Int J Clin Exp Med 2016;9(2):1330-1337
www.ijcem.com /ISSN:1940-5901/IJCEM0017474

Original Article

Development of an indirect ELISA using recombinant ompH protein for serological detection of *Riemerella anatipestifer* infection in ducks

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Received October 8, 2015; Accepted December 31, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: *Riemerella anatipestifer* (RA), the causative agent of duck septicemia, results in high mortality of ducklings and causes great economic losses in duck raising industry. The outer membrane protein H (OmpH) of RA, expressed in *E.coli* strain BL21, was first used as coating antigen to develop an indirect ELISA assay for serological detection of RA infection. The sensitivity test indicated that the detection limit of the OmpH protein based ELISA assay (OmpH-ELISA) was twice higher than that of the OmpA protein based ELISA assay (OmpA-ELISA), and 2600 times higher than that of the conventional tube agglutination (TA) assay. In addition, the OmpH-ELISA assay displayed no cross-reaction with *E.coli, S.anatum,* duck hepatitis virus (DHV), avian influenza virus (AIV), *Pasteurellamultocida* (*P.multocida*) and duck plague virus (DPV) antisera, while positive with RA ATCC and RA CH-2 antisera. For detection of clinical serum samples, 115 of 156 samples were positive by OmpH-ELISA (73.7%), 105 serum samples (67.3%) were positive by OmpA-ELISA assay, only 89 serum samples (57.05%) were detected positively by TA assay. All of these features suggested that the OmpH protein is a conserved protein among different serotypes of RA, and could be used as an ELISA coating antigen for serological detection of RA infection.

Keywords: *Riemerella anatipestifer*, omph, elisa

Introduction

Riemerella anatipestifer (RA), a gram-negative nonmotile bacillus, belongs to the family of *Flavobacteriaceae* in rRNA superfamily V [1, 2]. As a host specific pathogen, RA infects poultry species primarily, especially ducks, and caused high morbidity and mortality in ducklings. Due to the data from the world poultry council (http://www.internationalpoultrycouncil.org/industry/industry.cfm), Asia is the top producer of duck meat and owns the largest population of commercial ducks in the world. However, contagious disease such as serositis (caused by RA) severely impaired the rising business and resulted in great economic losses [3, 4].

Measures to limit the prevalence of RA such as thoroughly sterilizing of farm and strict quarantine of suspect birds seem helpful but not long lasting. Antibiotic therapy is functional, but only before systemic infection happened. Thus, fast and reliable diagnostic technique of RA infection is an urgent need for the early treatment. Till now, several methods have been developed to detect RA [5, 6]. The polymerase chain reaction (PCR) based technique needs costly thermal cycler, which reduced its field application. The whole cell based ELISA method was only able to detect some serotypes of RA, while the tube-agglutination assay required different types of positive serum.

The outer membrane protein H (OmpH) (also known as Skp), possessed the general characteristic of the porin [7, 8], was a conserved and protective outer membrane protein in *Pasteurellamultocida* somatic serotypes [9]. In

this study, we develop a simple, fast and cost-effective indirect ELISA assay for detection of RA infection. The coated antigen of OmpH proteins in this assay was obtained by cloning the *ompH* gene (encoded the outer membrane protein H, OmpH) of RA strain CH-1 into the vector plasmid and expressed in an *E.coli* strain BL21. The purified OmpH proteins were then used as ELISA antigen for serological detection of RA infection.

Materials and methods

Serum samples

The negative serum and duck antisera of RA CH-1, RA CH-2, RA ATCC 11845, *E.coli*, *Salmonella anatum* (*S.anatum*), duck hepatitis virus (DHV), duck plague virus (DPV), avian influenza virus (AIV) and *Pasteurellamultocida* (*P.multocida*) were identified, and were available in our lab. The clinical test serums were collected from ducks at different raising farms and stored at -70°C until used.

Bacterial strains, plasmids, and culture conditions

The T vector pMD19, expression vector pET32-a(+), *E.coli* strains *DH5α* and BL21 used in this study were purchased from Takara (Takara, Dalian, China) and Invitrogen (Invitrogen, Carlsbad, California, USA). RA strains CH-1 and CH-2 were isolated, identified and stored in our lab. RAATCC 11845 was purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA). The *E.coli* strains were routinely grown in LB broth or LB agar. When required, ampicillin was added to the medium at the final concentration of 100 μg/ml. RA strains CH-1, CH-2 and ATCC 11845 were grown in Trypticase Soy Broth (TSB) or Trypticase Soy Agar (TSA) (Difco, Franklin Lakes, New Jersey USA).

Plasmid construction

DNA fragment encoding the outer membrane protein H (*OmpH*) were amplified by PCR from RA strain CH-1, using specific primer sets of P1 (5'-GGATCCATGAAAAAATTAAGCGTATTGTTTGC-3') (*BamHI* site underline) and P2 (5'-CTCGAGATTTTACATTATTGAGATGCCCTATTG-3') (*XhoI* site underline). The *BamHI* and *XhoI* digested plasmid of pMD19 and *OmpH* PCR products were ligated and transformed into *E.coli* DH5α following the standard techniques [10]. Selection for ampicillin resistant transformant resulted in construction of plasmid pMD19-*ompH*. To generate the recombinant expression plasmid pET32a-*ompH*, both *BamHI* and *XhoI* digested plasmid of pMD19-*ompH* and pET32a(+) were ligated and transformed into *E.coli* BL21, using ampicillin as a positive selection. The resultant strain, containing the recombinant expression plasmid of pET32a(+)-*ompH*, was named as BL21X.

Expression, purification of ompH proteins

To obtain the OmpH proteins, 5 ml of an overnight culture of Strain BL21X was added to 500 ml fresh LB-Ampicillin broth for propagation at 37°C. When OD_{600} reach -0.5, isopropyl-β-d-thiogalactoside (IPTG) was added to the culture at the final concentration of 0.4 mmol/L. After incubation at 37°C for 9 hours, the bacterial pellets were harvested by centrifugation (7000 rev/min, 5 min) and resuspended in 50 ml Tris-HCl (20 mmol/L pH 8.0). Followed by sonication for 10 min (600 w, 30 sec/time), the supernatant was separated by centrifugation (12000 rev/min, 10 min) and submitted to 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE). The insoluble precipitate was dissolved in 8 mol/L urea, and was analyzed by 12% SDS-PAGE. The yields of OmpH proteins were increased by optimizing the inducing time (5, 7 and 9 h), temperature (25°C, 30°C and 37°C) and IPTG concentration (0.4, 0.8 and 1.2 mmol/L).

Based on the results of SDS-PAGE, the expressed OmpH proteins were purified from the lysed cell supernatantusing Ni^{2+}-agarose (GE healthcare, Hong Kong, China). Briefly, ~50 ml of supernatant were loaded into a Ni^{2+}-agarose column. Then, the OmpH proteins which bound to the Ni^{2+}-agarose was eluted using elution buffer (20 mmol/L Tris-HCl, 250 mmol/L imidazole). The concentration of the purified protein was measured by the Bradford method [11], and analyzed by 12% SDS-PAGE as before.

Immunobloting analysis

To validate the immunoreactivity of the expressed OmpH proteins, an immunoblotting assay was performed as described previously

[12]. Briefly, OmpH protein samples separated by SDS-PAGE were transferred into a polyvinylidene difluoride (PVDF) membrane (Millipore, Darmstadt, Germany) by following standard protocols. After blocking with 1% BSA at 37°C for an hour, the membrane was incubated with rabbit anti-RA antibody (prepared in our lab) at 4°C overnight. The OmpH proteins were finally being detected using goat anti-rabbit IgG conjugated with horseradish peroxidase (KPL, Gaithersburg, USA).

Development and optimization of the ompH-ELISA

To develop an OmpH protein based indirect ELISA method (OmpH-ELISA) for serological detection of RA infection, 100 μl purified OmpH proteins, diluted in 0.05 mol/L carbonate-bicarbonate buffer (pH 9.6), were added to 96-well ELISA plates (Corning, NY, USA) and coated overnight at 4°C. The next day, plates were washed five times with PBS-T (phosphate buffered saline with 0.5% Tween 20) and blocked with 1% bovine serum albumin (BSA) in PBS-T at 37°C for an hour. Then, 100 μl of 2-fold dilutions of the test serum was added to the wells and incubated at 37°C for an hour. Goat anti-duck IgG conjugated with horseradish peroxidase (HPR) were used to determine the OmpH specific IgG antibody in test serum. A positive reaction was defined as an OD_{450} value > x+3s (x = average of negative controls and s = standard deviation of the negative control sera).

The working condition of OmpH-ELISA assay was optimized by a conventional checkerboard titration method. The maximum difference value between positive and negative serum (P/N) at OD_{450} nm was selected for testing the experimental serum samples [13].

The cut-off value, sensitivity and specificity of ompH-ELISA

30 unimmunized duck serum, collected from a single flock of 50 one-month old specific pathogen free (SPF) Peking ducks, were used to determine the cut-off value of the OmpH-ELISA assay. Also, 30 unimmunized duck serum were showed negative by OmpA-ELISA [14]. A mean value plus 3 times standard deviation of the test serums were defined as the cut-off value.

The sensitivity of OmpH-ELISA was determined by performing the ELISA assay under the optimal conditions with 2-fold dilutions of RA CH-1 antisera from 1:325 to 1:41600. The highest dilution that produced an OD_{450} value > the cut-off value was considered as the detection limit of the OmpH-ELISA assay. Comparison detection sensitivity among OmpH-ELISA assay, OmpA-ELISA assay and TA assay.

To validate the detection specificity, duck antisera of different pathogens were submitted to OmpH-ELISA assay. The OD_{450} value of each sample was compared to the cut-off value, while RA CH-1 antisera and PBS diluents were served as the positive and blank controls, respectively.

Repeatability and reproducibility of the ompH-ELISA

To assess the repeatability and reproducibility of the OmpH-ELISA assay, five positive serum samples were tested in a single experiment with five different plates or in five independent experiments with same positive serum to determine the intra-assay variability (repeatability) and inter-assay variability (reproducibility), respectively. The coefficient of variation (CV) was defined as standard deviation (SD)/mean OD_{450} value. All experiments were performed in triplicate.

Detection of clinical serum samples

A total of 156 clinical serum samples, obtained from 3 independent duck raising farms, were tested by OmpH-ELISA assay, OmpA protein based ELISA (OmpA-ELISA) assay and conventional tube agglutination (TA) assay. The OmpH-ELISA assay was performed under the optimal condition as before. For the TA assay and OmpA-ELISA assay, they were carried out according to report described previously [14, 15].

Data processing

All of the raw data were input into the Microsoft Excel (version 2010) or GraphPad Prism (version V5.01) software for rearrangement and analysis.

Results

The optimal expression condition and immunoblotting analysis of ompH proteins

In order to increase the production of OmpH proteins, we optimized the expression condi-

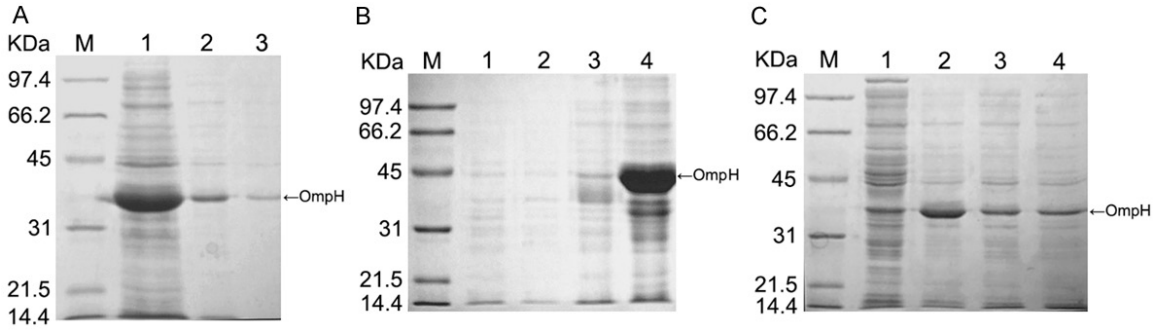

Figure 1. The expression of the recombinant OmpH proteins at different conditions. A. Optimization of cultural temperature. Lane M, protein marker; Lane 1, 25°C; Lane 2, 30°C; Lane 3, 37°C. B. Optimization of the IPTG concentration. Lane M, protein marker; Lane 1, no IPTG; Lane 2, 0.4 mM IPTG; Lane 3, 0.8 mM IPTG; Lane 4, 1.2 mM IPTG. C. Induction time optimization. Lane M, protein marker; Lane 1, 0 h; Lane 2, 9 h; Lane 3, 7 h; Lane 4, 5 h.

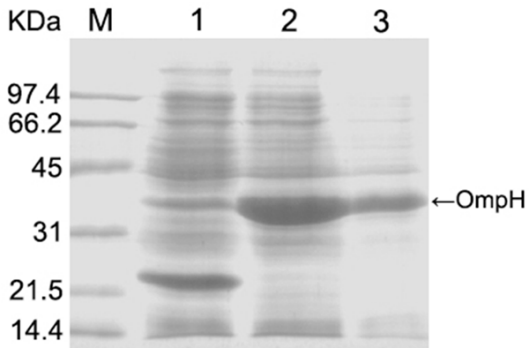

Figure 2. 12% SDS-PAGE analysis of the soluble and insoluble fractions of recombinant OmpH. Lane M, protein marker; Lane 1, blank pET32a(+) vector plasmid; Lane 2, supernatant; Lane 3, precipitate.

tions. As results shown in **Figure 1**, the best yield of OmpH proteins were achieved by culturing the cells of BL21X at 25°C (**Figure 1A**), with final IPTG concentration of 1.2 mmol/L (**Figure 1B**) and incubated for 9 h (**Figure 1C**). To locate the expressed OmpH proteins, both supernatant and precipitate of the lysed BL21X cells were submitted to SDS-PAGE. From the results, most of the OmpH proteins were observed in supernatant (**Figure 2**).

Next, we sought to validate the immunoreaction of the recombinant OmpH proteins. To this end, we first purified the OmpH proteins by Ni^{+} chromatography (**Figure 3**), the purified proteins were submitted to immunoblotting analysis. The results indicated that only a 38 kDa band of OmpH proteins was observed as we expected (**Figure 4**), which suggested that the OmpH proteins which expressed by strain BL21X still retained the natural immunoreaction of RA OmpH proteins.

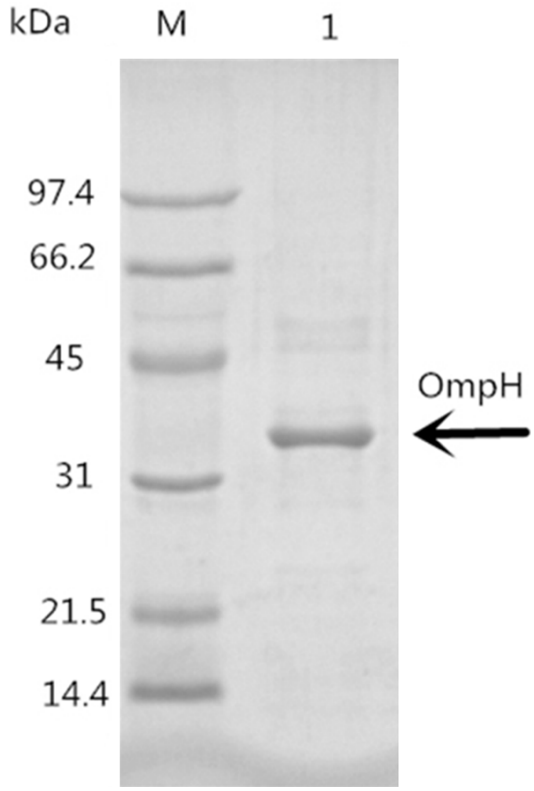

Figure 3. The SDS-PAGE analysis of the purified recombinant OmpH proteins. Lane M, protein marker; Lane 1, purified recombinant OmpH proteins.

Optimization of ompH-ELISA

The working condition of OmpH-ELISA was determined by performing the ELISA assay under 2-fold titration of antigen, serum as well as the secondary antibody, respectively. At a pre-determined dilution of secondary antibody (1:800), the optimal concentration of coated antigen (OmpH proteins) and serum titration

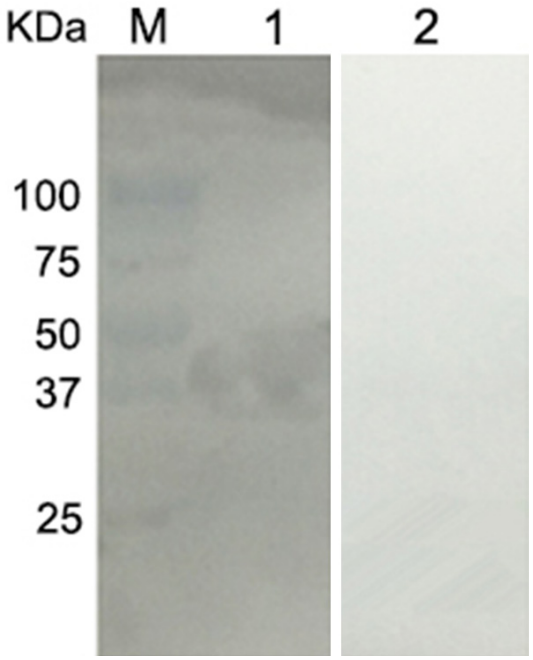

Figure 4. Identification of the immunoreactivity of the recombinant OmpH proteins by immunobloting assay. Lane M, protein marker; Lane 1, unpurified OmpH proteins analyzed by the rabbit anti-RA-CH-1 monoclonal antibody; Lane 2, negative control.

were 8.0 ng/μl and 1:100, respectively. Under the optimal antigen and serum working condition, the best titration of the goat anti-duck antibody was calculated as 1:400.

Determination the cutoff value, sensitivity and specificity of ompH-ELISA

By testing of 30 unimmunized duck serum samples, the cut-off value of the OmpH-ELISA assay was calculated as 0.37. Then, we determined the detection sensitivity of the OmpH-ELISA assay using series 2-fold dilutions of RA CH-1 antisera. As data showed in **Figure 5**, the detection limit of the OmpH-ELISA was 1:20800, which was 2 times higher than that of the OmpA-ELISA and 2600 times higher than that of the TA assay (data not showed).

To evaluate the specificity of the OmpH-ELISA assay, the negative serum and antisera of RA CH-1, RA CH-2, RA ATCC 11845, *E.coli*, S.*anatum*, AIV, *P.multocida*, DHV and DPV were tested under the optimal ELISA condition. From the results in **Figure 6**, the OD_{450} value obtained from the PBS, negative serum, antisera of *E.coli*, *S.anatum*, AIV, *P.multocida*, DHV and

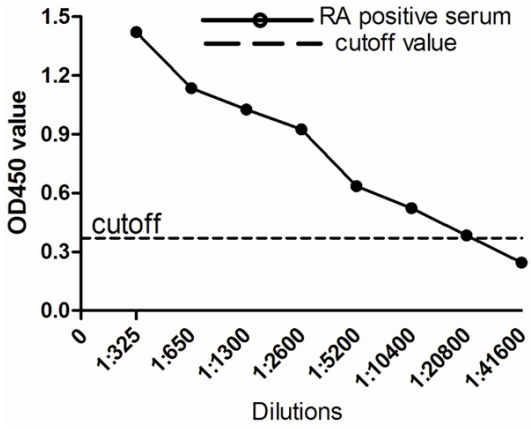

Figure 5. Sensitivity assessment of OmpH-ELISA assay. Different concentrations of the serum sample (from 1:325 dilution to 1:41600 dilution); the concentration of coated OmpH protein was 8.0 μg/100 μl. Specks represent means of absorbance from triplicate wells.

DPV were all below the cut-off value. In comparison, RA CH-1, RA CH-2 and ATCC 11845 antisera gave OD_{450} values greater than the cut-off value.

Repeatability and reproducibility of ompH-ELISA

To assess the reproducibility of OmpH-ELISA assay, the intra-assay variability (repeatability) and inter-assay variability (reproducibility) were confirmed. From the results, both inter- and intra-assay variability of this assay were lower than 10%, indicating that the OmpH-ELISA was highly stable and reproducible [16].

Detection of clinical serum samples

As results presented in **Table 1**, all of the 156 clinical serum samples, 105 specimens were positive by OmpA-ELISA assay (67.3%), 115 samples were positive by OmpH-ELISA (73.7%). In contrast, only 89 serum samples (57.05%) were detected positively by TA assay (**Table 1**).

Discussion

The precise diagnosis of RA infection is of great important, since false alarm may delay the treatment and result in death of the sufferer. Traditional method for detection of RA infection, relied on the isolation and identification of these bacteria, was time consuming and unsuited for field application [17]. PCR based

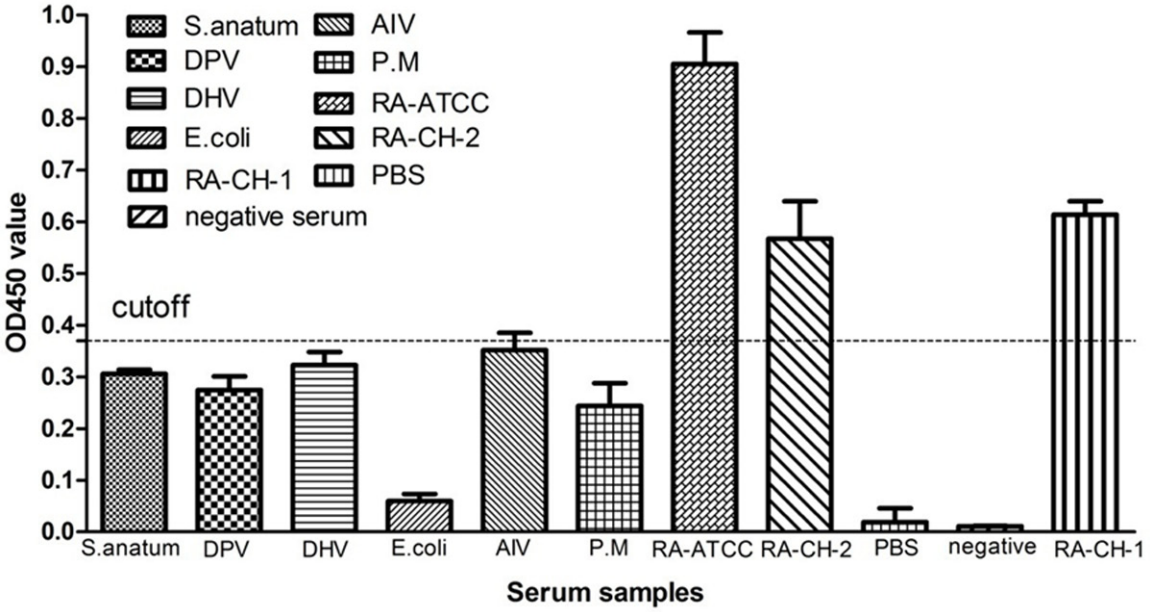

Figure 6. Specificity evaluation of OmpH-ELISA assay. Different antisera were tested by OmpH-ELISA assay. Each column represents a corresponding serum while the dashed line represents the cutoff value of the OmpH-ELISA assay.

Table 1. Comparison of the OMPs-ELISA, OmpH-ELISA and tube-agglutinstion assay for detection of RA infection

Tube-agglutination test	OmpA-ELISA			OmpH-ELISA		
	Positive (+)	Negative (-)	Total	Positive (+)	Negative (-)	Total
Positive (+)	89	0	89	89	0	89
Negative (-)	16	51	67	26	41	67
Total	105	51	156	115	41	156

method were restrained to the detection of suspect isolates, not the clinical sample per se [18, 19]. The slide agglutination (SA) assay was low in sensitivity [20].

In the present study, we developed a simple, reliable and time-saving indirect ELISA method for serological detection of RA infection. It was the first time to prove that the OmpH protein has immunogenicity in *Riemerella anatipestifer* instead of *Pasteurella Multocida* [21]. In addition, the amino acid sequence of OmpH protein in different RA serotypes was high homology (similarity > 95%), which suggested the OmpH protein a potential common antigen to detect the infection caused by different RA serotypes. To gain OmpH proteins *in vitro*, a recombinant plasmid of pET32a(+)-*ompH* was constructed and transformed into *E.coli* strain BL21 to generate BL21X. The expression of recombinant OmpH proteins in BL21X was enhanced by optimizing the cultural temperature, duration and IPTG concentration. After obtaining the recombinant OmpH proteins, we purified them by Ni^{+} chromatography, since the unpurified proteins would compete the binding site with coating antigen and resulted in nonspecific background [22]. The immunoblotting assay, using rabbit anti-duck IgG HPR conjugates, indicated that the purified recombinant OmpH proteins possessed good immunoreactivity and could be specific recognized by RA CH-1 antisera.

As the excessive antigen and serum would increase the non-specifity of the indirect ELISA assay [23], a checkerboard titration method was employ to optimize the working condition of both coated antigen and primary antibody (serum) of OmpH-ELISA assay. At a pre-determined dilution of secondary antibody (1:800), the slightest nonspecific background was observed when OmpH proteins were coated at 800 ng per well and the serum was diluted at 1:100. Under the optimal coated antigen and serum, we further confirmed the amount of

goat anti-duck IgG antibody, since too much conjugate also resulted in high background [24]. The dilution of secondary conjugate was finally determined as 1:400.

To date, more than 21 serotypes of RA have been identified, in which type 1 and 2 are the most prevalent serova in China [25]. In this study, the antisera against RA CH-1 (type 1), CH-2 (type 2) and RA ATCC 11845 (type 6) were detected positively, using the OmpH-ELISA assay. In contrast, no cross-reaction was found in serum against *E.coli*, *S.anatum*, *P.multocida,* AIV, DHV and DPV indicating that the OmpH-ELISA assay was high specificity. Except for the specificity, a high sensitivity of this assay was also observed.

A reliable detection method should be stable and repeatable; the OmpH-ELISA assay was shown to be well reproducible as previous published ELISA assay for detection of other duck disease [26, 27]. In detection of clinical serum samples, 105 of 156 specimens were positive by OmpA-ELISA assay (67.3%), 115 of 156 samples were positive by OmpH-ELISA (73.7%). In comparison, only 89 serum samples (57.05%) were detected positively by TA assay.

In conclusion, this study not only provided a strategy for obtaining and purifying the OmpH proteins, but developed a recombinant OmpH protein based indirect ELISA assay for serological detection of RA infection. As the high specificity, sensitivity and reliability of this ELISA assay, it could be a promising method for usage in field condition.

Acknowledgements

This work was supported by grants National science and technology support program(No. 2015BAD12B05), from Integration and Demonstration of Key Technologies for Duck Industrial in Sichuan Province (2014NZ0030), China Agricultural Research System (CARS-43-8) and Innovative Research Team Program in Education Department of Sichuan Province (12TD005/2013TD0015).

Disclosure of conflict of interest

None.

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