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Enzyme-linked immunosorbent assay for the detection of antibodies against the extracellular domain of the OxaA membrane protein of *Mycoplasma suis*

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Abstract: Epidemics of swine infected with *Mycoplasma suis*, a hemotrophic pathogen, cause substantial economic losses to the swine industry worldwide. The complementary DNA sequences encoding two predicted extracellular domains of the OxaA protein of *M. suis* were obtained using polymerase chain reaction and standard molecular cloning methods. The OxaA domains were expressed separately in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins, designated as GST-A and GST-B. The antigenicity of the GST-A and -B recombinant proteins was confirmed by western blotting. The GST-A and -B proteins were approximately 33.9 kDa and 34.6 kDa in size, respectively. An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies against the OxaA protein of *M. suis* using the GST-B protein. The efficacy of the GST-B-based ELISA was compared to a previously described ELISA method based on the analysis of serum samples from 150 swine, and the *M. suis*-positive rates were 22.7% and 17.3%, respectively. The GST-B-based ELISA demonstrated significantly higher levels of specificity, sensitivity, and stability for the serodiagnosis of *M. suis* infection in swine than those of the previously described method.

Key words: *Mycoplasma suis*, *OxaA* gene, expression, ELISA

1. Introduction

Mycoplasma suis is a hemotrophic *Mycoplasma* that colonizes the red blood cells of a wide range of vertebrates. Formerly classified as the rickettsial bacterium *Eperythrozoon suis*, a comparative sequence analysis of the *16S RNA* gene supported the reclassification of *M. suis* (1), and the *M. suis* genome was published in 2011 (2,3). The homology of the sequence of the *OxaA* gene is 100% between *Mycoplasma suis* KI-3806 and the Illinois type. OxaA is considered to be a membrane protein that can be utilized as one of the potential candidates for the diagnosis of *M. suis* and the development of a vaccine against *M. suis*. The *OxaA* gene of *M. suis* is 1026 nucleotides in length and encodes a 341-amino acid membrane protein that may be involved in the crucial adhesion step in the life cycle of the bacterium (4).

The currently available diagnostic tools for the detection of *M. suis* include indirect enzyme-linked immunosorbent assay (ELISA) (5,6) and real-time polymerase chain reaction (PCR) (7), but these methods have certain limitations (8,9). The recombinant expression of the OxaA protein for the production of *M. suis* antigens is confounded by suboptimal protein folding that might be related to the transmembrane region of the protein. However, structural studies of the OxaA protein have not been reported. Our preliminary computational analysis indicated that amino acids 9 to 63 and 122 to 176 of the OxaA protein comprise two extracellular domains, designated as A and B, respectively.

In our current study, we express the predicted extracellular domains of the OxaA protein of *M. suis* as the glutathione-S-transferase (GST) fusion proteins, GST-A and GST-B, in *Escherichia coli*. The antigenicity of the recombinant GST-A and -B proteins was confirmed by our analysis, and our GST-B-based ELISA demonstrated a high level of efficacy for the detection of antibodies against the OxaA protein of *M. suis*.

2. Materials and methods

2.1. Molecular cloning of the *OxaA* gene

Genomic DNA was isolated from *M. suis* collected in Yanbian Prefecture in Jilin Province, China. The sample we used was *M. suis*-positive blood, which was stored at –20 °C. The full-length complementary DNA (cDNA) sequence of the *OxaA* gene (GenBank accession no. CBZ40902) was amplified using PCR with the P1 and P2

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primers (Table 1) (4). Thermal cycling was performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s and 58 °C for 45 s, and a final extension step at 72 °C for 90 s. The PCR products (10 µL) were analyzed by electrophoresis on a 1% agarose gel and ethidium bromide staining. The *OxaA* cDNA was inserted into the pMD18-T bacterial plasmid and propagated in *E. coli* using standard molecular cloning methods. The DNA sequence of the *OxaA* gene of the Yanbian strain was determined by Shanghai Invitrogen Biotech Company (Shanghai, China).

The regions of the *OxaA* gene encoding the A and B extracellular domains of the OxaA protein were amplified using PCR with the P3 and P4 and the P5 and P6 primer sets, respectively (Table 1). Because of a difference in codon preference between *M. suis* and *E. coli*, the TGA codons in the *OxaA* sequence were replaced by TGG codons in the primers used for the PCR amplifications. The cDNAs coding for the A and B domains were inserted into the pMD18-T plasmid to produce the pMD18-T-A and -B plasmids, and the DNA sequences of each were determined. The pMD18-T-A and -B plasmids were digested with the *Bam*HI and *Eco*RI restriction endonucleases for 2 h at 37 °C. The cleaved cDNAs coding for the A and B domains were ligated into the pGEX-4T-1 prokaryotic expression plasmid to produce the pGEX-4T-A and pGEX-4T-B plasmids, respectively, using T4 DNA ligase at 16 °C for 12 h. The ligation products were used to transform competent BL21 (*DE3*) *E. coli*.

2.2. Protein expression

For protein expression, 50 µL of BL21 (DE3) *E. coli* transformed by OxaA was inoculated in 5 mL of ampicillin-resistant LB medium and cultured at 37 °C overnight. The next day, 500 µL of the cultured medium was inoculated into 50 mL of ampicillin-resistant LB medium and cultured until the OD_{600} value reached 0.5–0.6, and expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich, St. Louis, MO, USA). Protein expression occurred during a 12-h incubation at 30 °C. The recombinant fusion proteins were purified using the GST-tagged Protein Purification Kit (Beijing TransGen Biotech, Beijing, China), and the concentrations of the purified products were determined using a Bradford colorimetric assay.

2.3. Western blotting

For polyacrylamide gel electrophoresis (PAGE) analysis, the expression cultures were centrifuged at 10,000 × *g* for 1 min.

The cells were pelleted in a microfuge tube and resuspended in 100 µL of sample loading buffer containing 1% sodium dodecyl sulfate (SDS) and 0.2 M 2-mercaptoethanol. The samples were boiled for 5 min. The heated mixture was centrifuged for 1 min at 10,000 × *g*, and the lysis supernatants and the purified proteins were analyzed by SDS-PAGE (12%) at 80 to 120 V. The lysis supernatants and the purified proteins were also analyzed by immunoblotting using a mouse anti-GST tag monoclonal antibody (Invitrogen, Biotech Co., Shanghai, China) and *M. suis*-positive and *M. suis*-negative pig antisera were provided by the Laboratory of Veterinary Microbiology, Yanbian University. Anti-OxaA antibody reactivity was detected using a horseradish-peroxidase (HRP)-conjugated goat antimouse antibody or an HRP-conjugated rabbit antipig antibody (Beijing Solarbio Science & Technology, Beijing, China).

2.4. Optimization and validation of the GST-B-based ELISA for the detection of anti-OxaA antibodies

An ELISA was developed based on the GST-B protein (henceforth referred to as the rELISA) for the detection of IgG against the OxaA protein of *M. suis* in pig serum. Nunc microtitration plates (Thermo Scientific, Waltham, MA, USA) were coated with the purified GST-B protein in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed three times with PBS containing 0.3%

Table 1. Oligonucleotide primers used for the PCR amplification of cDNAs encoding the OxaA protein and the extracellular domains.

No.	Sequences	Length of the PCR products (bp)
P1	TTTAGTGTTACATTTTGCGGAA	1561
P2	ATCCCTCTCAAACTCCTACAAG	
P3	CG*GGATCCTCTATGTTCACCTCATATGTTTTTCACTCAAATGAAGCTGGAGTTGGTCT	181
P4	CG*GAATTCCCCATTTCCTGAACTAACTAAAGGCCAGTAAGCCCACGGATTTGGAGTCC	
P5	CG*GGATCCAGAGCTCAATATTATTCAGATTTAC	181
P6	CG*GAATTCAGGTTTTAGACCATGTTTTTTAC	

**Bam*HI and *Eco*RI restriction endonuclease sites.

Tween-20 (PBST), and each well was blocked with 200 µL of 3% skim milk for 2 h at 37 °C. After three washes with PBST, 100 µL of dilute *M. suis*-positive or *M. suis*-negative serum was added to each well, and the plates were incubated for 1 h at 37 °C. After three washes with PBST, 100 µL of a 1:1000 dilution of an HRP-conjugated rabbit antipig antibody was added to each well, and the plates were incubated at 1 h at 37 °C. After three washes with PBST, the HRP substrate, OPD (Sigma-Aldrich), was added to each well, and the plates were incubated in the dark for 10 min. The HRP reaction was stopped with 2 M vitriol, and the absorbance of the well contents was measured at 492 nm using a Multiskan MK3 computer-assisted microplate reader (Thermo Scientific).

Serum samples from swine infected with *E. coli*, *Streptococcus*, *M. hyopneumoniae* of swine, swine fever, and *Toxoplasma gondii* were tested using the rELISA to assess cross-reactivity. The sera from swine infected with *M. suis* were used as a positive control. All the sera used in this research were saved by the Preventive Veterinary Laboratory, Yanbian University, China (10). All of the assays were performed in triplicate. Serum samples from 15 swine from an animal farm at Yanbian University were analyzed using the rELISA. The mean of the coefficient of variation was determined to assess the precision of the rELISA for each antigen based on the results for 150 serum samples from randomly selected swine at a slaughterhouse in the Yanbian prefecture, and the results were compared with those of a previously described ELISA that used a purified antigen (henceforth referred to as pELISA) (10). The cut-off value for each rELISA was defined as the sum of the mean and 3 standard deviations (SDs) of the absorbance value of the *M. suis*-negative serum samples.

3. Results

The 1561-bp DNA sequence containing the full-length of the *OxaA* gene of the Yanbian strain of *M. suis* was submitted to the GenBank database (accession no. JN656209). The sequencing analysis of the *OxaA* gene of the Yanbian strain revealed 21 nucleotide-base substitutions and one amino acid mutation, and the nucleotide and amino acid homologies were 97.9% and 99.7%, respectively. The sequences of the cDNAs coding for the extracellular domains did not differ from the corresponding sequences in the full-length *OxaA* cDNA for the Yanbian strain.

The SDS-PAGE analysis and Coomassie brilliant blue staining showed that the purified GST-A and -B proteins were approximately 33.9 and 34.6 kDa is size, respectively, and the concentrations of the purified proteins were 1.24 and 1.31 mg mL^{-1}. In the western blotting analysis, the mouse anti-GST tag antibody and the *M. suis*-positive pig sera produced a band that corresponded to approximately 34 kDa (Figures 1A and 1B).

Much of the GST-A and -B proteins aggregated in intracellular inclusion bodies, rendering them difficult to purify. Because a higher level of the GST-B protein was present in the cell-lysis supernatant, the GST-B protein was used for the development of the rELISA for the detection of anti-OxaA antibodies in *M. suis* infected swine. To determine the specificity and sensitivity of the rELISA, different antigen concentrations and serum dilutions were tested, as shown in Table 2. The maximum P/N value was obtained when the concentration of the antigen was 4.0 µg mL^{-1} with a 2-h incubation period. The serum dilution was 1:200 with a 1-h incubation period, and the IgG HRP dilution was 1:1000 with a 1-h incubation period

The cut-off value was 0.221, based on the sum of the mean (0.1402) and three SDs (0.0269) of the absorbance values of the *M. suis*-negative serum samples (n = 108).

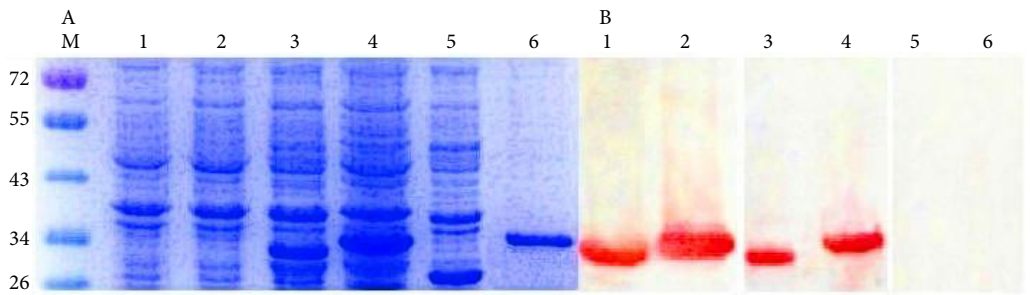

Figure 1. SDS-PAGE (A) and western blotting (B) analysis of the extracellular domains of the OxaA protein of *M. suis* expressed in *E. coli*. A: Lane 1, pGEX-4T-A before induction; lane 2, pGEX-4T-B before induction; lane 3, pGEX-4T-A at 12 h after induction; lane 4, pGEX-4T-B at 12 h after induction; lane 5, *E. coli* control at 12 h after induction; and lane 6, purified GST-B protein. B: Lane 1, GST-A protein and anti-GST tag antibody; lane 2, GST-B protein and anti-GST tag antibody; lane 3, GST-A protein and *M. suis*-positive pig serum; lane 4, GST-B protein and *M. suis*-positive pig serum; lane 5, GST-A protein and *M. suis*-negative pig serum; and lane 6, GST-B protein and *M. suis*-negative pig serum.

Table 2. Optimization of the GST-B-based ELISA for the detection of anti-OxaA antibodies in swine serum.

CCA		DS											
		50×	100×	200×	400×	800×	1600×	3200×	6400×	12,800×	25,600×	GST control	Blank
1.0 μg/mL	+	1.821	1.672	1.508	1.351	1.112	0.914	0.662	0.444	0.195	0.032	0.028	0.002
	–	0.148	0.112	0.043	0.011	0.001	0.005	0.002	0.000	0.002	0.000	0.026	0.003
	P/N	15.05	19.78	29.56	26.42	21.64	17.68	12.64	8.28	3.30	0.04	-	-
2.0 μg/mL	+	2.021	1.889	1.543	1.528	1.202	1.017	0.744	0.395	0.121	0.056	0.031	0.001
	–	0.176	0.119	0.031	0.023	0.003	0.012	0.002	0.000	0.003	0.001	0.027	0.002
	P/N	13.53	20.63	30.22	29.92	23.40	19.70	14.24	7.26	1.78	0.48	-	-
4.0 μg/mL	+	2.553	2.277	1.813	1.690	1.323	1.192	0.839	0.441	0.137	0.077	0.030	0.003
	–	0.194	0.111	0.042	0.019	0.002	0.002	0.001	0.009	0.002	0.001	0.029	0.001
	P/N	15.37	27.70	35.60*	33.14	25.80	23.18	16.12	8.16	2.08	0.88	-	-
8.0 μg/mL	+	2.219	1.975	1.699	1.637	1.370	0.916	0.651	0.511	0.130	0.050	0.028	0.002
	–	0.177	0.101	0.039	0.019	0.001	0.000	0.000	0.002	0.004	0.000	0.032	0.000
	P/N	15.10	28.19	33.38	32.14	26.80	17.72	12.42	9.62	2.00	0.40	-	-

Note: +, positive; -, negative; ×, dilution; DS, dilution of serum; CCA, concentration of coating antigen. The conjugation was 1:1000. P/N = (number of positives – number of blanks – average number of GST control)/(number of negatives – number of blanks – average number of GST control). If N < 0.05, the N of P/N = 0.05. *: Maximum P/N value.

Thus, the rELISA samples with an absorbance of 0.221 or less were considered as *M. suis*-negative, and those with an absorbance higher than 0.221 were considered to indicate *M. suis*-positive results. In the cross-reactivity analysis, the absorbance of the samples from pigs infected with *E. coli*, *Streptococcus*, *M. hyopneumoniae* of swine, swine fever, and *T. gondii* were lower than 0.221.

The sensitivity and specificity of the rELISA was evaluated based on a comparison with the pELISA results. The results showed that the *M. suis*-positive rate was 22.7% (34/150) and 17.3% (26/150) for the rELISA and pELISA, respectively. The *M. suis*-positive rate for rELISA was 5.4% higher than that of the pELISA. The overall agreement between the rELISA and the pELISA was 76.2% (17.3/22.7; Table 3).

4. Discussion

To date, laboratory diagnostics for detecting *M. suis* have produced inconsistent results, and the diagnosis of *M. suis* infection has often relied on the microscopic examination of peripheral blood smears to determine the attachment of *M. suis* to erythrocytes. For the rELISA, the specific value of P/N was at its maximum with an antigen concentration of 4.0 μg mL^{-1}, a serum dilution of 1:200, and an IgG-HRP dilution of 1:1000. No cross-reactivity with *E. coli*, *Streptococcus*, *M. hyopneumoniae*, swine fever, or *T. gondii* was observed. The *M. suis*-positive rate for the rELISA was 5.4% higher than that of the pELISA. Thus, the rELISA demonstrated higher levels of specificity, sensitivity, and stability for the serodiagnosis of *M. suis* infection in swine.

Table 3. Comparison of the GST-B-based ELISA and the purified antigen ELISA for the analysis of serum samples from 150 swine.

rELISA	Purified antigen ELISA		
	Positive	Negative	Total
Positive	17.3% (26/150)	5.4% (8/150)	22.7% (34/150)
Negative	0% (0/150)	77.3% (116/150)	77.3% (116/150)
Total	17.3% (26/150)	82.7% (124/150)	100% (150/150)

In recent years, a number of nationwide epidemics of *M. suis* infection have occurred that caused great economic losses to the swine industry. To control the disease, future studies of the pathogen are needed. The sequence of the *16S RNA* gene of *M. suis* is now available in the GenBank database. The sequences of five other *M. suis* genes have been previously reported, including a 1.8-kb DNA sequence (11), HspA1 (12), MSG1 (5), *app* (6), and *α-enolase* (13). Hoelzle et al. (14) produced an *M. suis* genomic library using the shotgun method and identified *MSG1*, which codes for the *M. suis* surface protein, MSG1. The amino-acid sequence of the MSG1 protein was shown to be homologous with glyceraldehyde-3-phosphate dehydrogenase, and the *MSG1* gene has been shown to be highly conserved in different *M. suis* isolates. A previous study also showed that *E. coli* expressing MSG1 on their surface adhered to porcine erythrocytes (1).

Membrane protein OxaA, belonging to the OxaA family, is a kind of protein integrated into the membrane. It interacts with the transmembrane region of new membrane proteins to assist their integration into the membrane. OxaA is expressed in *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, and *Mycoplasma capricolum*. In *M. suis*, the structure and function of OxaA and its potential application in immunity are unclear. Therefore, as the only confirmed membrane protein, it is important to study its biological characteristics and immunological application.

The cultivation of *M. suis* is confounded by the fastidious properties of the bacterium, and the codon preference of *M. suis* differs from that of *E. coli*. In *M. suis*, the TGA codon is translated as tryptophan, whereas TGA functions as a stop codon in *E. coli*. Therefore, TGA codons in the *M. suis* sequence were replaced with TGG codons in primers used for the PCR amplification of the cDNAs to ensure the accurate translation of the A and B extracellular domains in the BL21 (*DE3*) *E. coli* expression host. The ELISA and western blotting analyses indicated that a high level of antigenicity was maintained in the recombinant GST-B protein.

Zhang et al. (10) purified an *E. suis* antigen using Sephadex G-200 gel-filtration chromatography and used the protein to develop an ELISA for the detection of antibodies against *E. suis*. A comparison of their ELISA with an indirect hemagglutination test using serum samples from 78 swine showed that the ELISA demonstrated a higher level of sensitivity, specificity, and stability for the serodiagnosis of *E. suis* in swine.

In our current study, we developed an ELISA for the detection of antibodies against *M. suis* using the recombinant GST-B protein as the antigen (rELISA), and it demonstrated a higher level of efficacy for the clinical detection of anti-OxaA antibodies in swine serum compared with that of the previously described ELISA method (pELISA) (10). The prokaryotic expression system is an economic and efficient method of producing protein antigens, and many diagnostic reagents have been produced using various bacterial expression systems. The recombinant GST-A and -B proteins demonstrated acceptable antigenicity, and the rELISA may be effective for the diagnosis of *M. suis* infection in swine. Future studies of the extracellular domains of the OxaA protein are warranted to elucidate the pathogenic mechanisms of *M. suis*.

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