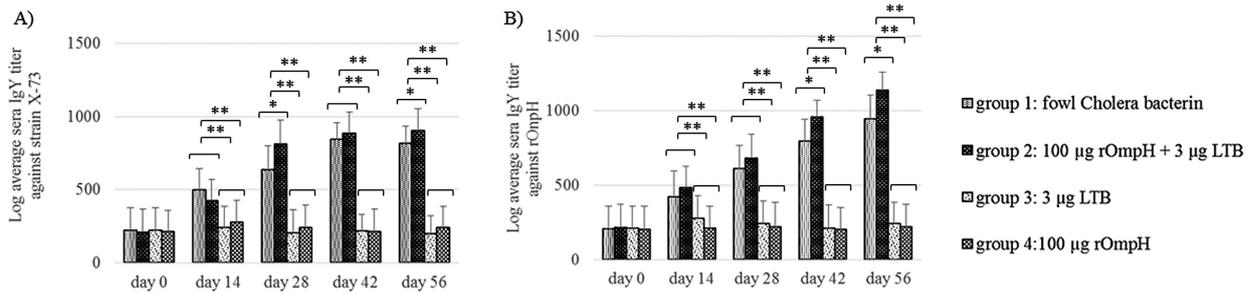


Fig. 3. Serum IgY profile of duck immunized with different vaccine formulation against A) *P. multocida* strain X-73 and B) rOmpH by indirect ELISA; * $P < 0.05$, ** $P < 0.01$.

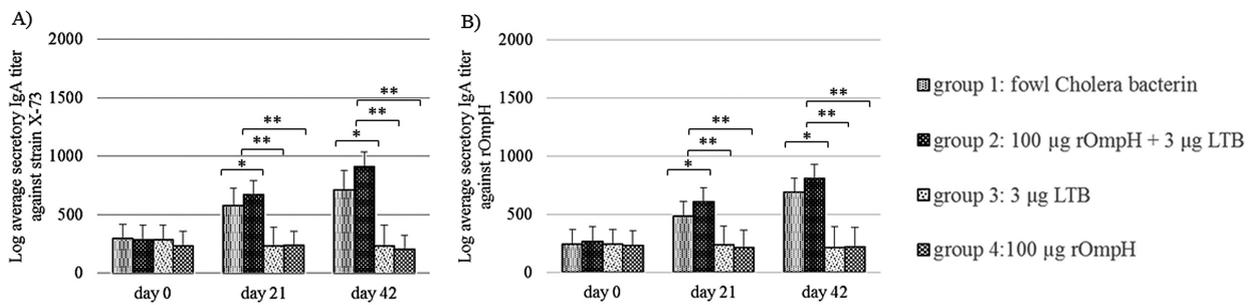


Fig. 4. Serum IgA profile of duck immunized with different vaccine formulation against A) *P. multocida* strain X-73 and B) rOmpH by indirect ELISA; * $P < 0.05$, ** $P < 0.01$.

rOmpH among control groups. Except for day 0, the serum IgY titer in fowl cholera bacterin or rOmpH-LTB based intranasal vaccine at each time point were significantly higher than control groups.

Secretory IgA profile: Secretory IgA titers profile was detected in all groups of ducks, as illustrated in Fig. 4. The secretory IgA titer profile against *P. multocida* strain X-73 (Fig. 4A) and rOmpH (Fig. 4B) were similar to each other. There were no differences in antibody titers of pre-immunized sera between groups at day 0. After the first immunization, the antibody titer levels in the ducks immunized with fowl cholera bacterin and rOmpH-LTB-based intranasal vaccine were observed at day 21 and continuously increased until the end of the experiment. In the control groups, no response was observed in antibody titers to both *P. multocida* strain X-73 and rOmpH antigen throughout the experiment. Interestingly, secretory IgA titers derived from the intranasal vaccine after first immunization were not only significantly higher than secretory

IgA titers derived from both control groups ($P < 0.01$) but also significantly higher than the fowl cholera bacterin group ($P < 0.05$) at each time point.

Lymphocyte proliferation assay (LPA): The LPA data are shown in Fig. 5. As observed, there was no response in PBMCs among all of the pre-immunization groups when stimulated with both avian *P. multocida* strains X-73 (Fig. 5A) and rOmpH (Fig. 5B). This indicates that there is no evidence of ducks being exposed to etiologic agents causing fowl cholera. After the first vaccination, the SI values of the duck immunized with rOmpH-LTB-based intranasal or fowl cholera bacterin vaccines were statistically significantly higher than the control groups ($P < 0.05$) at each time point. For both control groups, the SI values were not significantly different, even when stimulated with antigens of the bacteria and their recombinant proteins throughout the experiment.

Protectivity: A total volume 100 μ l of bacterial suspension containing 3.5×10^3 CFU of strain X-73 was administered intranasally. The

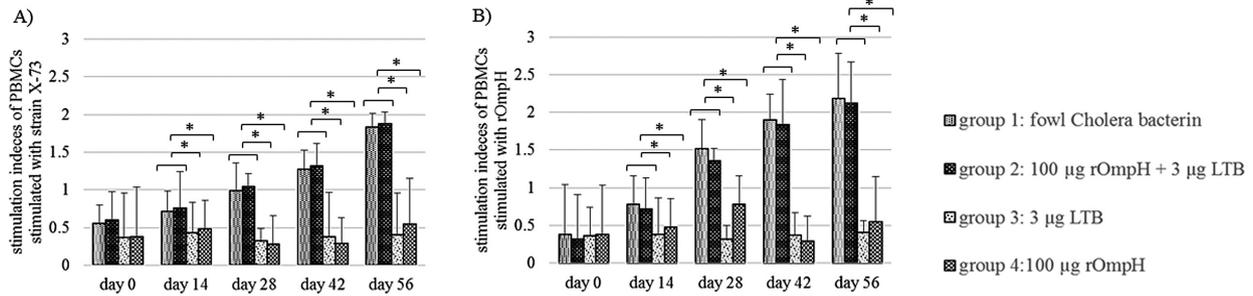


Fig. 5. Stimulation indices of duck PBMCs immunized with different vaccine formulation against A) *P. multocida* strain X-73 and B) rOmpH by LPA; * $P < 0.05$.

Table 2. Protections conferred in ducks vaccinated with formulations upon challenge-exposure to a live *P. multocida* strain

Group	Vaccine formulation (per dose)	No. of survivors / challenged with <i>P. multocida</i> strain X-73 (% protection) ^a
1	Fowl cholera bacterin	8/10 (80) ^b
2	100 µg rOmpH + 3 µg LTB	9/10 (90) ^b
3	3 µg LTB	0/5 (0)
4	100 µg rOmpH	0/5 (0)

^a Ducks were challenge-exposed intranasally with 0.1 ml of bacterial suspension containing 3.5×10^3 CFU/ml of strain X-73

^b Statistical significant as compared to control groups, $P < 0.05$.

protectivity level in the ducks immunized with the rOmpH-LTB-based intranasal vaccine was 90%, while in those immunized with the fowl cholera bacterin vaccine the corresponding level was 80% (Table 2). There was no significant difference between the protection conferred by the intranasal fowl cholera and bacterin vaccines. No survivors were observed in the LTB or purified rOmpH without LTB immunized ducks exposed to the bacterial strain.

Clinical signs, gross lesions, and bacterial isolation: Among challenge-exposed ducks group, there was no behavioral change observed in all ducks post-immunization in all treatment groups. In the control groups, ducks manifested the clinical signs at 6–8 h after the challenge exposure including depression, anorexia and severe diarrhea. During the period 8–12 h, the severity was developed rapidly resulting in the death of several ducks, and all of the ducks in this group died within 24 h. In addition, one duck in fowl cholera bacterin-immunized group and two ducks in the rOmpH-LTB-based intranasal vaccine-

immunized group strated to depression and anorexia at 10 h after the challenge exposure. These ducks then died within 24 h. The depression and loss of appetite were observed after challenge exposure manifested for a few days in the survivor ducks and the symptoms of the disease was improve after that.

The necropsy results demonstrated traditional lesions of fowl cholera in all of the dead ducks in this study including multiple necrotic foci in the liver and/or spleen, lung congestion, lung edema, multiple petechiae in the liver, hemorrhage in the small intestine, splenomegaly, fibrinopurulent peritonitis and salpingitis in all the duck carcasses.

P. multocida isolates were not found on plates at pre-immunization and pre-challenge without any clinical sign in ducks. After challenge-exposed with *P. multocida* strain X-73, the dead ducks were also subjected to isolation and identification of *P. multocida*. The results showed that *P. multocida* was recovered in pure cultures from the specimens of all the dead ducks.

Unfortunately, the bacterial serotyping was not performed in this study. Conversely, there was no *P. multocida* colony growth on agar medium cultured from nasal discharge or rectal swabs of the survivors.

Discussion

Recently, fowl cholera caused by *P. multocida* is distributed worldwide. Fowl cholera disease affects the poultry industry, incurring economic losses due to loss of products. Currently, the commercial vaccine including inactivated and live vaccines have several limitations¹⁶⁾. Therefore, using a modern vaccine as recombinant mucosal vaccine need to be developed for a safe and effective vaccine¹⁷⁾. The OmpH which mediates bacteria adherence to host cells during the initial stage of bacterial infection²⁷⁾ is considered to be a potential vaccine candidate antigen for fowl cholera disease²⁶⁾. Because it provided a cross-protection for mice and chickens against challenges with avian *P. multocida* strains²⁶⁾.

Previous studies have shown that the antisera against rOmpH of *P. multocida* inhibited their adhesion to chicken embryo fibroblast (CEF) cells³⁰⁾. Likewise, the present study has shown that *P. multocida* adhesion was statistically significantly decreased in DEF cells treated with rOmpH immunized sera. This results indicated that rOmpH purified by electroelution can conserve part of the immunogenicity of the rOmpH protein, which can induce antibodies to inhibit *P. multocida* adhesion to DEF cells. The results emphasize that the rOmpH plays an important role in the bacterial adhesion¹⁾.

It is known that *P. multocida* causes disease by infecting or entering through the mucosal surface in the upper respiratory tract of poultry species²²⁾. Thus, the first line of the host defense mechanism is produced against inhaled antigens and as a consequence is possibly the most effective route for vaccination using both peripheral and mucosal immunity⁵⁾. Therefore, the intranasal

vaccine, mucosal vaccines, was developed in this study. It provided more mucosal immune response closely resembles the natural immunity on the mucosal surface than the injectable vaccine^{5,32)}. To increase mucosal vaccine efficacy, the LTB adjuvant was incorporated into the intranasal vaccine. The result revealed that our intranasal vaccine with LTB as an adjuvant could elicit a strong immune response indicated by IgG or secretory IgA titers. The LTB could be a factor that supported the improvement of systemic and mucosal responses^{4,7,19)}. Moreover, ducks immunized with only LTB adjuvant showed no adverse effects such as behavior changes. Importantly, it showed a low level of duck sera IgY and secretory IgA. These findings ensure that rOmpH-LTB-based intranasal vaccine was no adverse effects. Therefore, it is considered to be an effective adjuvant for mucosal vaccine formulation^{6,23,29,31)}.

The IgY is the predominant immunoglobulin form in avian sera, and secretory IgA is produced locally by plasma cells situated at mucosal surfaces and plays an important role in mucosal immunity¹⁵⁾. In accordance with the serum IgY titer and secretory IgA titer, the results indicated that immunization with the rOmpH-LTB-based intranasal vaccine was capable of eliciting the production of both serum IgY and secretory IgA in ducks as well as the previous study in chicken^{29,31)}. Moreover, the SI indices of intranasal vaccine throughout the experiment showed the capability of cell-mediated immune response by inducing lymphocyte proliferation against *P. multocida* strain X-73 and their recombinant proteins. In regard to the protectivity, the rOmpH was recently employed to formulate an intranasal fowl cholera vaccine, which gave efficient protectivity in layers against artificial *P. multocida* infection^{29,31)}. Protectivity levels of the rOmpH-LTB-based intranasal vaccine in our study demonstrated that the intranasal vaccines conferred efficient protection in ducks as well.

In conclusions, ducks immunized with the rOmpH-LTB-based intranasal fowl cholera vaccine

can produce efficient antibody which reduces adhesion to DEF cells. The intranasal vaccine also successfully induce high levels of lymphocyte proliferation and produced homologous serum IgY and secretory IgA. These results suggest that the present intranasal fowl cholera vaccines cooperated with the efficient mucosal adjuvants is capable to induce a specific antibody against artificial avian *P. multocida* strain challenge in Khaki Campbell ducks. However, the rOmpH will be formulated with the commercial adjuvant because the LTB can not be used for the commercial production.

Conflict of interest

None of the authors has a conflict of interest to declare.

Acknowledgments

This research was financially supported by the National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand, grant no. P-14-50643 and additional funding through the Chiang Mai University Research Administration Office which provide the budget to the Excellence Center in Veterinary Bioscience, Chiang Mai University, Thailand. Miss Pichayanut Poolperm is a Ph.D. student under the Research and Researchers for Industries (RRi), Thailand Research Fund (TRF), grant no. PHD58I00042.

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