



Evaluation of the protective immunity of *Riemerella anatipestifer* OmpA

Xinxin Xu¹ · Yaohui Xu² · Shuang Miao¹ · Pan Jiang¹ · Junsheng Cui¹ · Yanshan Gong¹ · Panpan Tan¹ · Xiaoli Du¹ · Nazrul Islam¹ · Qinghai Hu¹

Received: 5 August 2019 / Revised: 21 November 2019 / Accepted: 3 December 2019 / Published online: 21 December 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Riemerella anatipestifer is responsible for an economically important disease of commercially raised ducks. No or only few cross-protection was observed between different serotypes of *R. anatipestifer* strains, and so far no protective antigen in this bacterium has been identified. OmpA is a predominant immunogenic protein of *R. anatipestifer*, and within the 1467 bp *ompA* ORF (*ompA1467*), there is another 1164 bp ORF (*ompA1164*) with the same C-terminal. In this study, our results showed that the full sequence of *ompA1467* from some *R. anatipestifer* strains with different serotypes shared the same amino acid sequence. Animal experiments showed that the soluble recombinant protein rOmpA1164, but not rOmpA1467, could provide partial protective immunity against challenge. Moreover, there was no significant difference in protective immunity between ducklings immunized with Th4^{ΔompA} bacterin and those immunized with Th4 bacterin. In addition, OmpA1467 was the main existing form of OmpA in *R. anatipestifer* cells by gel electrophoresis and western blot analyses. The results suggested that OmpA1467 was not a protective antigen of *R. anatipestifer*, and antibodies against proteins other than OmpA play a critical role in the process of anti-*R. anatipestifer* infection.

Keywords *Riemerella anatipestifer* · Outer membrane protein A · Protective immunity · Expression · Monoclonal antibody

Introduction

Riemerella anatipestifer infection, which causes disease primarily in domestic ducks, accounts for major economic losses to the duck industry worldwide as a result of high mortality, weight loss, and condemnation of carcasses (Ruiz and Sandhu 2013). To date, 21 *R. anatipestifer* serotypes have been identified (Pathanasophon et al. 2002). Of these, serotypes 1, 2, 6, and 10 have been responsible for most major outbreaks in

China (Cheng et al. 2003; Hu et al. 2001). Once the bacterium infects a duck flock, it can become endemic, and eradication can be difficult, with repeated infectious episodes possible. However, little is known about the protective antigens of *R. anatipestifer*.

Outer membrane protein A (OmpA) is a main protein of a large array of Gram-negative bacteria. OmpA of *Escherichia coli* and other enterobacteria is reportedly a multifaceted protein that functions as an adhesin and invasin, acts as both an immune target and evasin, and serves as a receptor for several bacteriophages (Smith et al. 2007). In some bacteria, such as *Haemophilus parasuis* (Tian et al. 2011), *Klebsiella pneumoniae* (Kurupati et al. 2011), *Salmonella enterica* serovar Typhimurium (Lee et al. 2010), and *Shigella flexneri* 2a (Pore et al. 2011), OmpA can induce a protective immune response. In *R. anatipestifer*, the open reading frame (ORF) of the *ompA* gene is 1467-bp, so we called it *ompA1467* in this study, while within this ORF, there is another 1164-bp ORF that shared the same C-terminal with *ompA1467*, and we called it *ompA1164* (Fig. S1). Our previous results showed that OmpA of *R. anatipestifer* was an important virulence factor (Hu et al. 2011). In addition, OmpA is a predominant

Xinxin Xu and Yaohui Xu contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00253-019-10294-3>) contains supplementary material, which is available to authorized users.

✉ Qinghai Hu
huqinghai@caas.cn

¹ Shanghai Veterinary Research Institute, The Chinese Academy of Agricultural Sciences, 518 Ziyue Road, Shanghai 200241, China

² College of Veterinary Medicine, Henan University of Animal Husbandry and Economy, 6 North Longzihu, Zhengzhou 450046, China

antigenic determinant of *R. anatipestifer* (Subramaniam et al. 2000). However, the recombinant OmpA (residues 101–488 aa) of serotype 15 *R. anatipestifer* strain 110/89 could not protect against subsequent challenge with the virulent serotype 15 strain, although specific antibodies were detected successfully in infected ducks (Huang et al. 2002). Nevertheless, the recombinant OmpA1467 (residues 33–466 aa) of serotype 2 *R. anatipestifer* strain Rf153 was reported to be able to induce protection against challenges with both homologous and heterologous strains (60% and 50% for Rf153 and serotype 1 strain Rf63, respectively) (Zhai et al. 2013). In addition, the homology of *R. anatipestifer ompA1164* nucleotide sequence has been analyzed (Tsai et al. 2005; Yu et al. 2008), and the identity of the *ompA1164* sequence of 15 Taiwan strains and eight reference strains and two other sequences retrieved from GenBank was 88.1–100.0% (Tsai et al. 2005). If OmpA1467 could induce protective immunity, partial cross immunity among different serotypes of *R. anatipestifer* strains may be found. However, very little or no cross-protection has been observed among different *R. anatipestifer* serotypes (Sandhu 1979). So, the results of the different studies seem to be confused and inconsistent as to whether OmpA in *R. anatipestifer* confers protective immunity. Therefore, the aim of the present study was to compare the protective immunity of rOmpA1467 and rOmpA1164 against virulent challenge and to evaluate the protective immunity of *ompA* deletion mutant.

Materials and methods

Bacterial strains and growth conditions

R. anatipestifer strains CH3 (serotype 1), WJ4 (serotype 1), Th4 (serotype 2), Yb2 (serotype 2), NJ-3 (serotype 2), HXb2 (serotype 10), YXb1 (serotype 10), and SX (serotype not determined) were isolated and identified by Qinghai Hu 10–20 years ago. *R. anatipestifer* type strain ATCC11845 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The *ompA* deletion *R. anatipestifer* mutant Th4 Δ *ompA* was constructed previously in our lab, and 865-bp (+247 to +1111 nt) of *ompA1467* ORF was deleted, and no OmpA protein was detected from Th4 Δ *ompA* by western blot (Hu et al. 2011). All *R. anatipestifer* strains were cultured in tryptic soy broth (TSB; Difco Laboratories, Franklin Lakes, NJ, USA) or on tryptic soy agar (TSA) at 37 °C under an atmosphere of 5% CO₂. For selective growth of bacterial strains, kanamycin (50 µg/mL) or spectinomycin (60 µg/mL) was added as needed.

Sequence analysis of *ompA1467* ORF from different serotypes of *R. anatipestifer* strains

The sequences of the *ompA1467* ORF of *R. anatipestifer* strains Th4, YXb1, and SX were amplified by PCR using the primers *ompA* P1 plus *ompA* P2 for *ompA1467* (*ompA* P1: 5'-GCGATTAAGGAGAGAGAAGCAA-3'; *ompA* P2: 5'-TTTTATCCCAACGAGCCATC-3'). The PCR products were sequenced by Shanghai Huagene Biotech Co., Ltd. (Shanghai, China). These nucleotide sequences of *ompA1467* have been deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) under Accession No. MF458999-MF459001. The full genome of WJ4, HXb2, CH3, and Yb2 has been sequenced by our lab or other lab in our institute and released to GenBank database under accession No. CP041029, CP011859, CP006649, and CP007204. The *ompA1467* sequences of other strains used in this study, such as ATCC11845, CH-1, CH-2, RA-GD etc., were retrieved from the GenBank database. The amino acid sequence homology of *ompA1467* from *R. anatipestifer* strains with different serotypes was aligned and analyzed by the Clustal W method enclosed within MegAlign program of Lasergene 7.01 software (DNASTAR Inc., Madison, WI, USA).

Expression of OmpA1164 and OmpA1467 in *Escherichia coli*

The full *ompA1467* gene of *R. anatipestifer* encodes 488 amino acids. The prediction results of the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) showed that the signal peptides of OmpA1467 and OmpA1164 were 21 and 23 amino acids in length, respectively. Two different lengths of *ompA* ORFs encoding for OmpA1467 (residues 22–488, OmpA22–488) and OmpA1164 (residues 125–488, OmpA125–488), without the signal peptides, were amplified from *R. anatipestifer* strain Th4 by PCR using the primer pairs *ompA1467* P1 plus *ompA* P2' and *ompA1164* P1 plus *ompA* P2' (*ompA1467* P1, 5'-ATGGATCCCAGACTACTAGC AATCCTTGTT-3'; *ompA* P2', 5'-GCTACTCGAGATAC TAATTATTTTCTTTTCTTTTACTACTTTT-3'; *ompA1164* P1, 5'-TAGGATCCAACGAAGATGCA TGGTTTGAC-3'), respectively. Then, the sequenced *ompA1164* and *ompA1467* were linked to the expression vector pET30a (+) (Novagen, Inc., Madison, WI, USA) and then expressed in *E. coli* BL21 (DE3) cells (Novagen, Inc.). The bacteria were collected by centrifugation and lysed by sonication. The inclusion bodies were washed with washing buffer (50 mM Tris (pH 8.0), 300 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 5 mM DTT), and dissolved with solubilization buffer (50 mM Tris (pH 8.0), 300 mM NaCl, 8 M Urea, 20 mM imidazole buffer), and then the recombinant proteins were purified by Ni-IDA affinity chromatography

(DetaiBio, Nanjing, China), and the target proteins were eluted with different concentrations of imidazole. For rOmpA1467 refolding, the eluted proteins with high purity by SDS-PAGE analysis were dialyzed at 4 °C in refolding buffer 1 (1 × PBS (pH 7.4), 4 mM GSH, 0.4 mM GSSG, 2 mM EDTA, 0.4 M L-Arginine, 2 M Urea), while for rOmpA1164 refolding, refolding buffer 2 (50 mM Tris(pH 8.0), 150 mM NaCl, 4 mM GSH, 0.4 mM GSSG, 2 mM EDTA, 0.4 M L-Arginine, 2 M Urea) was used. After refolding, the rOmpA1467 and rOmpA1164 proteins were finally dialyzed into storage buffer 1 (1 × PBS, 10% Glycerol, pH 7.4) and storage buffer 2 (50 mM Tris (pH 8.0), 150 mM NaCl, 10% Glycerol), respectively. The purified proteins were detected by SDS-PAGE and western blotting using anti-His tag antibody (Beyotime Biotechnology, Shanghai, China).

Post-refolding analysis

Ultracentrifugation and circular dichroism were performed to analyze whether the rOmpA1467 and rOmpA1164 proteins were correctly refolded. For ultracentrifugation, the rOmpA1467 and rOmpA1164 protein solutions were centrifuged at 30,000 g for 3 h at 4 °C in an Optima L-100XP Beckman ultracentrifuge, and the concentrations of protein solution before ultracentrifugation (C1) and after ultracentrifugation (C2) were measured by BCA Protein Assay kit (Pierce, Thermo Scientific, IL, USA), and C2/C1 ratio was calculated. To examine the secondary structures of the refolded rOmpA1467 and rOmpA1164 proteins, circular dichroism analysis was performed on a Chirascan™ circular dichroism spectrometer (Applied Photophysics, UK) in a 0.5-mm path length quartz cuvette under N₂ at room temperature. The UV absorption and CD spectra were recorded over the wavelength range 190–260 nm using a slit bandwidth of 1.0 nm and a scanning speed of 0.5 s per point. The CDNN CD spectra deconvolution software Version 2.1 was utilized to determine the secondary structure content of the rOmpA1467 and rOmpA1164 proteins.

Enzyme-linked immunosorbent assay (ELISA)

The antibodies from ducklings or hybridoma supernatants were measured by indirect ELISA using 96-well microplates as described previously (Prieto et al. 2003), with a coating antigen of whole *R. anatipestifer* cells at a concentration of 10⁶ cells/well or soluble recombinant protein at a concentration of 1.2 µg/mL. Background noise was corrected by subtracting the absorbance of negative control wells. All samples were tested in triplicate, and the results are presented as the mean ± the standard error of the mean. In addition, the highest dilution of the serum (positive/negative ratio of ≥ 2.1) was recorded as the ELISA titer.

Immunization and challenge

One-day-old Cherry Valley ducklings, in which no serum antibodies against *R. anatipestifer* Th4 was detected by indirect-ELISA as described above, were purchased from Jinhu Duck Farm (Jiangyin, Jiangsu province, China).

To evaluate the protective immunity of the recombinant OmpA1467 (rOmpA1467) and rOmpA1164, 8-day-old Cherry Valley ducklings were divided into four groups (18–19 ducklings per group) and immunized once intramuscularly with 100 µg of rOmpA1164 or rOmpA1467, or 1 × 10⁹ colony-forming units (CFU) of formaldehyde-inactivated Th4 cells, or phosphate-buffered saline (PBS), which were emulsified with ISA 70 V adjuvant (SEPPIC, Paris, France). Sera were collected 14 days after immunization, and the sera antibodies were detected using an enzyme-linked immunosorbent assay (ELISA) with rOmpA1467 or Th4 bacterial cells as the coating antigen. At post-immunization day 15, all ducklings were challenged with 3 × 10⁹ CFU of Th4 cells. Ducklings that became moribund were killed humanely and counted as dead and then subjected to *R. anatipestifer* identification. The mortality of the ducklings was recorded daily for a period of 10 days after challenge.

To further evaluate the role of OmpA in the protective immunity of whole *R. anatipestifer* cells, 8-day-old ducklings were immunized with the inactivated *ompA* deletion mutant Th4Δ*ompA* or wild-type Th4 cells and challenged with wild-type Th4. Briefly, Th4Δ*ompA* or Th4 cells were washed with PBS, inactivated with 0.3% (v/v) formaldehyde. The ducklings were allocated to one of the three groups (16 ducklings per group) and immunized subcutaneously with 2 × 10⁹ CFU of inactivated Th4 or Th4Δ*ompA* cells, or 500 µL of PBS as negative control on days 5 and 18, respectively. On day 30, all ducklings were challenged with 3 × 10⁹ CFU of the Th4 bacteria. Moribund ducklings were killed humanely. *R. anatipestifer* was isolated from the blood or liver tissues of all ducklings either after death or the termination of the experiment (10 days post-challenge). Duck sera before challenge were collected for the detection of the antibodies with ELISA.

Preparation of monoclonal antibodies against rOmpA1164

To prepare monoclonal antibodies against rOmpA1164, 6-week-old BALB/c mice were intraperitoneally immunized with 100 µg of rOmpA1164 with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, MO, USA), followed by booster immunization with 100 µg of rOmpA1164 with Freund's incomplete adjuvant twice at 2-week intervals. Three days after the final booster, spleen cells from the immunized mice were fused with mouse myeloma SP2/0-Ag14 cells (ATCC, VA, USA), and the hybridoma

supernatants were screened with indirect ELISA using whole Th4 cells or rOmpA1164 as coating antigen. The serum from rOmpA1164-immunized mice and serum from naïve mice were used as positive and negative control. The positive cells were subcloned by limiting dilution and further characterized by western blot. Positive hybridoma cells were subcloned two to three times by limiting dilution. Six hybridoma cell lines were then established, and culture supernatant of these hybridoma cell lines was collected. The reactivity of these monoclonal antibodies to rOmpA1164 and rOmpA1467 was detected by western blot.

Expression of OmpA1467 and OmpA1164 in *R. anatipestifer* cells

R. anatipestifer strains ATCC11845, CH3, WJ4, NJ3, Yb2, Th4, HXb2, and YXb1, which belong to different serotypes, were grown on TSA agar for 24 h at 37 °C under 5% CO₂. The cells were harvested into sterile PBS, washed and pelleted by centrifugation, and then resuspended in PBS and adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0. OmpA expression of these strains was measured by SDS-PAGE and western blot using monoclonal antibody 2A4 as the first detection antibody as described above. The *ompA* deletion mutant Th4Δ*ompA*, rOmpA1164, and rOmpA1467 were used as control.

Statistical analysis

The statistical significance of the data was determined using survival analyses and survival curve program or one-way ANOVA and Tukey's multiple comparison test within GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). A probability (*p*) value of <0.05 was considered statistically significant.

Results

OmpA1467 from some *R. anatipestifer* strains with different serotypes shared the same amino acid sequence

In this study, our results showed that *ompA1467* of *R. anatipestifer* strains with different serotypes shared the same amino acid sequence. OmpA1467 from strains Th4, CH3, CH-1, WJ4, YXb1, RA-GD, ATCC11845, NCTC11014, and RCAD0133, which belong to different serotypes, shared 100% amino acid identity, and so did strains SX, CH-2, Rf153 and 153, 17 and Yb2 (Fig. 1).

Expression and purification of the recombinant OmpA1467 and OmpA1164

In this study, the OmpA1467 and OmpA1164 without signal peptide were expressed as inclusion bodies in *E. coli*. The recombinant fusion proteins His-OmpA1467 (rOmpA1467) and His-OmpA1164 (rOmpA1164) were obtained after washing, dissolution, purification by affinity chromatography, and refolding. Only one clear band of recombinant proteins rOmpA1467 and rOmpA1164 occurred by SDS-PAGE and western blot (Fig. S2).

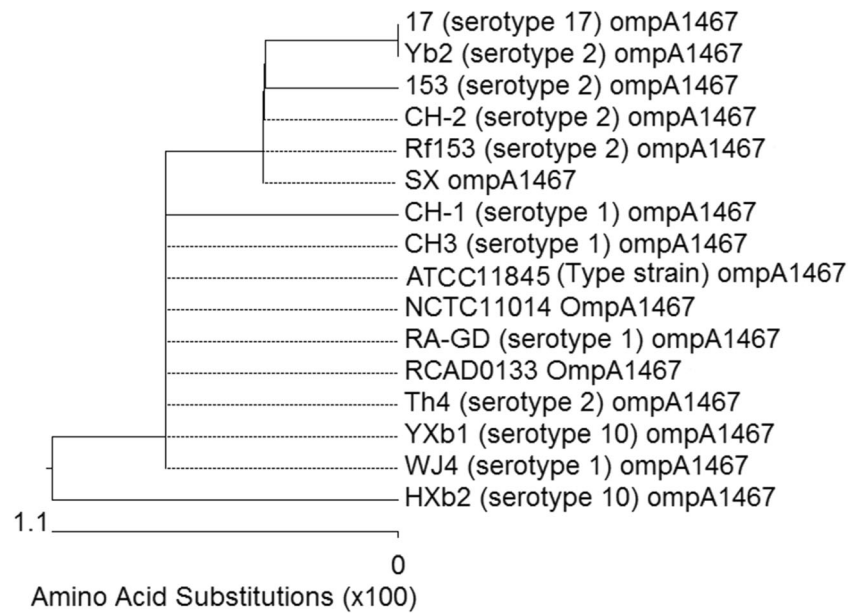
Refolded rOmpA1467 and rOmpA1164 are soluble and include secondary structural elements

After refolding, the rOmpA1467 and rOmpA1164 protein solutions were centrifuged, and the concentration ratios of rOmpA1467 and rOmpA1164 proteins after and before ultracentrifugation were 83.3% and 84.7%, respectively. The results indicated that 83.3% rOmpA1467 and 84.7% rOmpA1164 folded proteins were soluble, and over 80% rOmpA1467 and rOmpA1164 proteins were folded correctly. The second structures of rOmpA1467 and rOmpA1164 were further analyzed by circular dichroism. The CD of rOmpA1467 revealed 6.2% α-helices, 54.1% β-sheets, 16.3% β-turn, and 28.6% random coil, while rOmpA1164 appeared to contain 15.8% α-helices, 36.4% β-sheets, 20.6% β-turn, and 30.3% random coil (Fig. S3). The results indicated that compared to the secondary structures of rOmpA1467, rOmpA1164 contained more α-helices and β-turn but less β-sheets.

rOmpA1164, but not rOmpA1467, provided partial protective immunity against virulent Th4 challenge

As shown in Fig. 2a, 16 (84.2%) of 19 ducklings immunized with inactivated Th4 whole cells and 8 (44.4%) of 18 ducklings immunized with the soluble recombinant OmpA1164 were protected against virulent Th4 challenge. However, only 4 (21.1%) of 19 ducklings immunized with the soluble recombinant OmpA1467 survived after challenge, which was the same as group PBS. There was significant difference in protective immunity between groups rOmpA1467 and rOmpA1164 (*p* < 0.05). The results suggested that rOmpA1164, but not rOmpA1467, provided partial protective immunity against virulent Th4 challenge. Serum samples were collected from ducklings from each group, and the antibody titers were measured using rOmpA1467, rOmpA1164, or Th4 whole cells coated ELISA plates. As shown in Fig. 2b, when rOmpA1467 or rOmpA1164 proteins were used as the coating antigen, the serum antibodies titer from rOmpA1467-immunized ducklings was significantly higher than those from rOmpA1164 or Th4 cells-immunized ducklings (*p* < 0.05).

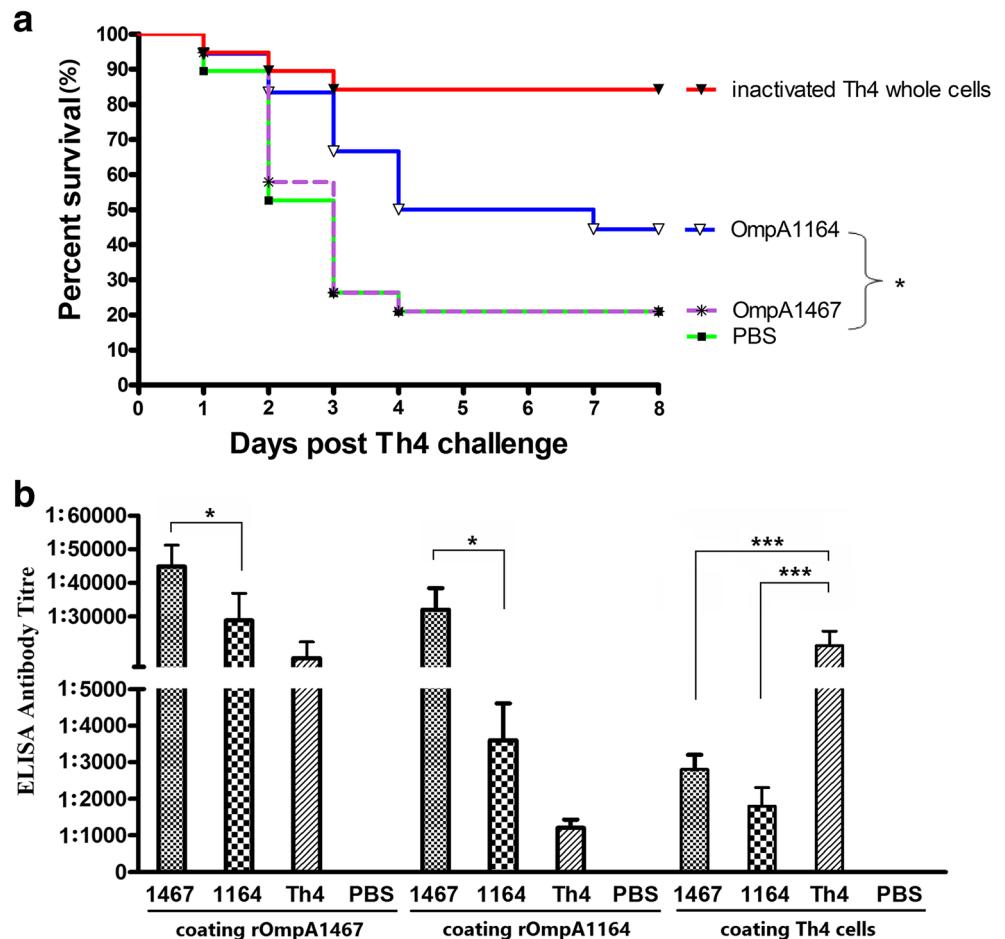
Fig. 1 OmpA1467 from some *R. anatipestifer* strains with different serotypes shared the same amino acid sequence. Amino acid sequence of OmpA1467 ORF from *R. anatipestifer* strains with different serotypes, including some retrieved from the GenBank database, was aligned and analyzed by Clustal W method enclosed within MegAlign program of Lasergene 7.01 software. A phylogenetic tree was built up based on the amino acid sequence of OmpA1467. The bar below indicates the distance between sequences. Units indicate the number of substitution events



When Th4 cells were used as the coating antigen, there was no significant difference in the serum antibodies titer from rOmpA1467- and rOmpA1164-immunized ducklings ($p > 0.05$), but the serum antibodies titer from Th4 cells-

immunized ducklings was significantly higher than those from rOmpA1467- and rOmpA1164-immunized ducklings ($p < 0.0001$). It suggested that high antibody titer of OmpA1467 in the serum of rOmpA1467-immunized

Fig. 2 The rOmpA1164, but not rOmpA1467, induced protective immunity against challenge. **a** Ducklings were immunized with 100 μ g of rOmpA1164 or rOmpA1467, or 1×10^9 CFU of formaldehyde-inactivated Th4 cells, or PBS, which were emulsified with ISA 70 V adjuvant. Two weeks later, the ducklings were challenged with virulent Th4 cells. The mortality of the ducklings was recorded daily for a period of 10 days after challenge. Survival curves were drawn using GraphPad Prism 5 software. Asterisks indicate statistically significant differences between two groups ($*p < 0.05$). **b** The titers of sera from ducklings immunized with rOmpA1467 and rOmpA1164 was measured using ELISA coated with rOmpA1467, rOmpA1164, or whole Th4 cells, respectively



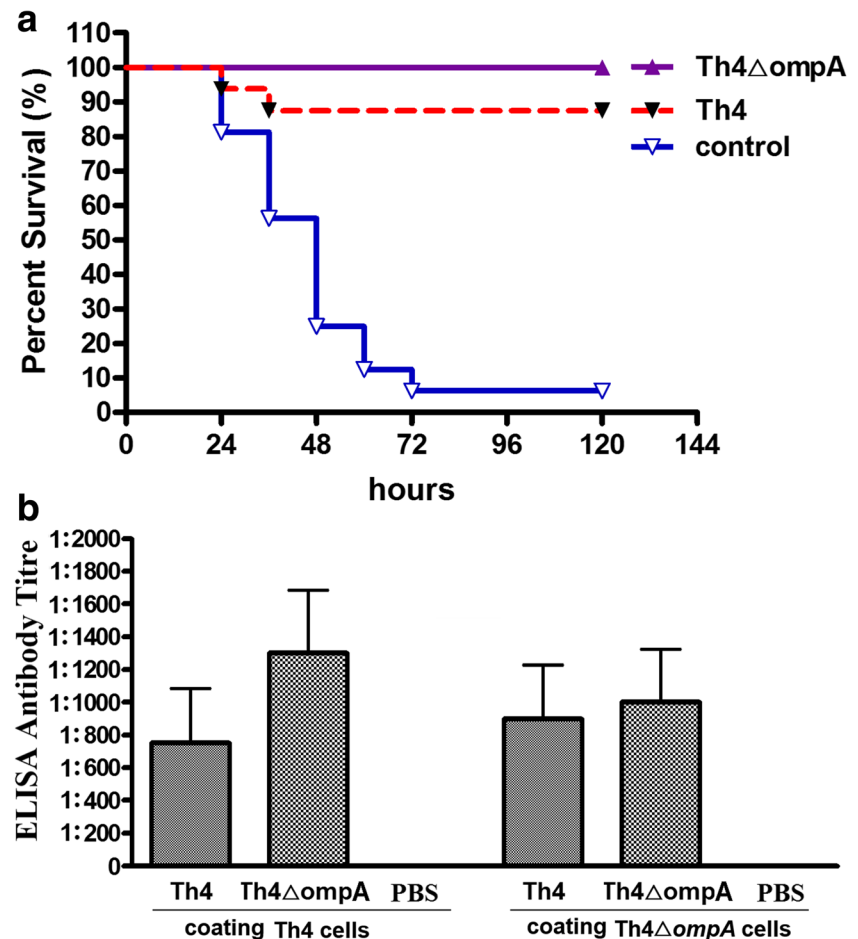
ducklings could not protect immunized ducklings from virulent challenge. The immunization-challenge experiment was performed twice, and the data from one representative experiment was shown.

Protection of the Th4 Δ ompA-immunized ducklings from challenge with virulent isolates

We further compared the protective immunity of the *ompA* deletion mutant Th4 Δ ompA with the wild-type Th4. As shown in Fig. 3a, all 16 (100%) ducklings immunized with the Th4 Δ ompA bacterin were protected from challenge with virulent Th4, and most showed no clinical signs of *R. anatipestifer* infection, with the exception of one that displayed a very brief episode of transient depression. In the group immunized with the Th4 bacterin, 14 (87.5%) of 16 ducklings were protected from the challenge with no clinical signs of disease, while the other two showed signs of mild depression and died at 24 and 36 h, respectively, after challenge. No bacteria were isolated from the blood or liver tissues of live-immunized ducklings at 10 days post-challenge. Of the 16 ducklings in the PBS control group, 15 died from challenge by day 5. The remaining

duck showed severe depression after challenge but had recovered at day 5 post-challenge. There was no significant difference in conferred protection from immunization with Th4 Δ ompA and Th4 bacterin ($p > 0.05$), but the protective rate of Th4 Δ ompA bacterin was higher than that of Th4 bacterin. Serum samples were collected from eight ducklings from each group, and the antibody titers were measured using ELISA plates coated with either whole cells of Th4 Δ ompA or the Th4 bacterin. When Th4 cells were used as the coating antigen, there was no significant difference in the serum antibodies titer between the Th4 and Th4 Δ ompA groups ($p > 0.05$, Fig. 3b). Likewise, when Th4 Δ ompA cells were used as the coating antigen, there was no significant difference in the mean ELISA titers between the Th4 and Th4 Δ ompA groups ($p > 0.05$, Fig. 3b). Interestingly, sera from the Th4 Δ ompA group had a higher antibody tendency than that from Th4 group with the coating antigen as Th4 Δ ompA or Th4. This was in accord with the results of immunization-challenge experiments in ducklings. It suggested that, in both Th4 and Th4 Δ ompA-immunized ducklings, sera antibodies to proteins other than OmpA are the main components of antibodies against *R. anatipestifer*, and these antibodies contain neutral

Fig. 3 There was no significant difference in protective immunity between inactivated *ompA* deletion mutant Th4 Δ ompA or wild-type Th4 cells. **a** Ducklings were immunized with inactivated Th4 Δ ompA or wild-type Th4 cells and then challenged with wild-type Th4 2 weeks later. The mortality of the ducklings was recorded daily for a period of 10 days after challenge. Survival curves were drawn using GraphPad Prism 5 software. **b** The titers of sera from ducklings immunized with the Th4 Δ ompA mutant and wild-type Th4 were measured using ELISA coated with whole Th4 or Th4 Δ ompA cells



antibodies against *R. anatipestifer* infection. The immunization-challenge experiments were performed twice, which yielded similar results.

Monoclonal antibodies against rOmpA1164

We generated mAbs against rOmpA1164 by traditional hybridoma method and obtained six mAbs against rOmpA1164. In this study, we focused on the property of two monoclonal antibodies 1C5 and 2A4. 1C5 and 2A4 are IgG1 subtype. The specificities of mAbs 1C5 and 2A4 were identified with rOmpA1164, rOmpA1467, total cell lysate of wild-type Th4, and *ompA* deletion mutant Th4 Δ *ompA* by western blot using the mAbs as primary antibodies. The results showed that 1C5 and 2A4 could react with rOmpA1164, rOmpA1467, and Th4 cells, but not Th4 Δ *ompA* cells. It also suggested that 1C5 and 2A4 were specific to OmpA and recognized the common epitope on OmpA1164 and OmpA1467. In addition, mAb 2A4 was better to detect OmpA1164 and OmpA1467 than 1C5 due to clean signal and less smear below the specific band in the western blot (Fig. S4). Therefore, 2A4 was chosen to detect OmpA expression in *R. anatipestifer*.

OmpA1467 was the main existing form of OmpA protein in *R. anatipestifer* cells

To determine in what form (OmpA1467 or OmpA1164) does OmpA protein exist in *R. anatipestifer* cells, the OmpA1467 and OmpA1164 proteins were detected in different serotypes of *R. anatipestifer* strains which were cultured on TSA agar by western blot analysis. As shown in Fig. 4, the results showed that all the tested *R. anatipestifer* with different serotypes, except *ompA* deletion mutant Th4 Δ *ompA*, expressed OmpA1467, and OmpA1467 in these strains gave very strong band compared with that of other bands in western blot using

mAb 2A4 as the primary antibodies, while other bands were very weak except CH3. It suggested OmpA1467 was the prominent form of OmpA in *R. anatipestifer* strains.

Discussion

OmpA is a predominant antigenic determinant of *R. anatipestifer* (Subramaniam et al. 2000); thus some researchers have attempted to develop a subunit vaccine against *R. anatipestifer* based on OmpA (Chu et al. 2015; Gao et al. 2014). However, whether or not OmpA is a protective protein in *R. anatipestifer* remains unclear because of the conflicting results in previous reports.

The results of the present study showed that OmpA1164, but not OmpA1467, induced protective immunity, which may be due to the differences in the three-dimensional (3-D) structures of these two proteins. The N-terminal domain of OmpA1467, but not that of OmpA1164, could form an intact eight-stranded β -barrel by SWISS-MODEL (<https://swissmodel.expasy.org/>, Fig. S5). It suggested that some epitopes which could induce protective immunity may be exposed to the surface of OmpA1164. In previous report, rOmpA102–488 failed to induce protective immunity, and the recombinant OmpA102–488 proteins dissolved in 6 M urea were used to immunize ducklings (Huang et al. 2002), which may lead to improper protein refolding and/or incorrect exposure of the protective epitopes. In another report, OmpA33–466 could induce protective immunity, but not OmpA1467 (OmpA22–488) in our study. This may also due to the difference of the protein structure, OmpA33–466 cannot form an intact eight-stranded β -barrel as OmpA22–488 by SWISS-MODEL.

In this study, our results showed that OmpA1467 was the prominent form of OmpA in *R. anatipestifer* strains, and the amount of OmpA1164 was very low or none, while

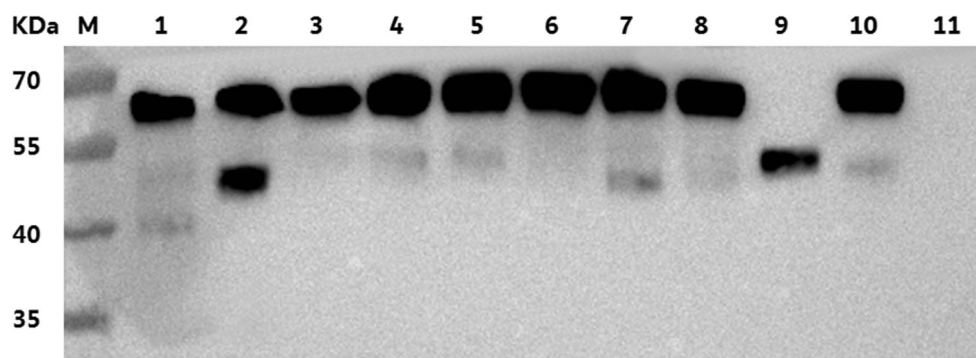


Fig. 4 OmpA1467 was the main existing form of OmpA protein in different *R. anatipestifer* strains. *R. anatipestifer* with different serotypes were grown on TSA, and OmpA expression was measured by SDS-PAGE and western blot using monoclonal antibody 2A4 as the first detection antibody. rOmpA1164, rOmpA1467, and the *ompA*

deletion mutant Th4 Δ *ompA* were used as control. M. Prestained protein Marker. Lane 1. ATCC11845; Lane 2. CH3; Lane 3. WJ4; Lane 4. NJ-3; Lane 5. Yb2; Lane 6. Th4; Lane 7. HXb2; Lane 8. YXb1; Lane 9. rOmpA1164; Lane 10. rOmpA1467; Lane 11. Th4 Δ *ompA*

OmpA1164, but not OmpA1467, could induce protective immunity. Therefore, even *ompA1467* ORF or *ompA1164* ORF from many different serotypes of *R. anatipestifer* strains shared the same amino acid sequence, only very poor or none cross-protection has also been found between different serotypes of *R. anatipestifer* strains (Sandhu 1979). Moreover, when Th4 cells were used as the coating antigen, there was no significant difference in the serum antibodies titer from rOmpA1467- and rOmpA1164-immunized ducklings ($p > 0.05$), but the serum antibodies titer from Th4 cells-immunized ducklings was significantly higher than those from rOmpA1467- and rOmpA1164-immunized ducklings ($p < 0.0001$). It suggested that, in both Th4 and Th4 Δ *ompA* -immunized ducklings, sera antibodies to proteins other than OmpA were the main components of antibodies against *R. anatipestifer*, and these antibodies contained neutral antibodies against *R. anatipestifer* infection.

In addition, the role of OmpA on protective immunity in whole *R. anatipestifer* cells was also evaluated using an *ompA* deletion mutant. The results showed that there was no significant difference in protection between Th4 Δ of the Th4 Δ *ompA* bacterin was higher than that of the Th4 bacterin. The following reasons may account for this phenomenon: (i) OmpA1467 is the main existing form of OmpA in *R. anatipestifer* strains, but it could not induce protective immunity; (ii) there were some unknown protective antigens which play important roles in protective immunity of *R. anatipestifer* infection; (iii) OmpA deletion leads to immune refocusing and hidden protective epitopes may be exposed (Tobin et al. 2008); and (iv) other unknown reasons. Otherwise, these results suggest that deletion of the *ompA* gene in a vaccine candidate may be beneficial to its protective function. In fact, this is not an isolated phenomenon. The outer membrane proteins (OMPs) of *Pasteurella multocida* are widely recognized as important immunogens that contribute to disease pathogenesis and protection against challenge (Basagoudanavar et al. 2006). However, the protection afforded by vaccination with *P. multocida* OMPs alone was adversely affected by the addition of the recombinant PmOmpA to the vaccine preparation. Nonetheless, targeted inactivation of the *ompA* gene in *P. multocida* 232 represents a potential step toward the development of an effective vaccine candidate (Dabo et al. 2008).

In summary, rOmpA1164, but not rOmpA1467, induced protective immunity. The findings of this study will help to uncover the biological characteristics of OmpA and to develop novel vaccines against *R. anatipestifer* infection.

Funding information This study was founded by the National Natural Science Foundation of China (Grant numbers: 31772770, 31472224, 31272590, and 31072156).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical statement Ducklings were housed in cages under a 12-h light/dark cycle with free access to food and water. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (Permit Number: 15–12).

References

- Basagoudanavar SH, Singh DK, Varshney BC (2006) Immunization with outer membrane proteins of *Pasteurella multocida* (6:B) provides protection in mice. *J Vet Med A Physiol Pathol Clin Med* 53(10):524–530
- Cheng A, Wang M, Chen X, Zhu D, Huang C, Liu F, Zhou Y, Guo Y, Liu Z, Fang P (2003) Epidemiology and new serotypes of *Riemerella anatipestifer* isolated from ducks in China and studies in their pathogenic characteristics. *Chin J Vet Sci* 23(4):320–323
- Chu CY, Liu CH, Liou JJ, Lee JW, Cheng LT (2015) Development of a subunit vaccine containing recombinant *Riemerella anatipestifer* outer membrane protein A and CpG ODN adjuvant. *Vaccine* 33(1):92–99
- Dabo SM, Confer A, Montelongo M, York P, Wyckoff JH 3rd (2008) Vaccination with *Pasteurella multocida* recombinant OmpA induces strong but non-protective and deleterious Th2-type immune response in mice. *Vaccine* 26(34):4345–4351
- Gao Y, Liu J, Wang W, Li Q, Zhang F, Ma H (2014) Cloning and expression of *Riemerella anatipestifer* OmpA with duck interleukin-2 fusion gene. *Chin J Vet Med* 50(8):42–46
- Hu Q, Han X, Zhou X, Ding C, Zhu Y, Yu S (2011) OmpA is a virulence factor of *Riemerella anatipestifer*. *Vet Microbiol* 150(3–4):278–283
- Hu Q, Zhang Z, Miao J, Liu Y, Liu X, Deen S (2001) The epidemiology study of *Riemerella anatipestifer* infection in Jiangsu and Anhui provinces. *Chin J Vet Sci Technol* 31(8):12–13
- Huang B, Subramaniam S, Frey J, Loh H, Tan HM, Fernandez CJ, Kwang J, Chua KL (2002) Vaccination of ducks with recombinant outer membrane protein (OmpA) and a 41 kDa partial protein (P45N) of *Riemerella anatipestifer*. *Vet Microbiol* 84(3):219–230
- Kurupati P, Ramachandran NP, Poh CL (2011) Protective efficacy of DNA vaccines encoding outer membrane protein A and OmpK36 of *Klebsiella pneumoniae* in mice. *Clin Vaccine Immunol* 18(1):82–88
- Lee JS, Jung ID, Lee CM, Park JW, Chun SH, Jeong SK, Ha TK, Shin YK, Kim DJ, Park YM (2010) Outer membrane protein A of *Salmonella enterica* serovar *Typhimurium* activates dendritic cells and enhances Th1 polarization. *BMC Microbiol* 10:263
- Pathanasophon P, Phuекtes P, Tanticharoenyos T, Narongsak W, Sawada T (2002) A potential new serotype of *Riemerella anatipestifer* isolated from ducks in Thailand. *Avian Pathol* 31(3):267–270
- Pore D, Mahata N, Pal A, Chakrabarti MK (2011) Outer membrane protein A (OmpA) of *Shigella flexneri* 2a, induces protective immune response in a mouse model. *PLoS One* 6(7):e22663
- Prieto CI, Rodriguez ME, Bosch A, Chirido FG, Yantorno OM (2003) Whole-bacterial cell enzyme-linked immunosorbent assay for cell-bound *Moraxella bovis* pili. *Vet Microbiol* 91(2–3):157–168
- Ruiz JA, Sandhu TS (2013) *Riemerella anatipestifer* infection. In: Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair

- VL(eds) Diseases of poultry, 13th ed. Wiley-Blackwell Press, p 823–828
- Sandhu T (1979) Immunization of white Pekin ducklings against *Pasteurella anatipestifer* infection. *Avian Dis* 23(3):662–669
- Smith SG, Mahon V, Lambert MA, Fagan RP (2007) A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett* 273(1):1–11
- Subramaniam S, Huang B, Loh H, Kwang J, Tan HM, Chua KL, Frey J (2000) Characterization of a predominant immunogenic outer membrane protein of *Riemerella anatipestifer*. *Clin Diagn Lab Immunol* 7(2):168–174
- Tian H, Fu F, Li X, Chen X, Wang W, Lang Y, Cong F, Liu C, Tong G (2011) Identification of the immunogenic outer membrane protein A antigen of *Haemophilus parasuis* by a proteomics approach and passive immunization with monoclonal antibodies in mice. *Clin Vaccine Immunol* 18(10):1695–1701
- Tobin GJ, Trujillo JD, Bushnell RV, Lin G, Chaudhuri AR, Long J, Barrera J, Pena L, Grubman MJ, Nara PL (2008) Deceptive imprinting and immune refocusing in vaccine design. *Vaccine* 26(49):6189–6199
- Tsai HJ, Liu YT, Tseng CS, Pan MJ (2005) Genetic variation of the ompA and 16S rRNA genes of *Riemerella anatipestifer*. *Avian Pathol* 34(1):55–64
- Yu CY, Liu YW, Chou SJ, Chao MR, Weng BC, Tsay JG, Chiu CH, Ching Wu C, Long Lin T, Chang CC, Chu C (2008) Genomic diversity and molecular differentiation of *Riemerella anatipestifer* associated with eight outbreaks in five farms. *Avian Pathol* 37(3):273–279
- Zhai Z, Li X, Xiao X, Yu J, Chen M, Yu Y, Wu G, Li Y, Ye L, Yao H, Lu C, Zhang W (2013) Immunoproteomics selection of cross-protective vaccine candidates from *Riemerella anatipestifer* serotypes 1 and 2. *Vet Microbiol* 162(2–4):850–857

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.