DEVELOPMENT OF AN ELISA USING RECOMBINANT OMPA PROTEIN FOR THE DETECTION OF *Pasteurella multocida* INFECTIONS IN RABBITS

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ABSTRACT

Pasteurella multocida, a gram-negative bacillus, is the causative agent of snuffles in rabbits, a disease which could incur much economic loss in the rabbit industry. Vaccination is not commercially available because of a lack of efficacy, antibiotics may be effective for resolving the symptoms in sick animals but usually do not clear the bacteria from colonized animals. Hence the detection of *P. multocida* in clinically healthy rabbit colonies is important for the control of this disease. An indirect enzyme-linked immunosorbent assay (ELISA) has been developed to facilitate early detection of *Pasteurella multocida* infections in rabbits. The antigen used was a recombinant protein, ompA, which was expressed in Escherichia coli as an N-terminal HIS fusion protein. The rompA-based ELISA successfully detected ompA antibodies in the sera of rabbits immunized with bacterin preparations of the C51-2-499 strain. Antibodies to ompA were also detected in the sera of 40 % (42 /106) which were collected from three different farms, but few of the rabbits (19%) were PCR positive. The rompA-HIS antigen did not cross-react with antibodies in sera from rabbits which were infected with other gram-negative and gram-positive bacterial pathogens, including *E.coli*, *Bordetella bronchiseptic*, *Pseudomonas aeruginosa* and *staphylococcus*. The rompA -based ELISA may be useful for the diagnosis of *Pasteurella multocida* infections in sick rabbits as well as for screening for carriers in research rabbit colonies.

Key words: *Pasteurella multocida*, ELISA, ompA.

INTRODUCTION

Infection with *Pasteurella multocida* is a significant cause of clinical disease in rabbits. Snuffles, a highly contagious pasteurellosis of rabbits primarily affects the upper respiratory tract with potential fatal consequences, such as septicemia, pneumonia, chronic rhinitis, and otitis media as well as multiple abscesses. Rabbits often get colonized with *P.multocida* for long durations without clinical signs, and the prevalence of *P.multocida* in clinically healthy animals had been estimated to range from 20% to 90% depending on the detection method employed (Sanchez et al., 2004). Healthy carriers or latent infections are common to all *P. multocida* infections and play a significant role in the epidemiology of these infections. Most serologic tests for detection of *P. multocida* infection have depended on the use of whole-cell lysates as antigens to detect the presence of antibodies in the sera of infected rabbits (Dziva *et al.,* 2008). These tests have limitations in that they may detect only the specific serotype of the bacterium used in development of the assay, snuffles is also caused by either serotype A or D and serotype F strains (Jaglic *et al.*, 2008).

P. multocida OmpA protein is conserved, immunogenic and an adhesion that binds host cells and host cell extracellular matrix molecules, suggesting that this antigen may be useful for the serological diagnosis of pasteurellosis(Dabo et al., 2003). In this study we reported on the use of an ompA enzyme-linked immunosorbent assay (ELISA) to detect *P. multocida* infection in healthy and clinically ill rabbits.

MATERIALS AND METHODS

Animals and experimental design

Negative control sera used in Western blot and ELISA assays were obtained from 40 rabbits, provided by Zhejiang University laboratory animal center, a whole-cell-lysate ELISA was performed as described by Kawamoto et al. in order to confirm the infection status of these rabbits (Kawamoto et al., 1994). Sera used for Pasteurella-positive controls were obtained by subcutaneous immunization of 4-weeks-old P. multocida free rabbits with 1.0 ml formalinzed bacteria mixed with an equal volume of adjuvant (Sepik Mountainside ISA773, France). After 2 and 4 weeks, the rabbits were boosted twice, using the same dosage of formalinzed bacteria. Sera were collected from the rabbits 2 weeks after the second boost. Field sera samples for the ELISA were obtained from three different commercial rabbitry in Zhejiang province.

Expression and purification of P. multocida recombinant OmpA

P. multocida strain C51-2-499 was originally obtained from China Institute of Veterinary Drugs Control and grown routinely in brain heart infusion (BHI) broth containing 5% (v/v) sheep blood at 37 ◦C in a 5% CO2 environment. The DNA fragment encoding the mature domain of *P. multocida* C51-2-499 OmpA was amplified from the genomic DNA and sub-cloned into pET28a (+) expression vector. The insert was confirmed by DNA sequencing and the resulting plasmid was used to transform competent *E.coli* BL21 (DE3). Expression of the recombinant protein was according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). The rPmOmpA protein was produced as inclusion bodies and purified under denaturing conditions according to the manufacturer's instructions. Purified rOmpA protein was electrophoresed in 5% stacking/10% resolving SDS polyacrylamide gel and then analysed by Western blot.

ELISA procedure

A checkerboard titration was performed to determine the optimal working dilution of the coating antigen, serum and horseradish peroxidase-labelled goat-anti- rabbit IgG (HRP-IgG) (Sigma, St. Louis, MO) using a 96-well ELISA plate. Antigen coating concentrations were 2 μg, 1.6 μg, 0.8 μg, 0.4 μg, 0.2 μg, 0.1 μg and 0.05 μg per well, and serum dilutions were 1:25, 1:50, 1:100,1:200, 1:400, 1:800, and 1:1600. The dilutions that gave the maximum difference between positive and negative serum (P/N) by absorbance at 450 nm were selected for large-scale testing of serum samples. Test sera included positive, negative and blank sample controls. The reaction temperature, time and other conditions were optimized by P/N value.

To evaluate repeatability, the co-efficient of variation (CV) was calculated between plates (inter-assay variation) and within the same plate (intra-assay variation) for 9 sera samples. For inter-assay CV, each sample was tested on three different plates on different occasions. For intra-assay CV, three replicates within each plate were assayed.

To validate specificity, four positive serums were diluted 1:200 with phosphate-buffered saline, mixed with purified rOmpA protein as described above (1:1 v/v ratio) and incubated for 24 h at 37°C, then tested by the indirect ELISA. Test sera included unprocessed positive serum and negative controls.

Four samples positive serologically for related rabbit bacteria including *E. coli*, *Bordetella bronchiseptica* *Pseudomonas aeruginosa* and *staphylococcus* were tested by the indirect ELISA to assess the degree of assay cross-reactivity.

Detection of field samples

Forty-two serum samples accompanying with swabs of lesions or nasal exudates were acquired from rabbits with clinical signs suggestive of pasteurellosis from three different farms for ELISA and PCR detection. The rOmpA -based ELISA was applied to the detection of antibodies to pasteurellosis in sera. The template for PCR was prepared by incubating sample swabs or control strains in BHI broth

at 37°C overnight. PCR was performed with primers P16sf (5-GAGTCTAGAGTACTTTAGGGAG-3') and P16sR (5'-ACTTTCTGAGATTCGCTC-3') to amplify 643 bp of the 16SrRNA gene. PCR was performed in a DNA thermal cycler in 50 μL reaction mix containing 5 μL 10× PCR buffer, 2 μL dNTPs Mix(2.5Mm), 1 μL each primer(10 pmol), 2 μL *P.multocida* DNA , 0.5 μL *Taq* polymerase mix (5 U) and 39.5 μL Sterilization water. The PCR cycling parameters consisted of 35 cycles of (30 s at 95°C, 30 s at 56°C, 30 s at 72°C). An additional extension step of 10 min at 72°C was added at the end of the last cycle.

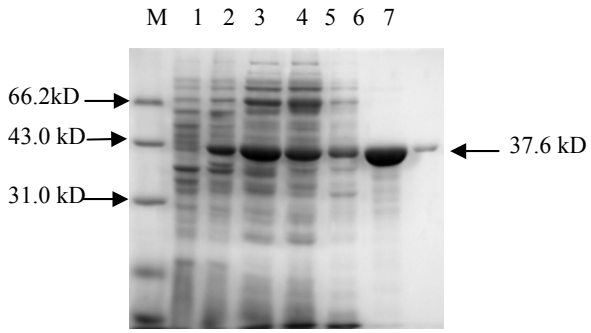
Statistical Analysis

To set negative/positive cutoff values, 40 negative samples were tested in duplicate by ELISA. Cut-off values were determined as the mean + 2 standard deviations (SDs) and mean + 3 SDs derived from the 40 negative samples.

RESULTS AND DISCUSSION

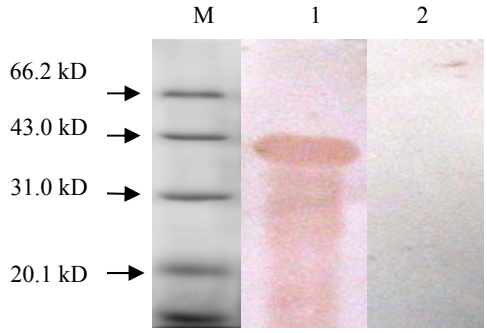
Expression and purification of P. multocida recombinant OmpA

A 1068 bp DNA fragment, comprising the complete sequence of OmpA was amplified from genomic DNA of *P. multocida* reference strain C51-2-499. The purified PCR products were cloned into pET-28a□+□, plasmids were transformed into *E. coli* BL21 (DE3) for expression of OmpA. The identity and orientation of the DNA insert were verified by DNA sequencing, the translation of the complete ORF produced a protein with 376 amino acids. The 37.6 kDa rOmpA fusion protein was expressed mainly as an insoluble protein. Optimal solubilization of the recombinant protein was obtained using 8M urea in lysis buffer. The immunoblot indicated the 37.6 kDa rOmpA protein reacted with sera from rabbits immunized with bacterin preparations of *P. multocida*, but didn't react with negative control serum from uninfected rabbits.



M: protein marker;1: negative control ; 2: His-OmpA protein before purification; 3: cracking solution;4: His-OmpA protein was eluted with 8 mol/ L urea in pH6.3; 5: His-OmpA protein was eluted with 8 mol/ L urea in pH5.5; 6: His-OmpA protein was eluted with 8 mol/ L urea in pH4.0; 7: His-OmpA protein was eluted with 8 mol/ L urea in pH4.0 for second time

Fig.1 SDS-PAGE result of recombination OmpA protein



M: protein marker; 1: Pm positivetive control serum; 2: Pm negative control serum

Fig.2 examination of the purified recombination ompA protein by Western-blotting

Development of ELISA assay

After optimisation, indirect ELISA was performed using the following procedure. Ninety-six well ELISA plates (Costar) were coated with 0.8 μg total purified fusion protein per well, diluted in carbonate buffer, and incubated overnight at 4□. After three washes with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST), plates were sealed with BSA (PBS containing1□BSA),

and incubated for 60 min at 37□. After three washes with PBST, serum samples were diluted 1:200 in dilution buffer (PBS containing 5% skimmed milk, 10% horse serum and 3% E. coli lysate), in a 100 μL volume per well, and incubated for 60 min at 37□. After three washes, horseradish peroxidase-conjugated goat anti- rabbit serum (Sigma) was added in the same dilution buffer at an appropriate working concentration, 100 μL per well, and incubated at 37ºC for 90 min. After three washes, color was developed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB, Sigma), and the reaction was stopped after 15 min with 50 μL 2.0 mol/L H_2SO_4. The OD_{450} was read with a microplate reader (Model 680. Bio-Rad).For the test system to be valid, we determined that the *ODpos* should be higher than 0.145, and the *ODneg* should be lower than 0.131. For values outside these limits, the test was repeated. The inter-assay CV ranged from 1.2% to 8.9%, and the intra-assay CV ranged from 2% to 9%. The mean OD_{450} of 4 positive samples unprocessed was 0.685, the mean OD_{450} of positive samples mixed with purified rOmpA protein was 0.26, the negative control samples was 0.12. No evidence of cross-reactivity with known positive sera to rabbit bacteria was observed□all tested sera gave values below the defined cut-off point.

Detection of field samples

The rOmpA -based ELISA was applied to the detection of antibodies to R. anatipestifer in sera obtained from 106 rabbits from three different farms. The OD_{450} of 42 samples were greater than 0.145, suggesting P. multocida exposure in 40% of these rabbits but few of the rabbits (19%) were PCR positive.

DISCUSSION

We report a recombinant protein, ompA, which was expressed in Escherichia coli as an N-terminal HIS fusion protein. *P. multocida* OmpA was first identified as a 36 kDa, two domain, heat-modifiable protein using monoclonal antibodies against OM vesicles (Carpenter et al., 2007). Immunoblot analysis using recombinant *P. multocida* OmpA expressed in E. coli revealed that the protein was both immunogenic and expressed in vivo. The immunogenic properties of OmpA allowed it to be used as an antigen for immunodetection of *P. multocida* infection in rabbits.

Many conventional methods are used for *P. multocida* diagnosis, these methods are variable, time-consuming and expensive. An indirect enzyme-linked immunosorbent assay (ELISA) greatly reduces the time required for diagnosis, and is specific and sensitive in the identification of rabbits infected with P. *multocida*. The rOmpA -based ELISA was applied to the detection of antibodies to *P. multocida* in sera obtained from 106 rabbits from three different farms. Although few of the rabbits (19%) were PCR positive, 42 of the 106 serum samples (40%) were positive for antibodies by the OmpA-I-ELISA. The result was broadly consistent with the findings of Sanchez et al (Sanchez et al., 2004). Some of these ELISA-positive rabbits probably had clinical infections caused by unrelated bacteria but may have had respiratory colonization with *P.multocida*, resulting in the OmpA antibodies. Other ELISA positive rabbits were probably chronically infected with *P. multocida* but may have cleared most of the bacteria or may have been treated with antibiotics or the swab samples were poorly preserved during shipping. While many of these ELISA-positive animals (17/22) exhibited symptoms of deep infection (nasal discharge, abscess, torticollis, head tilt, mandibular and ataxia). Many of the swabs might have contained too few bacteria for detection by the PCR. Some of the PCR-negative sick rabbits possessed high serum antibody titers, suggesting that the OmpA-I-ELISA may be more sensitive than PCR for the identification of infected animals.

CONCLUSIONS

In summary, the OmpA -I-ELISA established here was sensitive and specific for *P. multocida* antibody detection. The success of this study has builted up a solid base for developing a novel diagnostic methodology to the *Pasteurella multocida* infection in rabbits.

ACKNOWLEDGEMENTS

This work was supported by grant from the China agricultural research system (Code No. nycytx-44-3-2).

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