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Original article

Indirect enzyme-linked immunosorbent assay method based on *Streptococcus agalactiae* rSip-Pgk-FbsA fusion protein for detection of bovine mastitis

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Abstract

Objective: The aim of this study was to establish a rapid and accurate method for the detection of the *Streptococcus agalactiae* antibody (SA-Ab) to determine the presence of the bovine mastitis (BM)-causative pathogen.

Methods: The multi-subunit fusion protein rSip-Pgk-FbsA was prokaryotically expressed and purified. The triple activities of the membrane surface-associated proteins Sip, phosphoglycerate kinase (Pgk), and fibronectin (FbsA) were used as the diagnostic antigens to establish an indirect enzyme-linked immunosorbent assay (ELISA) method for the detection of SA-Ab in BM.

Results: The optimal antigen coating concentration was 2 μg/mL, the optimal serum dilution was 1:160, and the optimal dilution of the enzyme-labeled secondary antibody was 1:6000. The sensitivity, specificity, and repeatability tests showed that the method established in this study had no cross-reaction with antibodies to *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* in the sera. The results of the sensitivity test showed that a positive result could be obtained even if the serum dilution reached 1:12,800, indicating the high sensitivity and good repeatability of the method. The positive coincidence rate of this method was 98.6%, which is higher than that of previous tests established with the Sip or Pgk mono-antigen fusion protein, respectively, demonstrating the relatively higher sensitivity of this newly established method. The detection rate for 389 clinical samples was 46.53%.

Conclusions: The indirect ELISA method established in this study could provide a more accurate and reliable serological method for the rapid detection of *S. agalactiae* in cases of BM.

Key words: Streptococcus agalactiae, nulti-epitope fusion antigen, purification, ELISA detection

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Introduction

Bovine mastitis (BM) is an acute or chronic inflammation occurring in the breast tissues of dairy cows and is one of the most frequent diseases of dairy cows. Although numerous pathogenic microorganisms have been implicated in BM, *Streptococcus agalactiae* (SA) is the main causal pathogen of this disease (Bramley and Schultze 1991, Baseggio et al. 1997, Pinto et al. 2013, Wang et al. 2015a, Bosward et al. 2016). The detection rate of SA in mastitis milk samples from cows in Inner Mongolia of China was reported to be 70%, posing great hazards to human and animal health (Andersen et al. 2003, Duarte et al. 2004, Verhoeven et al. 2014). Therefore, establishing an improved diagnostic technique for SA-causing BM would have important significance for public health.

The key factors in the clinical prevention and control of BM are timely and accurate screening and diagnosis to guarantee effective disease control and support corresponding epidemiological investigations (Boddie et al. 1996, Boddie and Nickerson 2002, Sukhnanand et al. 2005, Wang and Liu 2015). Currently, the commonly used laboratory serological detection methods include an enzyme-linked immunosorbent assay (ELISA), agar diffusion test, or agglutination test (Meiri-Bendek et al. 2002, Vidovh et al. 2009, Jzrgensen et al. 2016). Among these tests, ELISA has certain advantages such as simplicity, convenience for batch testing, high throughput, high sensitivity, strong specificity, and cost-effectiveness. The SA membrane surface proteins surface immunogenic protein (Sip), phosphoglycerate kinase (Pgk), and the fibrinogen receptor FbsA are immunogenic proteins of this pathogen (Zanardi et al. 2014, Mahmmod et al. 2015, Vidanarachchi et al. 2015), which are good candidate for use as diagnostic antigens.

The aim of the present study was to evaluate the specificity and sensitivity of an SA-causative BM detection method based on detection of antigens for the three major surface proteins (Sip, Pgk, and FbsA). An indirect ELISA for the detection of SA antibody was developed based on the recombinant fusion protein rSip-Pgk-FbsA, which could provide a reliable detection technology for BM caused by SA. This method will have great significance for improving the production capacity of raw milk and the security of cattle cows.

Materials and Methods

Strain and serum

The recombinant bacteria with BL21/pET--30(+)-fps multi-subunit fusion antigens were constructed and preserved in the Institute of Milk-derived Pathogens, Inner Mongolia University for Nationalities. Serum samples that were positive and negative for SA, positive for the SA membrane surface-associated proteins (Sip, Pgk, and FbsA) and *Staphylococcus aureus*, and those of cows clinically diagnosed with BM were collected, prepared, or stored by the Institute of Milk-derived Pathogens, Inner Mongolia University for Nationalities. Sera that were positive for *Staphylococcus epidermidis*, *Streptococcus pyogenes,* and *Escherichia coli* were purchased from Shanghai Peptides Co., Ltd. A total of 389 serum samples from cows clinically diagnosed with BM were collected from dairy cow breeders and farms in Inner Mongolia Kezuohouqi, Keerqin District, and Zhaluteqi. SA strains were isolated from all of these dairy cows.

Main reagents

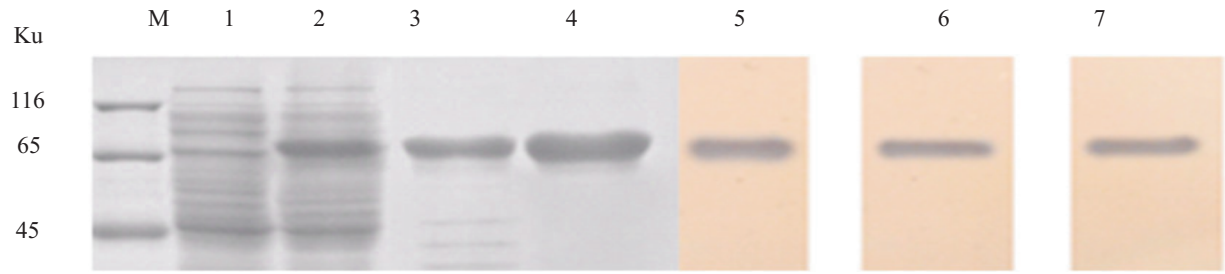
The low-molecular-weight protein marker ovalbumin (OVA), gelatin, and horse serum were all of domestic analytical grade. The HisTrapFF affinity chromatographic column, Q Sepharose HP ion-exchange column, and protein chromatograph AKTApurifier100 were purchased from GE Healthcare. The horseradish peroxidase-labeled rabbit anti-cattle secondary antibody was purchased from Wuhan Amyjet Scientific Inc. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate as a chromogenic reagent was purchased from Beijing Makewonderbio Co.

Expression and purification of multi-subunit fusion antigens

E. coli BL21 (DE3) cells were transformed with the pET-30(+)-fps plasmid with multi-subunit fusion antigens and cultured in Luria-Bertani medium (1:100 dilution). Cells were induced with 1 mM of isopropyl-β-d-thiogalactopyanoside for 4 h at 37°C, 225 rpm. After sonication, the products were subjected to Ni-affinity chromatography and anion-exchange chromatography to obtain the purified fusion protein rSip-Pgk-FbsA; the protein concentration was then determined and the protein was detected with western blotting (Wang et al. 2015b).

Establishment of the indirect ELISA method

To determine the optimal conditions of the indirect ELISA reaction, square matrix titration tests were performed using different coating concentra-



M – Protein Marker, 1 – Non induced recombinant *E. coli*, 2 – Total protein in recombinant *E. coli*, 3 – affinitz chromatography, 4 – Protein after anion-exchange chromatography, 5 – Reaction with sip antibody, 6 – Reaction with pgk antibody, 7 – Reaction with Fbsa antibody

Fig. 1. Expression, purification, and Western blot of the fusion antibody.

tions of the recombinant antigens, dilutions of the serum samples, blocking solutions, and reaction times, as well as various dilutions and reaction times of the secondary antibody under the same reaction conditions.

To determine the threshold for detection, 64 BM-negative bovine serum samples were subjected to the optimized indirect ELISA method. After statistical analysis, the mean optical density at 450 nm (OD450) and the standard deviation (SD) were obtained; according to statistical theory, results of OD450 ≥ mean + 3SD were determined to be positive, those of OD450 ≤ mean + 2SD were determined to be negative, and intermediate values were designated as suspicious.

Specificity test

The specificity of the fusion protein rSip-Pgk-FbsA antigen-based indirect ELISA method established in this study for SA detection was evaluated by applying the method to serum samples positive for *S. pyogenes*, *E. coli*, *S. aureus*, and *S. epidermidis*, as well as to both the SA-positive and -negative control serum samples.

Sensitivity test

The sensitivity of the method was tested with standard SA-positive serum samples prepared through a dilution gradient (from 1:50 to 1:25,600) with four repetitions for each dilution. The OD450 values were determined using the indirect ELISA method to establish the minimum dilution level needed for positive antibody detection.

Repeatability test

The intra- and inter-batch repeatability tests were performed using the optimized indirect ELISA method, and the data were subjected to statistical analysis.

Coincidence rate test

A total of 160 serum samples (including 73 positive serum samples) were tested in parallel using the ELISA method established in this study as well as the methods previously established in our laboratory using SA Sip and Pgk protein, respectively (Meiri-Bendek et al. 2002, Vidovh et al. 2009). The results were then compared to calculate the coincidence rate of the ELISA method established in this study.

Detection of clinical serum samples

The indirect ELISA method was then applied to detect 389 clinical serum samples collected in this study to investigate the positive rate of the SA antibody in the Inner Mongolia region.

Results

Expression and purification of the fusion protein rSip-Pgk-FbsA

The inductively expressed fusion protein rSip-Pgk-FbsA was subjected to affinity chromatography and ion-exchange chromatography, and its concentration was determined to be 2 mg/mL using the Bradford method. The western blot showed that the purified antigens exhibited reactions with the Sip-positive serum, Pgk-positive serum, and FbsA-positive serum (Fig. 1), indicating that the recombinant fusion protein rSip-Pgk-FbsA had triple activities.

Table 1. Optimized ELISA conditions.

	r sip-pgk-FbsA-coating protein	Blocking condition	Sample	Rabbit anti-cow HRP-IgG	Developing time
Optimized dilutions	2 μg/mL	2%OVA	1:160	1:6 000	
Reaction conditions	37°C/2 h	37°C/2 h	37°C/1 h	37°C/1 h	37°C/15 min

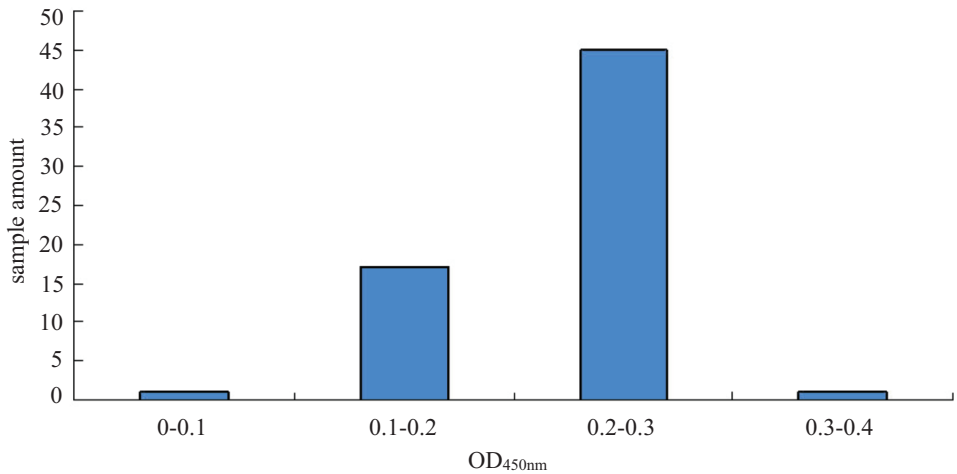


Fig. 2. Regional distribution of OD_{450nm} value of negative serums.

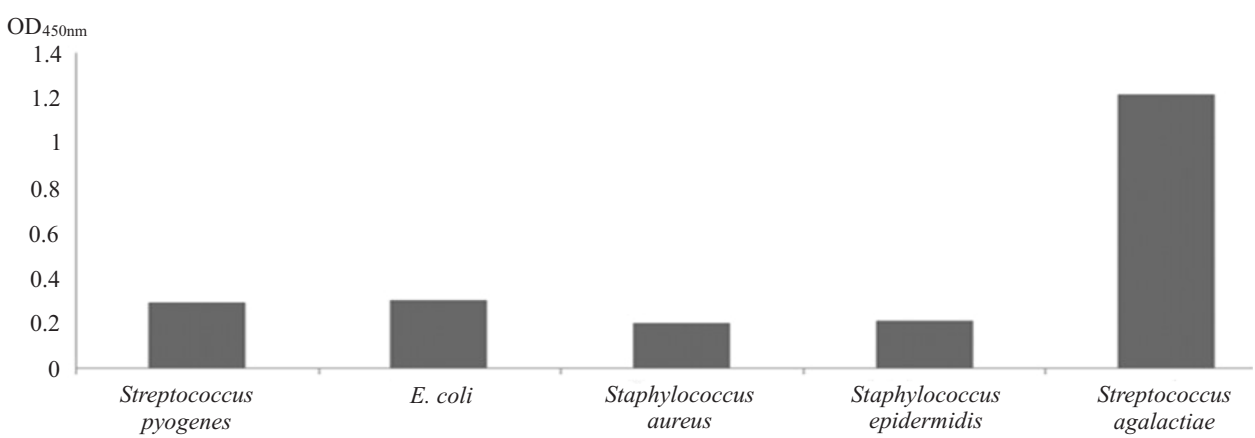


Fig. 3. Interaction of fusion subunit antigen with 5 kinds of positive serum.

Construction of a multi-subunit fusion antigen-based indirect ELISA method

The optimal ELISA conditions were determined using a checkerboard titration method. Microtiter plates were coated with the rSip-Pgk-FbsA protein diluted in coating buffer (50 mM carbonate buffer, pH 9.6) at a concentration of 0.2 μg/well and incubated for 2 h at 37°C. After three washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the plates were blocked with 2% OVA for 2 h at 37°C and then incubated with the serum diluted to 1:160 in PBS for 1 h at 37°C. After another washing step, 1:6000 diluted peroxidase-conjugated rabbit anti-cow IgG antibody was added and the mixture was incubated at 37°C for 1 h. The peroxidase reaction was visualized using the TMB-hydrogen peroxide solution as the substrate for 15 min at 37°C, and the reaction was stopped by adding 50 μL of 2 M sulfuric acid to each well (Table 1).

The 64 SA-negative bovine serum samples were then detected using the optimized ELISA method described above. The results showed that the highest OD450 value was 0.318 and the lowest value was 0.096, with a mean of 0.215 and SD of 0.043; the OD450 values of the negative samples were mainly distributed between 0.1 and 0.3 (Fig. 2). Further statistical analysis determined that the serum samples

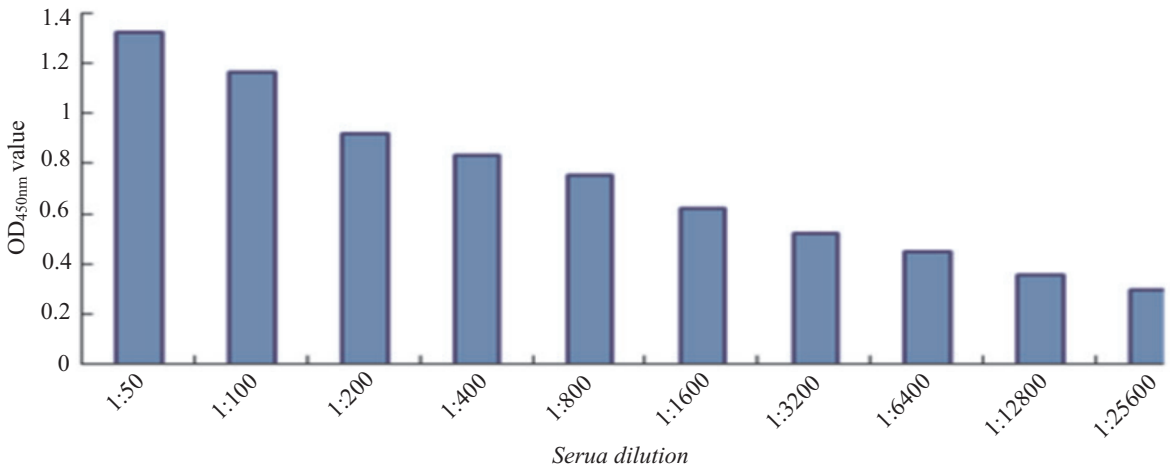


Fig. 4. Test of sensitivity.

Table 2. Results of repeatability test.

Serum number	Repeated number	Intra-batch test		Inter- batch test	
		$\bar{x} \pm 3SD$	CV (%)	$\bar{x} \pm 3SD$	CV (%)
1	8	1.090 ± 0.026	2.36%	1.357 ± 0.056	4.12%
2	8	0.833 ± 0.032	3.80%	0.788 ± 0.025	3.15%
3	8	0.525 ± 0.020	3.85%	0.528 ± 0.010	1.88%
4	8	0.374 ± 0.013	3.52%	0.336 ± 0.023	6.88%
5	8	0.215 ± 0.007	3.31%	0.235 ± 0.020	8.35%

Note: cv = (SD/ Mean) × 100%; during statistical analysis, CV should be less than 15%, otherwise it indicated the data were unstable.

with OD450 ≥ 0.344 could be judged as positive, those with OD450 ≤ 0.301 could be judged as negative, and those located within this range could be judged as suspicious requiring re-examination.

Specificity test

The detection using the ELISA method established in this study towards five different kinds of pathogen-positive serum samples showed that the OD450 values of all pathogen-positive sera except for the SA-positive sample were all less than 0.301, exhibiting negative results (Fig. 3). This finding indicated that the detection method established in this study had good specificity.

Sensitivity test

The standard SA-positive serum sample was subjected to gradient dilution to detect the sensitivity of the method. The results showed that when the serum was diluted 12,800 times, the OD450 value was 0.355 (Fig. 4), which still exhibited a positive result. However, when the serum was diluted 25,600 times, its OD450 value was 0.298, exhibiting a negative result. Therefore, the sensitivity of the method could reach 1:12,800, indicating that this method had high sensitivity for SA detection.

Repeatability test

Intra- and inter-batch repeatability tests were then performed to determine the repeatability of the optimized ELISA method. The results showed that the coefficients of variation of the intra- and inter-batch were less than 10% (Table 2), indicating that the ELISA method established in this study had good repeatability.

Comparison test

The ELISA established in this study was then compared with those established previously in our laboratory using single Sip or Pgk protein, respectively, through parallel detection of 160 serum samples. The results showed that the positive coincidence rate of

Table 3. The comparing detection of ELISA of the fusion antigens with mono-antigen.

Detecting method of ELISA	Positive cases	Negative cases	Test rate	Coincidence rate
ELISA of the recombinant fusion subunit antigen	72	88	45.63%	98.6%
ELISA of the sip outer membrane antigen	70	90	43.75%	95.9%
ELISA of the pgk antigen	67	93	41.88%	91.8%

Table 4. Test results of clinical cow mastitis serum samples.

Serum sample source	Sample number	Positive cases	Detection rate
Dairy farm A	56	27	48.21%
Dairy farm B	67	32	47.76%
Dairy farm C	84	40	47.62%
Dairy farm D	52	27	51.92%
Dairy farm E	60	25	41.67%
Dairy farm F	70	30	42.86%
Total	389	181	46.53%

the rSip-Pgk-FbsA fusion protein antigen-based ELISA method was 98.6%, which was higher than those established using single Sip or Pgk protein (95.9% and 91.8%, respectively; Table 3), indicating that the sensitivity of this recombinant multi-subunit fusion antigen-based ELISA method was superior.

Detection of clinical serum samples

The ELISA method established in this study was then used to test 389 serum samples collected from seven dairy cow farms (breeders). The results showed that the clinical SA infection rate was relatively high at greater than 40%, and the total detection rate of the samples was 46.53% (Table 4). This finding indicates that SA-based BM is a serious problem in the Inner Mongolia region, and that the ELISA method established in this study could be used for the clinical detection of BM in dairy cows.

Discussion

The membrane surface proteins Sip, Pgk, and FbsA are well-established immune-protective antigens of SA. Accordingly, certain ELISA detection methods have been established based on these relatively conserved single antigens, but the sensitivities of the methods targeting one single conserved antigen are not sufficiently high (Keefe 1997, Dogan et al. 2005, Pinto et al. 2014). In our previous study, we used the DNAstar software to analyze the antigen epitope sequences of Sip, Pgk, and FbsA3 of SA, respectively, and the dominant antigen genes of these three proteins were amplified and concatenated by overlap-extension PCR, followed by expression and purification to ultimately obtain the recombinant multi-subunit fusion protein rSip-Pgk-FbsA. The results of western blot showed that the constructed fusion antigen had the triple activities of these three proteins; therefore, it could be used as a diagnostic antigen for the ELISA detection targeting the serum antibodies of SA.

During establishment of this ELISA detection method, the purity of the antigen was found to have a greater influence on the ELISA results. Therefore, to improve the purity of the rSip-Pgk-FbsA fusion protein and reduce the impacts of the *E. coli* host proteins, we first used affinity chromatography to capture the recombinant protein, followed by anion-exchange chromatography; this procedure could significantly improve the purity of the recombinant protein, reaching more than 97%. To optimize the concentration of the coating antigen, dilutions of the primary and secondary antibodies, working duration of the primary and secondary antibodies, and other various experimental conditions, we used the multi-factor orthogonal matrix titration protocol, thus realizing the statistics-based optimization of various conditions to obtain the optimal reaction conditions.

The indirect ELISA method established in this study had no cross-reaction with serum samples positive for *S. pyogenes*, *E. coli*, *S. aureus*, and *S. epidermidis*, indicating that the method had good specificity. The sensitivity test showed positive results even when the serum dilution reached 1:12,800, indicating that it had high sensitivity. Moreover, the intra- and inter-batch stability test showed that the coefficient of variation values were all less than 10%, indicating that the ELISA method had good stability.

The ELISA detection method established in this study using the multi-subunit fusion antigen was then compared with the single Sip or Pgk protein-based ELISA method in detecting the same batch of serum samples, demonstrating that the new method had a higher coincidence rate and the sensitivity was further enhanced, thus exhibiting obvious advantages in epidemiological surveys. Detection of serum samples of clinically confirmed BM cases in the Inner Mongolia region using the ELISA method established in this study showed that the detection rate of SA was 46.53%. This indicates that SA infection is a serious problem in the partial Inner Mongolia regions; therefore, this pathogen appears to be the main source of BM in dairy cows in these regions, and monitoring should be strengthened in this area.

In summary, a new ELISA method for SA was established based on the fusion protein rSip-Pgk-FbsA as the coating antigen, which showed good sensitivity, specificity, and stability, indicating its applicability for clinical testing. This detection method provides a more accurate and reliable serological detection method for epidemiological surveys of SA in dairy cows, as well as providing a reliable experimental basis for the future development of an SA antibody detection kit.

Acknowledgments

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