FULL PAPER

Bacteriology

Ducks as a potential reservoir for *Pasteurella multocida* infection detected using a new rOmpH-based ELISA

Rongchang LIU[1], Cuiteng CHEN[1], Longfei CHENG[1], Ronghui LU[1], Guanghua FU[1], Shaohua SHI[1], Hongmei CHEN[1], Chunhe WAN[1], Jiansheng LIN[1], Qiuling FU[1] and Yu HUANG[1]*

[1]Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences, Fujian Animal Diseases Control Technology Center, Fujian Provincial Key Laboratory for Avian Diseases Control and Prevention, Fuzhou 350013, China

ABSTRACT. *Pasteurella multocida* is an important pathogen of numerous domestic poultry and wild animals and is associated with a variety of diseases including fowl cholera. The aim of this study was to develop an indirect enzyme-linked immunosorbent assay (ELISA) based on recombinant outer-membrane protein H (rOmpH) for detection of anti-*P. multocida* antibodies in serum to determine their prevalence in Chinese ducks. The *P. multocida* ompH gene was cloned into pET32a, and rOmpH was expressed in *Escherichia coli* BL21 (DE3). Western blotting revealed that purified rOmpH was recognized by duck antisera against *P. multocida*, and an indirect ELISA was established. During analysis of serum samples (*n*=115) from ducks, the rOmpH ELISA showed 95.0% specificity, 100% sensitivity and a 92.0% κ coefficient (95% confidence interval 0.844–0.997) as compared with a microtiter agglutination test. Among 165 randomly selected serum samples, which were collected in 2015 and originated from six duck farms across Fujian Province, China, anti-*P. multocida* antibodies were detected in 22.42% of apparently healthy ducks, including 25 of 90 sheldrakes (27.8%), eight of 50 Peking ducks (16.0%) and four of 25 Muscovy ducks (16%). Overall, the data suggest that rOmpH is a suitable candidate antigen for the development of an indirect ELISA for detection of *P. multocida* in ducks; moreover, our results showed that ducks could serve as a potential reservoir for *P. multocida* infection.

KEY WORDS: duck, indirect ELISA, *Pasteurella multocida*, reservoir, rOmpH protein

Pasteurella multocida is a gram-negative bacterial species and is a facultative commensal of the upper respiratory tract of humans and other animal species [6, 10]. *P. multocida* is classified into serogroups A, B, D, E and F based on its capsular composition, and serotypes 1 to 16 based on its lipopolysaccharide antigens [10]. Serogroup A strains are mainly associated with fowl cholera, whereas serogroup B and E strains are associated with hemorrhagic septicemia; progressive atrophic rhinitis is normally caused by serogroup D strains [4, 5]. Fowl cholera is one of the most important infectious diseases in birds, especially for waterfowl [16, 31]. Research shows that transmission can occur by bird-to-bird contact via aerosolized bacteria as well as through ingestion of bacteria in contaminated environments [1]. Nonetheless, some apparently healthy waterfowl species act as carriers and may play a key role in the initiation of outbreaks, whereas wetlands or shallow water environments are important for pathogen transmission and maintenance during outbreaks [2, 22, 23].

Antibody detection is a standard method for diagnosing diseases and evaluating the immune responses generated by vaccines. Among the many serological approaches, the enzyme-linked immunosorbent assay (ELISA) has become the most important method for laboratory use worldwide because it is fast, efficient, accurate, and inexpensive. The coating antigen is a major reagent for an indirect ELISA, and the accuracy of this test is dependent on the selection of antigens. Outer-membrane protein H (OmpH) is a crucial protein in *P. multocida*, because it is an adhesion protein that interacts with host cell receptors, which mediate the adhesion of bacteria to host cells during the initial steps of an infection, and OmpH is a major target of the host immune system [11]. In addition, OmpH is highly conserved among *P. multocida* serotypes and is the only *P. multocida* protein where a small number of gene fragments are similar to eukaryotic cilia or flagella [11, 13]. Numerous studies have shown that both natural OmpH and recombinant OmpH (rOmpH) elicit good protection from parental strains in immunized chickens or mice, and that

the induced antibody protection rate is similar to that of whole bacteria [13, 17, 27]. Nevertheless, subsequent research revealed that an indirect ELISA based on rOmpH greatly reduces the background titer as compared with *P. multocida* whole cells and has high sensitivity and specificity for detection of *P. multocida* antibodies in swine [15]. Accordingly, OmpH is a major outer antigen inducing an antibody response and is a suitable candidate for developing an ELISA.

Various studies have shown that healthy animals can harbor *P. multocida*, and apparently healthy animals can be a source of sporadic outbreaks. Muhairwa *et al.* (2000) first reported isolation of *P. multocida* from the cloacal mucosa of apparently healthy domestic poultry, and a study on the carrier rate of *P. multocida* in healthy commercial poultry in Denmark indicated that 37% of web-footed birds (Peking and Muscovy ducks as well as geese) and 38% of chickens carry *P. multocida* [19]. Mbuthia *et al.* documented the occurrence of *P. multocida*, with an isolation rate of 25.9% among healthy-looking ducks from free-range family poultry farms in a tropical setting (Kenya) [18]. In addition, serological research on *P. multocida* among healthy-looking cattle using a capsular-antigen-based ELISA from 2013 to 2014 in Egypt showed that as many as 20% of apparently healthy cattle tested positive for *P. multocida* [7].

Waterfowl are key hosts of *P. multocida* and are strongly involved in the maintenance and transmission of *P. multocida*. Although China is a major poultry-producing country, little is known about the prevalence of healthy carriers in China. In this study, rOmpH of *P. multocida* serotype A:1 was expressed in *Escherichia coli* BL21 and used as an antigen to develop an indirect ELISA (the rOmpH ELISA), whose performance was evaluated by comparison with a conventional microtiter agglutination test (MAT). Additionally, the prevalence of *P. multocida* was evaluated in ducks obtained in China during 2015.

MATERIALS AND METHODS

Strains and serum samples

The *P. multocida* serotype A:1 F39 strain was isolated from ducks in Fujian, China, in 2013 [3]. Serum samples were obtained from several sources: 35 serum samples were obtained from ducks immunized with the *P. multocida* F39 strain and were analyzed by a MAT and used as positive controls in all the assays; 80 serum samples obtained from ducks served as negative controls in all the assays, 30 of which were obtained from one specific-pathogen-free flock, whereas the remaining 50 were obtained from a commercial farm that had no history of *P. multocida* vaccination and which was assumed to be free of fowl cholera because of the complete lack of both clinical signs and lesions that are indicative of fowl cholera; the *P. multocida* infection status was determined by RT-PCR analysis of rectal swabs for *P. multocida* and by MAT in serum samples in a laboratory. (This farm was tested every four months during 2015.) One hundred and sixty-five serum samples were randomly selected on six open-backyard duck farms (*n*=20 to 30 samples per farm) located in three states (San ming, Long yan and Zhang zhou) across Fujian Province, China, in 2015.

Expression and purification of rOmpH

The *ompH* gene of the F39 strain was polymerase chain reaction-amplified and sequenced, and the *ompH* fragment, with a deleted signal peptide, was cloned into expression vector pET32a using previously reported primers and methods [8]. Briefly, after the recombinant OmpH-expressing plasmid was transfected into *E. coli* strain BL21 (DE3) pLysS, a positive clone was inoculated into a tube with Luria–Bertani broth, and rOmpH expression was induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.5 mM, followed by incubation at 30°C with shaking at 150 rpm. The expressed protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). rOmpH was purified by Ni-Sepharose 6 Fast Flow affinity chromatography (GE Healthcare AB, Stockholm, Sweden). Purified rOmpH was analyzed by SDS-PAGE and western blotting.

Development of the rOmpH ELISA

Assay optimization: To determine the optimal concentration of the antigen and the optimal serum dilution to be tested, different concentrations of purified rOmpH as well as different control serum concentrations were screened in checkerboard titration procedures. rOmpH was diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to concentrations ranging from 3.2 to 0.05 μg/well and was tested against varying serum dilutions (1:50 to 1:400), which were prepared in phosphate-buffered saline containing 0.05% of Tween 20 (PBST). The assay conditions were as follows: 96-well microtiter plates (Nunc; Thermo Fisher Scientific, Agawam, MA, U.S.A.) were coated overnight at 4°C with purified rOmpH at 200 ng/well. The plates were washed three times and incubated for 1 hr at 37°C with a blocking solution (5% non-fat dry milk in PBST). After three washes, serum samples diluted in PBST were added in duplicate into the wells, and then, the plates were incubated for 1 hr at 37°C. After three additional washes, a horseradish peroxidase-conjugated rabbit anti-duck immunoglobulin G antibody (KPL, Gaithersburg, MD, U.S.A.) was diluted as recommended by the manufacturer (1:5,000 in PBST) and added into the plate wells, followed by incubation at 37°C for 1 hr. Next, the plates were washed three times with PBST, and 100 μl of the peroxidase substrate tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO, U.S.A.) was added into each well. Then, the plates were incubated for 15 min in the dark, and the reaction was stopped by addition of 2N H_2SO_4. Optical density was read at 450 nm (OD_{450}) using an ELISA TP-Reader plate (Bio-Tek, Winooski, VT, U.S.A.).

Determination of the assay cutoff value: The cutoff was calculated by a receiver operator characteristic (ROC) analysis for maximum diagnostic sensitivity and specificity using samples classified as *P. multocida* positive (*n*=35) or negative (*n*=80 samples). The positive and negative samples used in the ELISA were tested by a MAT as described elsewhere [24]. The cutoff value was

Table 1. Details of the *P. multocida* strains used in this study

No.	Name	Country	Isolation year	Host	Serotype	Accession no.
1	P1059	U.S.A.	1996	Avian	A3	U52200
2	X73	U.S.A.	1996	Chicken	A1	U50907
3	P-1662	U.S.A.	1999	Chicken	A1	U52201.1
4	YDY	India	2004	Duck	A1	AY606823
5	HN13	China	2004	Pig	D	AY864815
6	HG	Korea	2004	Pig	D4	AY603962
7	XJ149	Xinjiang, China	2004	Cattle	A	JQ082509
8	C48-1	China	2006	Chicken	A5	EF027093
9	C44-1	China	2007	Pig	B2	EF635422
10	679–230	China	2007	Pig	B2	EF635421
11	P52	India	2007	Cattle	B2	EU016232
12	YAK	Xinjiang, China	2010	Yak	B	HM582885
13	C44-49	China	2010	Pig	D3	HM486501
14	XJNKY-1	Xinjiang, China	2012	Sheep	A	JX473020
15	XJNKY-12	Xinjiang, China	2012	Sheep	A	JX473022
16	F39	Fujian, China	2014	Duck	A1	-

selected to maximize the sensitivity and specificity while minimizing false negative and false positive results. The obtained value was further assessed using the cumulative data from all the other samples. The ROC was set up to determine the cutoff value of the *P. multocida* rOmpH ELISA in MedCalc for Windows, version 9.2.0.1 (MedCalc Software, Ostend, Belgium).

Assay specificity and a reproducibility test: The specificity of the *P. multocida* rOmpH ELISA was evaluated on serum samples with high antibody levels against *Riemerella anatipestifer* (*n*=3), *E. coli* (*n*=3), avian influenza virus H9 (*n*=3), Newcastle disease virus (*n*=3), duck hepatitis virus (*n*=3) or duck plague virus (*n*=3), which were prepared and preserved by our laboratory. The reproducibility of the *P. multocida* rOmpH ELISA was evaluated using six serum samples with different antibody titers. The coefficient of variation (CV) was used to evaluate the intra- and inter-assay variations. Each sample was tested in each of the three plates on three occasions to determine the inter-assay CV, and three replicates within the same plate were used to calculate the intra-assay CV.

Statistical analyses

All the measurements were made in triplicate. ROC curves, the area under the curve (AUC), the optimal cutoff point and inter-rater agreement (i.e., the κ coefficient) were determined by means of the MedCalc software version 9.2.0.1 for Windows (MedCalc Software).

RESULTS

Sequence analysis of the ompH region of P. multocida serotype A:1 isolate F39

Sequencing results showed that the *ompH* gene of *P. multocida* serotype A:1 F39 consists of 1,056 nucleotides. A sequence analysis indicated that the *ompH* gene of *P. multocida* serotype A:1 F39 shares high homology with other *P. multocida* serotypes, and the homologies of the nucleotide and amino acid sequences were 88.7–99.8% and 82.7–100.0%, respectively, among the other *P. multocida* serotypes (Tables 1 and 2). A multiple alignment of OmpH amino acid sequences suggested that the overall identity among the strains was as high as 90.2%. The *P. multocida* F39 strain showed maximal nucleotide identities with *P. multocida* strains X73/serotype A1/chicken (99.8%), YDY/serotype A1/duck (99.6%) and C48-1/serotype A5/chicken (99.7%), which cause fowl cholera (Table 2). A phylogenetic tree indicated that F39 clustered with strain X73/serotype A1/chicken, strain YDY/serotype A1/duck and strain C48-1/ serotype A5/chicken. Other strains, which are present in separate clusters, are distantly related to strain F39 (Fig. 1).

Expression and purification of rOmpH

Pasteurella multocida rOmpH was expressed in *E. coli* using conventional molecular techniques. The purification of rOmpH and its antigenicity were confirmed by SDS-PAGE (Fig. 2A) and western blotting (Fig. 2B) via visualization of the expected ~57-kDa protein band. Western blotting showed that purified *P. multocida* rOmpH reacted with duck anti-*P. multocida*-positive serum (1:100 dilution) (Fig. 2B).

Development of the rOmpH ELISA

The optimal concentration of purified rOmpH for plate coating was found to be 200 *n*g/well: at this concentration, positive and negative serum samples were better differentiated into reactants (testing positive) or nonreactants (testing negative) when diluted 1:100. Duck serum samples (*n*=115) were tested by a MAT, which revealed that 80 negative samples (69.6%) tested negative for

Table 2. Nucleotide sequence identity and the predicted amino acid sequences encoded by the *ompH* gene of *P. multocida*

|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Name
1		85.9	98.0	85.9	97.2	94.0	91.9	85.9	96.4	96.9	96.4	96.0	94.4	91.9	91.5	85.9	P1059
2	89.4		85.4	100.0	87.2	82.7	82.7	100.0	84.5	85.0	84.5	84.1	83.2	82.7	82.7	100.0	X73
3	96.7	88.5		85.4	95.2	95.2	89.8	85.4	97.2	85.4	97.2	96.9	95.6	89.8	89.4	85.4	P-1662
4	89.7	99.8	88.8		87.2	82.7	82.7	100.0	84.5	85.0	84.5	84.1	83.2	82.7	82.7	100.0	YDY
5	98.2	89.9	95.6	90.1		92.4	94.0	87.2	94.8	95.2	95.6	95.2	92.8	94.0	93.6	87.2	HN13
6	94.6	87.7	95.1	87.9	93.4		87.6	82.7	92.4	92.8	92.4	91.9	98.4	87.6	87.2	82.7	HG
7	95.7	89.0	93.2	89.2	96.9	91.6		82.7	90.2	90.7	91.1	91.5	88.1	99.2	98.8	82.7	XJ149
8	89.6	99.7	88.6	99.9	90.0	87.8	89.1		84.5	85.0	84.5	84.1	83.2	82.7	82.7	100.0	C48-1
9	97.1	88.3	95.7	88.5	96.4	92.4	94.1	88.4		99.6	99.2	98.8	92.8	90.2	89.8	84.5	C44-1
10	97.2	88.4	95.8	88.6	96.5	92.6	94.2	88.5	99.9		99.6	99.2	93.2	90.7	90.2	85.0	679-230
11	97.2	88.3	95.7	88.5	96.4	92.5	94.1	88.4	99.8	99.9		99.6	92.8	91.1	90.7	84.5	P52
12	97.2	88.3	95.7	88.5	96.4	92.4	94.3	88.4	99.8	99.9	99.8		92.4	91.5	91.1	84.1	YAK
13	94.5	87.7	95.0	87.9	93.3	99.5	91.5	87.8	92.3	92.5	92.4	92.4		88.1	87.6	83.2	C44-49
14	95.7	89.0	93.2	89.2	96.9	91.6	99.8	89.1	94.1	94.2	94.1	94.3	91.5		99.6	82.7	XJNKY-1
15	95.5	88.8	93.0	89.1	96.7	91.6	99.6	89.0	93.9	94.0	93.9	94.1	91.5	99.8		82.7	XJNKY-12
16	89.4	99.6	88.5	99.8	89.9	87.7	89.0	99.7	88.3	88.4	88.3	88.3	87.7	89.0	88.9		F39
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

Upper-right, amino acid homology; lower-left, nucleotide sequence homology.
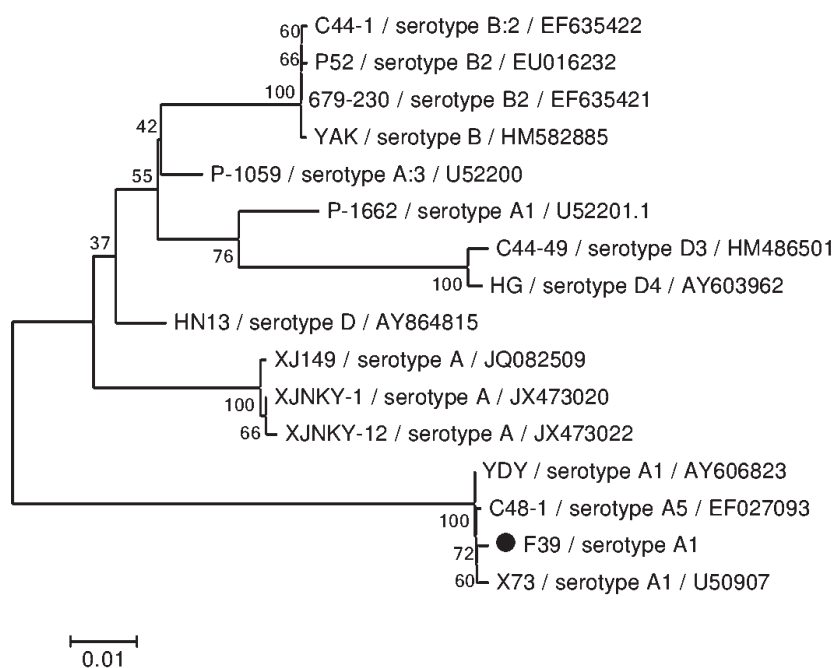
Fig. 1. A phylogenetic tree based on the *ompH* gene sequences of the *P. multocida* strain F39 and other *P. multocida* strains of different serotypes or from different hosts.

the *P. multocida* F39 strain (1:2 dilution), while 35 positive control samples (30.4%) tested positive. Compared with the MAT results, the AUC indicated that the rOmpH ELISA was on average 99.7% accurate (Fig. 3A). The 95% confidence interval (CI) of the AUC for the ELISA ranged from 96.2 to 100%, and the significance level area was 0.5 ($P<0.0001$). The ROC analysis indicated that the optimal cutoff point was OD_{450} of 0.202, corresponding to sensitivity of 100% and specificity of 95.0% (Fig. 3B and 3C); a κ coefficient of 0.920 (95% CI 0.844–0.997) was calculated between the MAT and the rOmpH ELISA. Of the 35 MAT-positive serum samples, all were labeled as true positives, because the ELISA readings were above the optimized cutoff. Among the 80 MAT-negative serum samples, four (5.00%) collected from a commercial farm were labeled as false positives, because the ELISA values were above the cutoff; 76 samples (95%) showed ELISA readings below the cutoff and were labeled as true negatives (Table 3 and Fig. 2C). The inter- and intra-assay CVs of six control serum samples tested by the rOmpH ELISA were less than 10%. The intra-assay CV ranged from 0.9 to 5.8%, while the inter-assay CV ranged from 1.4 to 7.1%, suggesting that the results were reproducible (Table 4). The specificity of the rOmpH ELISA was also evaluated using serum samples with high antibody levels
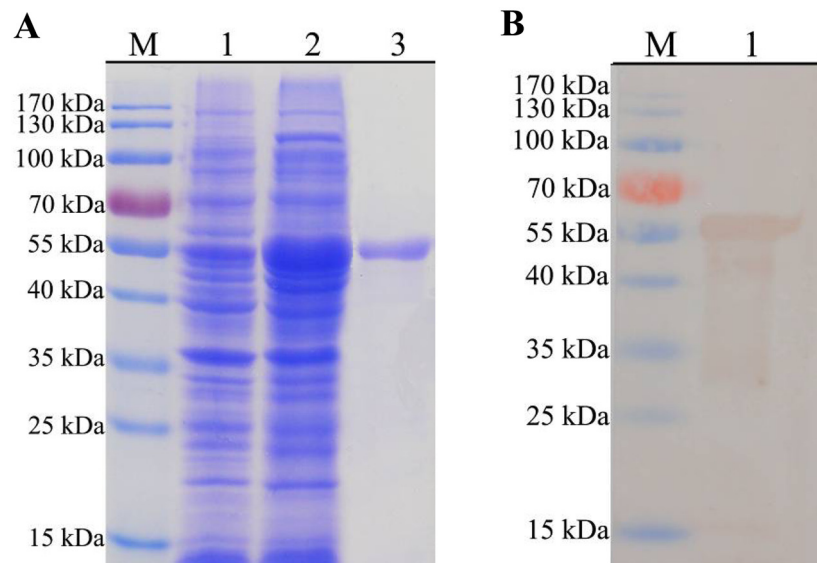
Fig. 2. SDS-PAGE and western blotting analysis of expressed and purified rOmpH. A) SDS-PAGE analysis of expressed and purified rOmpH. Lane M, protein molecular mass markers; lane 1, a sonicated whole-cell lysate of pET32a-ompH/BL21; lane 2, a sonicated whole-cell lysate of induced pET32a-ompH/BL21; lane 3, purified rOmpH. B) A western blot of purified rOmpH. Lane M, protein molecular mass markers; lane 1, purified rOmpH.

against *Riemerella* (n=3), *E. coli* (n=3), avian influenza virus H9 (n=3), Newcastle disease virus (n=3), duck hepatitis virus (n=3) or duck plague virus (n=3). Their OD_{450} values are 0.121 ± 0.008, 0.118 ± 0.009, 0.093 ± 0.042, 0.092 ± 0.060, 0.069 ± 0.034 and 0.071 ± 0.054, respectively. Among the 18 samples, none yielded OD_{450} higher than 0.202.

*Infection status among healthy-looking ducks in Fujian, China*

One hundred and sixty-five serum samples collected on six duck farms in Fujian, China, during 2015 were screened for anti-*P. multocida* antibodies. In apparently healthy sheldrake, Peking duck and Muscovy duck flocks, 37 positive samples were uncovered (37/165; 22.42%). The positive detection rates on individual farms ranged from 8.00 to 36.67%, with 25 of 90 sheldrakes (27.8%), eight of 50 Peking ducks (16.0%) and four of 25 Muscovy ducks (16%) testing positive, and the ages of the antibody-positive ducks ranged from 15 to 140 days (Table 5).

DISCUSSION

Infection with *P. multocida* is a major disease on duck farms; therefore, it is important to choose a reliable *P. multocida* detection method. The isolates used in this study have been shown to belong to serotype A:1, a serotype that has been reported to be among leading causes of severe cases of fowl cholera [12, 14, 21, 30]. In the present study, the *ompH* gene of *P. multocida* F39 (serotype A:1) was cloned, and a sequence analysis indicated that the *ompH* gene is conserved among all the *P. multocida* serotypes, in agreement with another study [26]. In addition, the *P. multocida* F39 strain shares maximal nucleotide identities with avian *P. multocida* strains causing fowl cholera (the nucleotide homologies were 99.6–99.8%), which are present in the same clusters of the phylogenetic tree.

In our study, the *ompH* fragment, with a deleted signal peptide, from the duck *P. multocida* F39 strain (serotype A:1) was cloned into pET32a and expressed in *E. coli* BL21. A positive clone was cultured in Luria-Bertani broth at 30°C, which maintains weak expression, thereby increasing the probability that the recombinant protein folds properly [28]. The expressed rOmpH protein has the expected size of ~57 kDa, corresponding to the 36.5-kDa OmpH protein and a 20.3-kDa histidine tag. The western blotting results showed that the purified protein reacts with duck anti-*P. multocida*-positive serum; this result indicated that rOmpH reacts with the antibodies induced by native OmpH.

To test the hypothesis that rOmpH is suitable as an antigen in an immunodiagnostic test for *P. multocida* in ducks, we developed a new indirect ELISA using purified rOmpH as an antigen. A comparison of rOmpH ELISA and MAT results using 115 reference serum samples indicated that the rOmpH ELISA is in excellent agreement with the MAT: 95.0% specificity, 100% sensitivity and a 92.0% κ coefficient (95% CI 0.844–0.997) when the optimal cutoff value was 0.202. To further study the reproducibility of the rOmpH ELISA, six serum samples with different antibody titers were examined on different occasions and in different batches of rOmpH ELISA plates. The results revealed that the inter- and intra-assay CVs were all less than 10%, suggesting that the rOmpH ELISA was reproducible. In addition, it has been determined previously that coinfections by *P. multocida* with other pathogenic
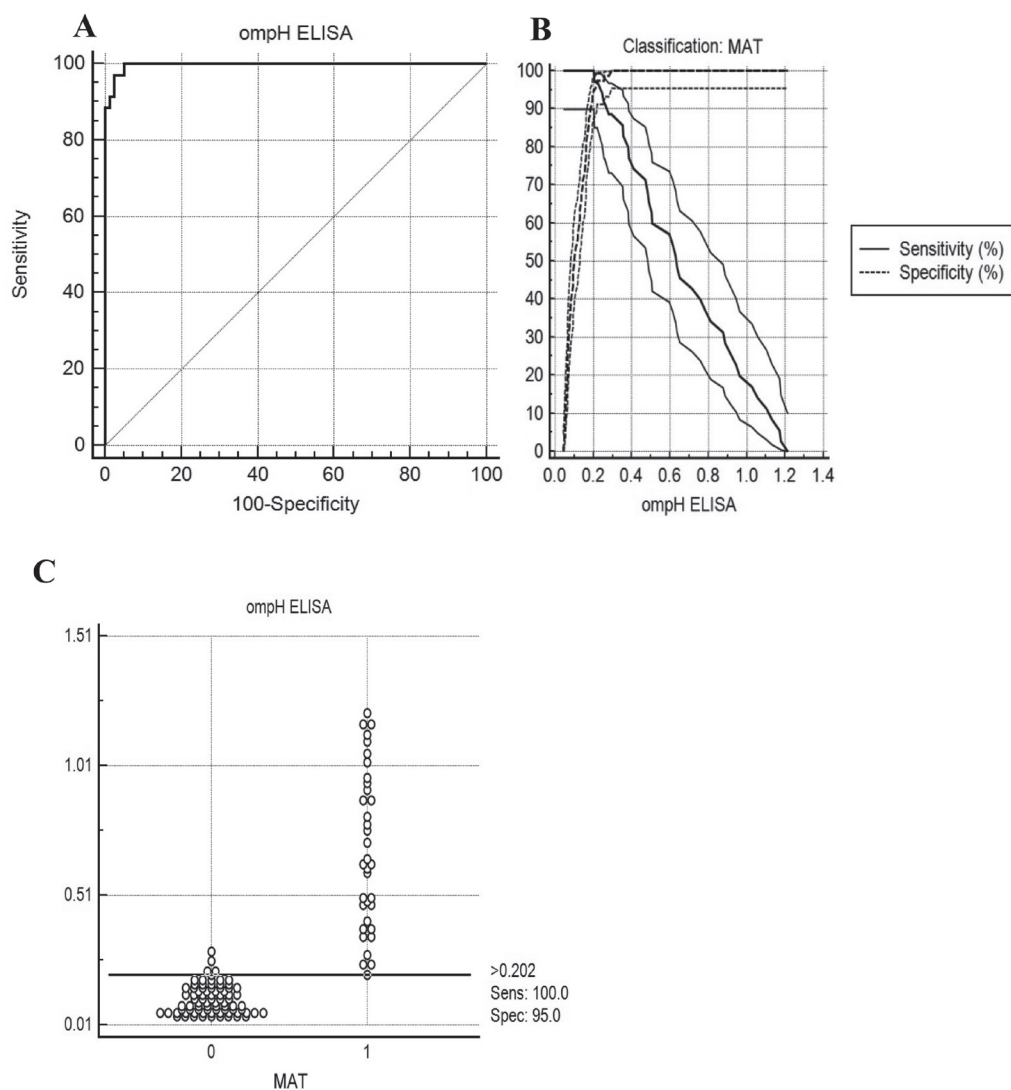
Fig. 3. ROC analysis of the rOmpH ELISA. A) ROC curves based on results of the rOmpH ELISA (*n*=115). B) The relation of ROC-based estimates of test sensitivity and specificity with the rOmpH ELISA cutoffs. C) An interactive dot diagram based on rOmpH ELISA outcomes in relation to the MAT (MAT negative=0 and MAT positive=1).

Table 3. Comparison of the indirect ELISA and the MAT

| ompH ELISA | MAT | | Total (%) |
	Positive	Negative	
Positive	35	4	39 (33.9)
Negative	0	76	76 (66.1)
Total (%)	35 (30.4)	80 (69.6)	115

The table presents the results on 115 serum samples analyzed by the MAT and the rOmpH ELISA. κ coefficient=0.920 (95% CI 0.844–0.997).

Table 4. Intra- and inter-batch reproducibility tests

| Serum samples | Intra-assay variability (CV) | | Inter-assay variability (CV) | |
	X ± SD	CV (%)	X ± SD	CV (%)
1	0.788 ± 0.007	0.9	0.797 ± 0.010	1.4
2	0.980 ± 0.022	2.2	0.971 ± 0.019	1.9
3	0.818 ± 0.006	0.7	0.829 ± 0.019	2.2
4	0.674 ± 0.018	2.7	0.655 ± 0.047	7.1
5	0.574 ± 0.034	5.8	0.600 ± 0.013	2.2
6	0.636 ± 0.030	4.8	0.627 ± 0.021	3.3

microorganisms are common [29]. Accordingly, specificity of the rOmpH ELISA was also evaluated using 18 serum samples with high antibody titers against six other pathogenic microorganisms. Among these serum samples, none yielded OD_{450} higher than 0.202 (the rOmpH ELISA cutoff value).

Nonetheless, selecting a single cutoff value on the basis of field samples may also result in a loss of sensitivity or specificity. Therefore, to address this issue, the established cutoff value of 0.202 was evaluated by means of the cumulative data on 90 samples originating from seronegative farms (classified as negative due to the absence of any *P. multocida*-positive ducks among the ducks

Table 5. The detection rate of anti-*P. multocida* antibodies in serum samples (collected during 2015) from healthy-looking ducks

Farm	Species	Age (day)	Sex	History	Positive/Total (Positive rate (%))
1	Sheldrake	~80	Female	apparent healthy	8/30 (26.67)
2	Peking duck	~30	Unknown	apparent healthy	6/25 (24.00)
3	Sheldrake	~120	Female	apparent healthy	6/30 (20.00)
4	Sheldrake	~140	Female	apparent healthy	11/30 (36.67)
5	Muscovy duck	~20	Unknown	apparent healthy	4/25 (16.00)
6	Peking duck	~15	Unknown	apparent healthy	2/25 (8.00)
Total					37/165 (22.42)

All the duck serum samples were obtained from open-backyard farms in six states across Fujian, China.

tested by RT-PCR for *P. multocida* on rectal swabs and MAT for serum samples) by calculation of the average OD and standard deviation (SD; data not shown). The cutoff value was computed as the average OD $\pm 3 \times$ SD, and our result showed that the adoption of this method yielded a lower cutoff value (0.186, 0.081 $\pm 3 \times$ 0.035) and greater sensitivity. Consequently, samples with an OD value between 0.186 and 0.202 may be considered inconclusive. Overall, the rOmpH ELISA uses a single dilution of the serum tested, thus offering considerable advantages over more labor-intensive assays, such as the MAT or an indirect hemagglutination test, and overcomes the drawbacks of a whole-cell-lysate–based ELISA, namely the high background value, which can lead to false positives.

Testing for serum antibodies against *P. multocida* is a useful diagnostic tool for detecting *P. multocida* because it is not always possible to isolate *P. multocida* from the cloaca or throat of seropositive animals, and *P. multocida* may persist in other organs in animals that are latent carriers [9]. To assess the prevalence of *P. multocida* in ducks in China, we screened 165 serum samples (collected on six open-backyard duck farms in 2015) for anti-*P. multocida* antibodies. The results showed that in apparently healthy flocks of sheldrakes, Peking ducks and Muscovy ducks, 37 positive samples were found (37/165). The positive detection rates on individual farms ranged from 8.00 to 36.67%, with 25 of 90 sheldrakes (27.8%), eight of 50 Peking ducks (16.0%) and four of 25 Muscovy ducks (16%) testing positive. These results suggest that there is some incidence of latent infections with *P. multocida* among ducks in China. On open-backyard farms, birds share drinking water and feed with other species, such as wild birds or feral cats, and can easily transmit pathogens on these farms; this situation makes domestic birds susceptible to *P. multocida* infections [25, 26]. Moreover, seropositive animals, including recovering or persistently infected *P. multocida* carrier ducks, constitute the biggest challenge to effective control of the disease. Because birds that recover become carriers of the disease and can remain infected for life, they become a reservoir of infection for further outbreaks, particularly in the presence of environmental stressors, such as injury, excitement, or changes in climate or nutrition [20]. Given this scenario, continuous improvements in duck *P. multocida* diagnosis and surveillance are crucial for disease containment.

In summary, in this study, we used rOmpH of *P. multocida* serotype A:1 as an antigen to develop a new indirect ELISA for detection of anti-*P. multocida* antibodies in ducks. This assay has potential applications in studies on the epidemiology of *P. multocida*. Ducks were shown here as a potential reservoir of *P. multocida* infection in serological analyses of apparently healthy flocks in Fujian province of China using the rOmpH-Based ELISA. In the future, these results will be confirmed in a more extensive serological study.

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