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Evaluation of long-term antibody response and cross-serotype reaction in ducks immunised with recombinant *Riemerella anatipestifer* outer membrane protein A and CpG ODN

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Abstract

Introduction: *Riemerella anatipestifer* (RA) infections can lead to high mortality in ducklings. Inactivated vaccines against RA are commercially available, but they fail to provide cross-protection against various serotypes. We have previously demonstrated that a subunit vaccine containing recombinant outer membrane protein A (rOmpA) antigen of serotype 2 formulated with CpG oligodeoxynucleotides (ODN) as the adjuvant was able to stimulate both humoral and cellular immunities. **Material and Methods:** In the present study, thirty healthy 7-day-old Pekin ducks were randomly assigned to three equal treatment groups: rOmpA-vaccinated, rOmpA + CpG-vaccinated, and control. Vaccine was injected intramuscularly and a booster dose of the same vaccine was given two weeks after primary immunisation. The long-term antibody response and cross-serotype reaction of this vaccine were evaluated in ducks. **Results:** Compared to ducks immunised with rOmpA alone, ducks immunised with rOmpA + CpG ODN had significantly ($p < 0.05$) increased serum antibody titre from two weeks until nine months after primary immunisation. In addition, expression of cytokines including interferon (IFN)-α, IFN-γ, interleukin (IL)-6, and IL-12 was significantly ($p < 0.05$) enhanced in PBMC of ducks immunised with rOmpA + CpG ODN two weeks after primary immunisation. Antibodies from ducks immunised with the rOmpA + CpG ODN vaccine could also detect RA serotypes 1 and 6 in Western blot analysis. **Conclusion:** Combination of rOmpA and CpG ODN could be a feasible strategy for developing a subunit RA vaccine with long term and broader-ranging protection.

Keywords: duck, *Riemerella anatipestifer*, vaccine, adjuvant, long-term antibody.

Introduction

Riemerella anatipestifer (RA) is a non-motile gram-negative bacterium belonging to the *Flavobacteriaceae* family (17, 18), which is pathogenic to ducks and geese, and may be to gallinaceous birds, particularly turkeys (15, 20). RA infection can result in poor feed conversion, increased condemnations, and high mortality rates in ducklings and goslings. Mortality of ducks under 8 weeks of age was reported to be between 15% and 75% (19). In total, 21 serotypes of RA have been identified based on slide and tube agglutination tests with antisera and no cross-serotype protection has been observed (10, 11). Results from previous studies revealed that RA serotypes vary in different countries or regions. For example, serotypes 1–10, 11, 13, 14, and 15 were reported in China (6), while serotypes 1, 5, 7, 10, 15, and 21 were found in Thailand (11). In Taiwan, serotypes 1, 2, 4, 5, 6 and other undetermined serotypes were noted (14, 22, 24). Taken together, serotypes 1, 2, and 10 are responsible for most of the major outbreaks (21, 22, 24).

Vaccination with inactivated or live RA has been shown to successfully confer protection against homologous strains of RA, but not heterologous ones (9, 12, 16). Due to the diversity of RA serotypes isolated from the field, a vaccine capable of providing cross-protection would be desirable. Under this

circumstance, using a molecule conserved among various RA serotypes as the antigen is imperative. The 42-kDa outer membrane protein A (OmpA) is the major virulence factor of RA responsible for cellular adhesion and invasion (4, 5). OmpA can be found in all RA serotypes with minor genetic heterogeneity among them (4, 5, 27). Some OmpA vaccines against bacterial infections have been tested, and although immunisation with the purified recombinant protein (rOmpA) induced production of antibodies in ducks (3, 7), administration of the preparations failed to effectively prevent morbidity.

Studies indicated that the efficacy of vaccines containing protein-based antigens such as rOmpA could be significantly enhanced by using CpG oligodeoxynucleotides (ODNs) as the adjuvant. CpG ODNs are able to induce more balanced and effective immune responses that enhance the immunogenicity of antigens. CpG ODNs can be classified into class A, B, or C, based on differences in structure and elicited immunological profile (13). Class B is the strongest in terms of stimulating humoral responses, making them potential vaccine adjuvants. However, the stimulating effect of CpG ODNs is species-specific. It is generally agreed that the optimal motif for murine and avian cells is GACGTT, and the most stimulatory motif for other species, including humans, bovine, ovine, and porcine, is GTCGTT (25, 26).

We previously demonstrated that CpG ODN plasmid constructs containing three copies of GACGTT motifs enhanced both humoral and cell-mediated immunity elicited by a rOmpA vaccine in ducks up to five weeks after the primary immunisation (3, 8). Results from the homologous challenge showed that ducks immunised with a rOmpA + CpG ODN vaccine had a 90%-reduced pathological score in comparison with the control. In the present study, long-term antibody production (up to nine months post-immunisation) and cross-serotype reaction of the antibody were investigated in ducklings immunised with the rOmpA + CpG ODN vaccine.

Material and Methods

RA serotyping. RA isolates were collected from field cases in southern Taiwan. PCR using 16S primers (F: 5′-CAGCTTAACGTAGAACTGC-3′ and R: 5′ TCGAGATTTGCATCACTTCG-3′) was performed for identity confirmation. Samples were sent to the Animal Health Research Institute of Taiwan for serotyping by gel-diffusion precipitin test.

Expression of rOmpA. Expression of rOmpA (serotype 2) was induced as described in our previous study (3). Bacterial DNA was extracted using the Blood & Culture Cell Genomic DNA Extraction Mini Kit (Geneaid, Taiwan). The OmpA gene was amplified and cloned into the pET32a vector (Novagen, Germany). *E. coli* strain BL21 (DE3) (Invitrogen, USA) was used for recombinant protein expression. The expected size of the recombinant OmpA protein was 65 kDa: OmpA at 42 kDa plus a tag of 23 kDa from the pET32a vector. The amount of rOmpA was quantified by regression with bovine serum albumin (Kirkegaard & Perry Laboratories, USA). Pyrotell lysate (Associates of Cape Cod, USA) was used to examine possible endotoxin contamination (>0.125 IU/mL).

Vaccine preparation. The rOmpA vaccine was prepared with or without CpG ODN adjuvant as described previously (3). Bacterial culture containing 100 µg of rOmpA was mixed with an equal volume of PBS (rOmpA) or 100 µg of CpG ODN in PBS (rOmpA + CpG). The final injection volume for each immunisation was 200 µL.

Animals and experimental design. Thirty healthy 7-day-old Pekin ducks were used in this study, obtained from the Livestock Research Institute, of the Taiwanese Council of Agriculture. The birds were randomly assigned to three equal treatment groups: rOmpA-vaccinated, rOmpA + CpG–vaccinated, and control. Vaccine in a 200 µL volume was injected intramuscularly and a booster dose of the same vaccine given two weeks after primary immunisation (W2). Ducks in the control group were injected with sterile saline. Blood samples were collected before immunisation (W0) and weekly until four weeks post-immunisation (W1, W2, W3, and W4). Thereafter, blood sample collection was performed on a monthly basis until nine months post immunisation (2M to 9M). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Pingtung University of Science and Technology, Taiwan.

RA-specific antibody responses. The RA-specific antibody levels of immunised ducks were examined using indirect ELISA as previously described (4). RA cell lysate was prepared by coating it onto 96-well plates at 0.5 µg/well. The scotopic to protopic (S/P) ratio was calculated as (test OD value − negative OD value) / (positive OD value − negative OD value).

Cytokine expression analysis. The mRNA levels of cytokine genes were analysed as previously described (4). Briefly, PBMCs from vaccinated ducks were collected and stimulated with RA antigen. Total RNA was extracted, RT-PCR performed, and relative quantitation carried out using GAPDH as the reference gene, where fold change = $[(E_{target}) \times (\text{control CP}_{target} - \text{treatment CP}_{target})] / [(E_{ref}) \times (\text{control CP}_{ref} - \text{treatment CP}_{ref})]$.

Cross-detection of antibodies. Cross-reactivity of antibodies to heterologous serotypes was examined by Western blot analysis. Different serotypes of RA, including serotypes 1, 2, 6, and RA56a (of unknown serotype), were cultured, denatured (90°C for 10 min), and loaded onto a 10% SDS acrylamide gel. After electrophoresis, separated proteins were transferred onto a P-immobilin PVDF membrane (Millipore, USA). The blots were blocked and incubated with the

duck sera (1:1000) before or after immunisation with serotype 2 rOmpA at room temperature for 1 h. After washing, the blots were incubated with goat anti-duck Horseradish peroxidase (HRP)-conjugated antibody (1:3000, Kirkegaard & Perry Laboratories) at room temperature for 1 h. Chemiluminescence assay was performed using the ECL kit (Amersham-Pharmacia Biotech, now GE Healthcare, UK).

Statistical analysis. Statistical analysis of all data was performed using the general linear model in the SAS statistical software package (Version 9.4; SAS Institute, USA). Differences among the treatment groups at each time point were analysed with Tukey multiple comparison tests. The results were presented as means ± standard deviation. Probability less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Expression of rOmpA. The expression level of rOmpA was quantified (Fig. 1A) and its immunogenicity and size (65 kDa) were confirmed by Western blotting using convalescent duck antisera (Fig. 1B and C).

Antibody responses. In comparison with unimmunised ducks, the production of serum RA-specific antibody ($p < 0.05$) increased significantly in immunised ducks (Fig. 2). Individuals immunised with rOmpA + CpG had significantly ($p < 0.05$) higher titres of RA-specific antibody in serum than unimmunised counterparts from 2 weeks to 9 months after primary immunisation. In addition, the level of RA-specific antibody in serum in ducks immunised with rOmpA + CpG was significantly ($p < 0.05$) higher than that of ducks immunised with rOmpA at most time points throughout the study period.

Expression of cytokines. Analysis of mRNA expression of selected cytokines, including IFN-α, IFN-γ, IL-6, and IL-12 was undertaken two (W2) and four (W4) weeks after primary immunisation. In response to the stimulation by rOmpA, PBMCs from ducks immunised with rOmpA + CpG showed significantly ($p < 0.05$) increased upregulation of these cytokines compared to those from ducks immunised with rOmpA at both time points (Fig. 3). Moreover, the upregulation of IFN-γ and IL-6 expression was greater at W4 in PBMCs from ducks immunised with rOmpA + CpG.

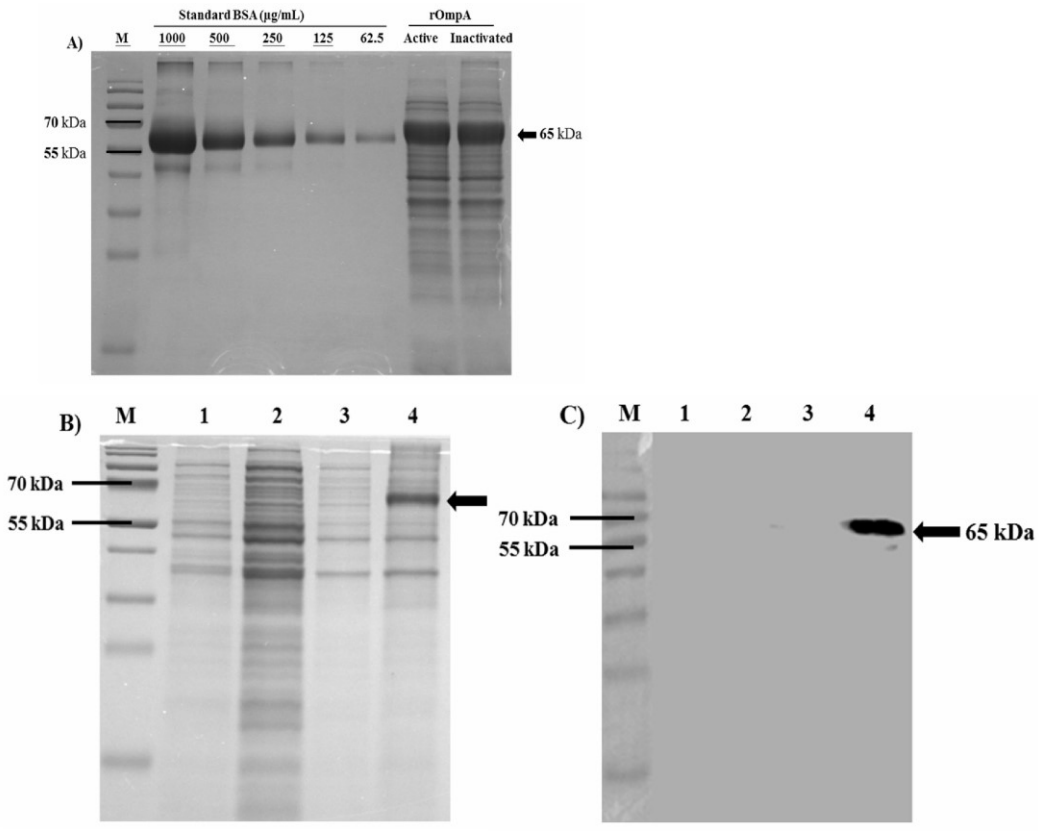

Fig. 1. Expression and immunogenicity analysis of recombinant OmpA. A – rOmpA separated on 10% SDS-PAGE for protein quantitation. Lanes 1–5 – BSA standards, lane 6 – lysate of *E. coli* expressing rOmpA induced for 4 h, lane 7 – lysate of *E. coli* expressing inactivated rOmpA, and lane M – gene marker. B – The 65 kDa of expressed rOmpA separated on 10% SDS-PAGE. Lane 1 – pET-32a incubated for 0 h, lane 2 – pET-32a incubated for 4 h, lane 3 – lysate of *E. coli* expressing rOmpA induced for 0 h, lane 4 – lysate of *E. coli* expressing rOmpA induced for 4 h, and lane M – gene marker. C – Western blot analysis with anti-RA duck sera. Lane 1 – pET-32a incubated for 0 h, lane 2 – pET-32a incubated for 4 h, lane 3 – lysate of *E. coli* expressing rOmpA induced for 0 h, lane 4 – lysate of *E. coli* expressing rOmpA induced for 4 h and M – gene marker

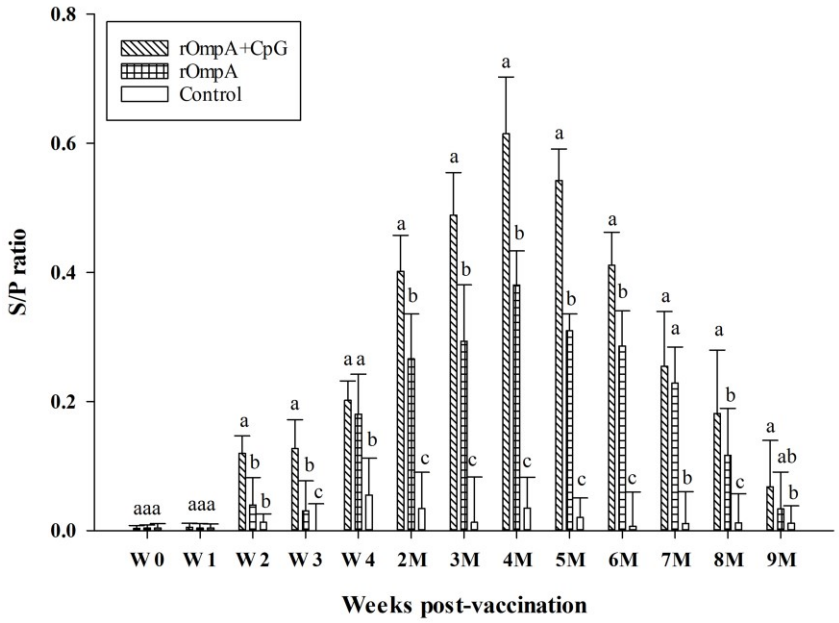

Fig. 2. RA-specific antibody level in immunised ducks. Different superscript letters indicate statistically significant difference ($p < 0.05$) between groups for each sampling time point

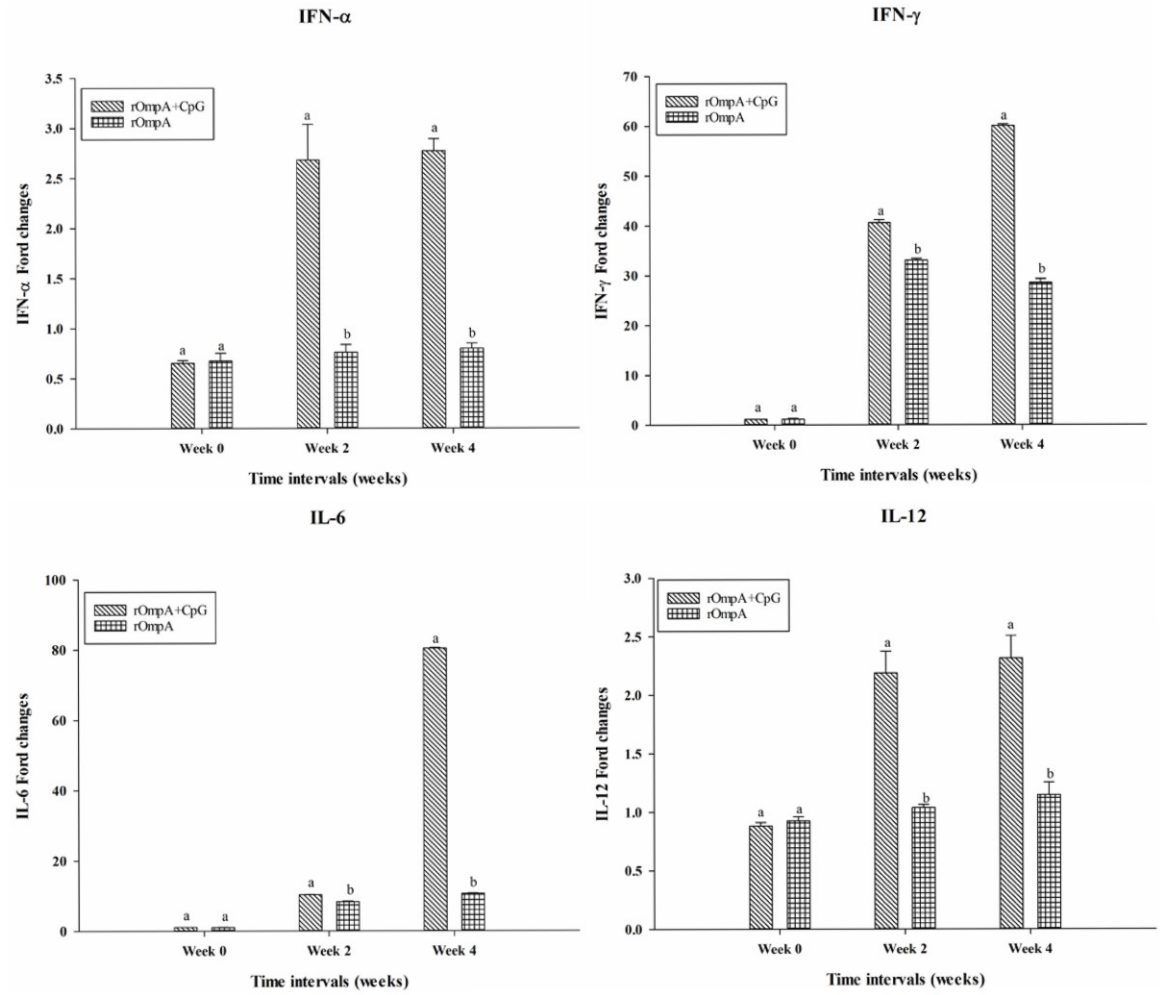

Fig. 3. Cytokine mRNA levels in immunised ducks. The mRNA expression levels of IFN-α (A), IFN-γ (B), IL-6 (C), and IL-12 (D) are shown as fold change relative to the saline control. Data are presented as the mean ± standard deviation of ducks in the treatment ($n = 5$). Different superscript letters indicate statistically significant difference ($p < 0.05$) between groups for the same sampling time point

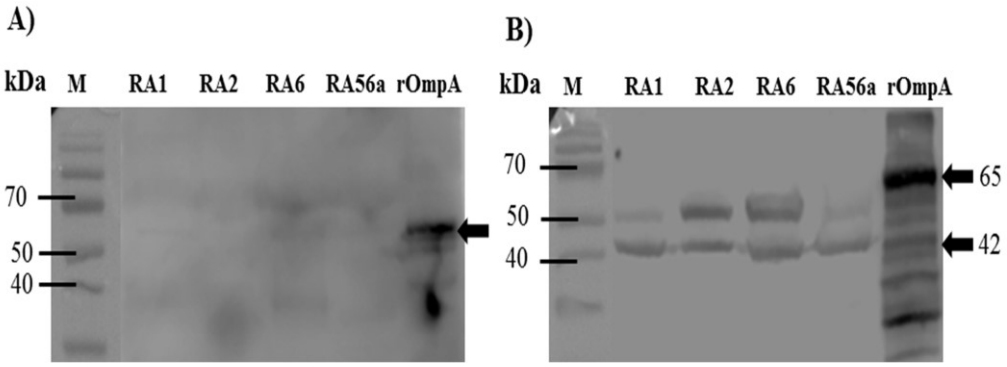

Fig. 4. Cross-serotype detection by antiserum from ducks immunised with rOmpA + CpG. A – Detection with antiserum of unimmunised ducks. B – Detection with antiserum of ducks immunised with rOmpA + CpG. RA1 – RA serotype 1, RA2 – RA serotype 2, RA6 – RA serotype 6, RA56a – RA of unknown serotype, rOmpA – rOmpA induced for 4 h, and M – protein ladder with labelled molecular weights. The positions of the 65-kDa rOmpA and the 42-kDa OmpA proteins are indicated by the arrows

Cross-serotype reaction of duck sera. The antisera from ducks immunised with rOmpA from serotype 2 showed cross-serotype reactivity to other serotypes of RA in Western blot analysis, including serotypes 1, 6, and strain 56a (of unknown serotype) (Fig. 4). The serum antibody elicited by immunisation with rOmpA (65 kDa) was able to react with all serotypes tested and show two distinct bands: 42 and 55 kDa.

Discussion

We previously demonstrated that immunisation with rOmpA of RA serotype 2 plus CpG ODN enhanced both humoral and cell-mediated immune responses in ducks up to 35 days after primary immunisation (3). The results of a homologous challenge revealed that the pathological score was reduced by 90% in immunised ducks. To further evaluate the application of this vaccine in the field, in the present study, serum RA-specific antibody was monitored for nine months in immunised ducks and its cross-serotype reactivity was examined. In ducks immunised with rOmpA + CpG ODNs, the RA-specific antibody started to increase 2 weeks after primary immunisation, peaked at four months, and gradually declined afterwards. The antibody profile revealed that immunisation with rOmpA + CpG ODNs is suitable for providing protection in commercial duck flocks. Breeder ducks start to lay eggs around four to six months of age, which is comfortably inside the duration of protection as immunisation with the vaccine could induce antibody production for nine months. This indicates that the vaccine could convey protection against RA infection in the life cycle stages from newborn ducklings to layers (1, 2, 15).

Since RA infections can be caused by different serotypes, we were interested in examining the cross-serotype reactivity of antibodies elicited by immunisation with rOmpA + CpG ODN (21, 24). Our results demonstrated that the antibody against serotype 2 rOmpA was able to react with serotypes 1, 6, and strain 56A (an isolate from Taiwan of unknown serotype). A Western blot of total RA antigens showed two distinct bands of 55 and 42 kDa, which were consistent with previous studies showing multiple bands of OmpA at various stages of processing (19). Recombinant OmpA expressed in *E. coli*, on the other hand, showed only one band (Fig. 1C), potentially due to a lack of the post-translational modification in *E. coli* which is found in RA (19). Since antibodies from ducks immunised with RA serotype 2 can also detect whole-cell lysates of serotypes 1 and 6, the rOmpA subunit vaccine also seems promising as a provider of cross-protection. Future challenge studies can verify its cross-protectivity. Our finding that ducks immunised with rOmpA + CpG ODN had significantly ($p < 0.05$) increased expression levels of IFN-α, IFN-γ, IL-6, and IL-12 indicated that the vaccine activated type 1 helper T (Th1) cells important for cellular immunity. The increase in IL-6 represented activation of type 2 helper T (Th2) cells, which are important for humoral immunity. The balance between Th1 and Th2 cells is important in the immune responses, suggesting that both humoral and cell-mediated immunities were induced after vaccination (23).

In conclusion, the results showed that rOmpA + CpG ODN vaccination through intramuscular injection was able to induce long-term antibody response in ducks lasting nine months. This long-term protection would be beneficial for mother ducks conferring passive immunity to ducklings. Furthermore, since antibodies from ducks immunised with RA serotype 2 can recognise a number of other serotypes, this rOmpA vaccine can potentially be cross-protective.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: All experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Pingtung University of Science and Technology (NPUST-104-012), Taiwan.

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