Development of an indirect ELISA for bovine mastitis using Sip protein of *Streptococcus agalactiae*

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(Received 31 Jan 2015; revised version 16 Apr 2015; accepted 22 Apr 2015)

Summary

The *sip* gene encoding for a conserved highly immunogenic surface protein of *Streptococcus agalactiae* was amplified using polymerase chain reaction (PCR) and subcloned into prokaryotic expression vector pET32a (+) and expressed as a recombinant protein in *E. coli* BL21 (DE3). An indirect enzyme linked immunosorbent assay (ELISA) was developed using the purified Sip protein as a coating antigen, which could identify *S. agalactiae* specific antibody in sera. The coating antigen at a concentration of 3.125 µg/ml, serum diluted to 1:160, and HRP-conjugated secondary antibody concentration at 1:4000 was found to be most effective in exhibiting positive result. The ELISA was found to be highly specific for *S. agalactiae* that may be used for the detection of the pathogen in mastitis cases, for epidemiological studies and for surveillance.

Key words: Bovine mastitis, ELISA, *Streptococcus agalactiae*

Introduction

Bovine mastitis (BM) is a common disease that results in significant decrease in milk quality and yield, thus causing considerable economic loss to the dairy industry worldwide (Bergseng *et al*., 2007) and affects public health (Keefe, 1997; Kossaibati and Esslemont, 1997). In recent years, *S. agalactiae* has also emerged as an important cause of BM (Kossaibati and Esslemont, 1997; Bu *et al*., 2009; Zhen *et al*., 2009). To ensure rapid diagnosis of the disease caused by this organism, it is critical to develop an effective method to detect *S. agalactiae*.

S. agalactiae belongs to group B streptococci, which can be divided into nine different serotypes based on capsular polysaccharides with almost no cross-protective immunity between each serotype (Gonzalez and Andreu, 2005; Bu *et al*., 2009; Noriyuki *et al*., 2012; Abarzúa *et al*., 2014). The key surface immunogenic protein (Sip) of *S. agalactiae* is a 25 amino acid monopeptide located on the surface of the bacteria. Since Sip is highly conserved among various *S. agalactiae* strains, it has been considered an important candidate for diagnostics (Brodeur *et al*., 2000; Bu *et al*., 2013). In the present study, we isolated *S. agalactiae* from milk from cows with BM at Horqin, Inner Mongolia, China. The genomic DNA of *S. agalactiae* was extracted and the *sip* gene was amplified by PCR and further cloned into expression vector. Following prokaryotic expression of Sip protein, an indirect ELISA was developed using the purified Sip as coating antigen to identify the *S. agalactiae* specific antibody. This method can be used for epidemiological investigation of mastitis by dairy industry.

Materials and Methods

Bacterial strain and vector

Reference standard strain of *S. agalactiae* (1886.C55901) was purchased from the China Institute of Veterinary Drugs Control. The test strain was isolated from the milk of sick cows with BM at Horqin, Inner Mongolia. *E. coli* JM109, *E. coli* BL21 (DE3), pMD18-T vector, and pET-30a (+) vector were purchased from TaKaRa Co. (Dalian, China).

Production of antibodies

Healthy rabbits (~ 2 kg), 6-week-old, were purchased from the Experimental Animal Center of Jiling University (Changchun, China). Rabbit polyclonal antibodies for *S. agalactiae*, *S. pyogene*, *E. coli*, and *Staphylococcus aureus* and purified vector tag protein were prepared in our laboratory as previously described (Liu, 2013).

Enzymes and reagents

*Bam*H I, *Hin*d III, T4 DNA ligase, and low molecular weight protein marker were purchased from Fermentas (Beijing, China). The MiniPrep kit, *Taq* DNA polymerase and DNA markers were purchased from TaKaRa (Dalian, China). The DNA gel extraction kit, FCA, FIA, OPD, and 4-CN were purchased from Sigma (Beijing, China). HRP conjugated goat anti-rabbit IgG

was purchased from Solarbio (Beijing, China). Ni-NTA agarose was purchased from QIAGEN (Beijing, China). 96-well microtiter plates were purchased from Costar (Harbin, China).

Primers

Based on the *sip* gene sequences of *S. agalactiae* (GenBank accession No. AF151361), the forward and reversed primers targeting the epitope sequence (81-975nt) were designed with the *Bam*H I and *Hin*d III restriction sites at the 5′ terminus. The primer sequences were as follows: P1 (5′-CAA GGA TCC GAA ACA GAT ACG ACG-3′); P2 (5′-CTG AAG CTT GAT ATT GGA GCC CTG C-3′). The amplified target sequence was 894 bp in length.

Preparation of antiserum

Streptococcus agalactiae, *S. pyogene*, *E. coli*, and *S. aureus* were grown overnight at 37°C in volume of 300 ml media and the bacteria harvested using centrifugation at 12000 × g for 10 min. The pellet containing the bacteria was resuspended in 10 ml of PBS (pH = 7.2), repeatedly frozen at -20°C, thawed at room temperature and finally sonicated. The cell lysate was emulsified with an equal volume of complete freund adjuvant (FCA)/ incomplete freund adjuvant (FIA) for inoculation. The test group of rabbits was inoculated with 10^8 CFU per animal on days 0, 14, and 28. The first immunization used complete Freund's adjuvant and the rest were prepared in incomplete Freund's adjuvant. For the control group, the bacterial solution was replaced with PBS (pH = 7.2). Five ml of blood was collected at pre-inoculation and at 14, 28, 42, 56 and 70 days after inoculation. The rabbit serum was collected and stored at -20°C until use.

Extraction of genomic DNA

A single *S. agalactiae* colony was selected and inoculated into 5 ml of LB medium containing 5% fetal bovine serum (FBS), and cultured on a shaker at 37°C for 16 h. Total DNA was extracted using DNA extraction kit according to the manufacturer's instructions (Sigma, China).

PCR

The 25 µL PCR reaction mixture consisted of 14.35 µL of ddH_2O, 2.5 µL of 10 × PCR buffer, 2 µL of dNTPs, 2 µL of each primers (10 µM), 2 µL of DNA template, and 0.15 µL of *Taq* DNA polymerase (5 U/µL). The PCR protocol was as follows: 95°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; 72°C for 10 min. 5 µL of PCR product was analyzed using 1% agarose gel electrophoresis.

Construction of recombinant plasmid

The amplified *sip* gene was inserted into the *Bam*H I/*Hin*d III site of prokaryotic expression vector pET30-a(+) using standard molecular techniques (Carson *et al*., 2012), resulting in a recombinant plasmid designated as *sip*-pET. The sequence of recombinant plasmid *sip*-pET was confirmed by DNA sequencing, then transformed into BL21 (DE3) by standard methods (Carson *et al*., 2012), and *sip*-pET positive BL21 was grown in LB media and at OD_{600nm} = 0.6, was induced with IPTG (0.25 mM to 1.25 mM) at different temperatures (16 to 37°C).

Protein purification and western blot

Recombinant proteins were purified using a Ni-NTA agarose protein purification kit according to the manufacturer's instructions (Qiagen). The recombinant protein was isolated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with rabbit anti-*S. agalactiae* antibody (1:160 dilution, provided by Dr. Guangxing Li) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:4000).

Development of indirect ELISA

The antibody titers of the serum were determined using the established ELISA. The sera of four rabbits immunized with purified Sip antigen were collected at 0, 14, 28, 42, 56, 70 days post immunization. The sera of two rabbits immunized with purified vector Tag protein served as control. The optimal concentration of coating antigen and the optimal serum dilution were determined using square matrix titration. Briefly, the antigen was diluted to 25 µg/ml in 0.05 M carbonate buffer (pH = 9.0), followed by doubling dilution. They were filled with 200 µL of blocking solution (5% dry milk in 0.01 M PBS with 0.05% Tween 20) and incubated at 4°C overnight. The positive and negative sera were double diluted by PBS (pH = 7.2) starting from 1:10. Experiments were designed using the indirect ELISA program as previously described (Bu *et al*., 2013), and the optimal concentrations of coating antigen and the optimal serum dilution were determined based on the ratio of the OD_{492nm} values of positive and negative serum (P/N) >2. To determine the optimal concentration and reaction time of HRP-conjugated secondary antibody, HRP conjugated goat anti-rabbit IgG was diluted at 1:1000 to 1:8000. At each dilution, the IgG reaction was conducted at 37°C for 30 min, 60 min, and 90 min, respectively. Under optimized conditions, square matrix titration was performed according to the indirect ELISA program. Statistical analysis was performed to calculate the mean value (X) and standard deviation (SD) of the OD_{492nm} values of 36 serum samples. The threshold value was determined as X + 3 SD. The cross reaction between the positive serum of *S. agalactiae*, *S. pyogene*, *E. coli*, and *S. aureus* was investigated using ELISA to analyze the specificity of the antigen.

Detection of mastitis cases

We detected the sera antibody of 45 clinical mastitis cases from 4 dairy farms in Tongliao city area of China with the established method. PCR method was used to detect the *S. agalactiae* DNA in the mastitis cases milk samples at the same time to validate its reliability.

Results

Construction and verification of recombinant expression plasmid

The *sip* gene of *S. agalactiae* was amplified by PCR and the PCR product (894 bp) was observed when electrophoresed in 1% agarose gel (Fig. 1). The amplified PCR product was inserted into the *Bam*H I and *Hin*d III sites of pET-30a vector and the sequence of recombinant plasmid *sip*-pET was confirmed by DNA sequencing.

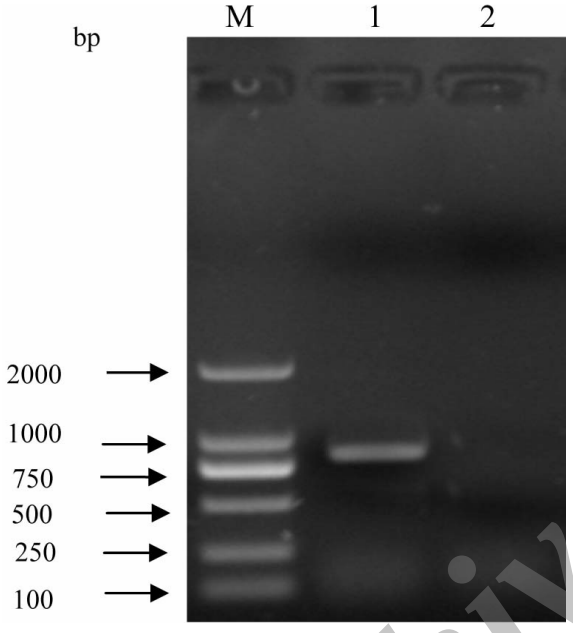

Fig. 1: Amplification of *sip* gene by PCR. Lane 1: DL2000 DNA marker, Lane 2: PCR product, and Lane 3: Negative control

Protein expression and western blot

The recombinant bacteria carrying the *sip*-pET plasmid were induced with IPTG, and harvested and sonicated as previously described (Liu *et al*., 2009; Liu, 2013). The proteins in the supernatant and the pellet were analyzed using SDS-PAGE that showed the presence of a 40 KD protein as expected. The protein was detected in both the supernatant and the pellet. Maximum Sip protein expression was observed at 16°C when induced with IPTG at 1.0 mm/L, following 6 h induction. Using Bandscan software, the Sip protein accounted for 43.8% of total protein (data not shown). SDS-PAGE analysis revealed that the protein had high purity (Fig. 2A). Western blot analysis revealed that the Sip protein could be detected with anti-Sip antiserum (Fig. 2B).

Compared to the control group, the antibody titer of the rabbit serum was significantly increased. Particularlyat 56 days after inoculation, the titer of the antibody in the serum reached the peak of 1:3200. At 70 days, the titer was still as high as 1:3000 (Fig. 3). These results indicated that our indirect ELISA method was suitable for the measurement of antibody titer in the peripheral serum.

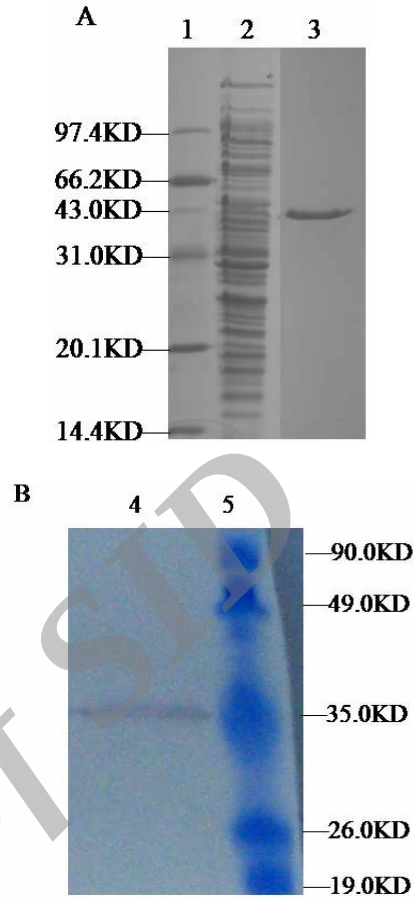

Fig. 2: Purification and western blotting analyses of the recombinant protein. A: SDS-PAGE of recombinant Sip protein before purification and after purification, and B: Western blot of recombinant sip purification protein. Lanes 1, 5: Low molecular weight protein marker, Lane 2: Recombinant Sip protein on the pre-column, Lane 3: After washing, and Lane 4: Result of western blotting

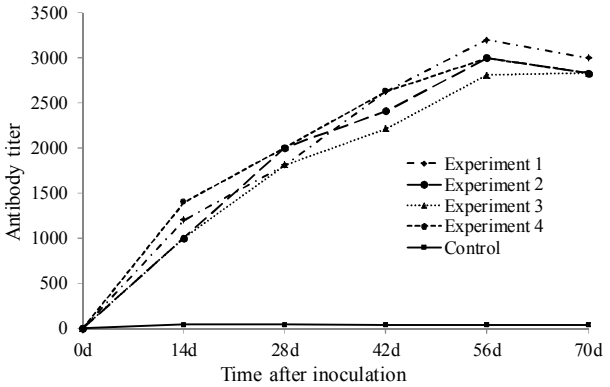

Fig. 3: Determination of antibody levels of immunized rabbits

Development of indirect ELISA

The square matrix titration results showed that the optimal antigen coating concentration was 3.125 µg/ml, namely, 0.3125 µg per well, and the optimal dilution of serum was 1:160. The optimal concentration was 1:4000, and the optimal reaction time was 60 min. The mean value of the OD_{492nm} values of 36 samples was 0.184,

and the SD value was 0.022. The result would be positive if OD_{492nm} ≥0.25, when the difference between the OD_{492nm} values of the standard positive serum and the negative serum was higher than 0.05, P/N ≥2.0 and OD_{492nm} ≥0.25.

Specificity of the ELISA

The ELISA using the Sip antigen was tested with rabbit anti *S. pyogene*, *E. coli*, and *S. aureus* sera, all of which exhibited negative results, indicating that the purified antigen was highly specific for the Sip protein from *S. agalactiae*.

Detection of mastitis cases antisera

The established indirect ELISA method was used for detection of sera antibody of the 45 clinical mastitis cases. The results showed that the 35 cases were positive, up to 75.6%. The results were consistent with the results of PCR, reaching 100%

Discussion

The average rate of incidence of BM is approximately 40% worldwide, resulting in about $35 billion economic loss per year (Xi *et al*., 2013). It has been widely accepted that *S. aureus*, *S. agalactiae*, and *E. coli* are three major pathogens for bovine mastitis in China (Chen *et al*., 2011). Currently, antibiotics are widely used as therapeutics for combating BM. It is important to detect the pathogen involved in BM so effective treatment can be provided in a timely manner to save the cows and prevent economic loss.

The Sip protein of *S. agalactiae* has been established as a conserved surface protein that can be used as a candidate antigen for the detection of the pathogen (Dmitriev *et al*., 1999). In the present study, we cloned a fragment of the DNA encoding the epitope of the Sip protein in the region between 27-325 amino acid that exhibited high antigenic index prediction by DNAMAN software (data not shown). Expression of this protein *in vitro* as observed in SDS-PAGE analysis revealed that the target protein accounted for 43.8% of the total bacterial protein, and the soluble target protein accounted for 48.7% of the total bacterial soluble protein. Western blot confirmed the protein to be highly reactive with its antibody. Using the purified Sip protein, an indirect ELISA was developed. We optimized the conditions of the indirect ELISA that was established to be highly sensitive. The coating antigen at a concentration of 3.125 µg/ml, serum diluted to 1:160, and HRP-conjugated secondary antibody concentration at 1:4000 was found to be most effective, exhibiting positive result threshold P/N ≥2.0 and OD_{492nm} ≥0.25. The specificity of the ELISA against anti-sera generated using *S. pyogenes*, *S. aureus* and *E. coli*, showed the indirect ELISA assay was highly specific for *S. agalactiae*. This method can be reliably used for detection of *S. agalactiae*, in epidemiological studies and for surveillance of BM caused by *S. agalactiae*.

Acknowledgements

This research was supported by the National Science Foundation of China (31060352; 31260600). Thanks to Professor J. Chang of Zhejiang University and X. F. Ren of Northeast Agricultural University for reading our manuscript.

Conflict of interest

There is no conflict of interest.

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مقاله کامل:

توسعه روش الایزا غیر مستقیم برای ورم پستان گاوی با استفاده از پروتئین Sip *استرپتوکوکوس آگالاکتیه*

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(دریافت مقاله: ۱۱ بهمن ۱۳۹۳، پذیرش نهایی: ۲ اردیبهشت ۱۳۹۴)

ژن کد کننده پروتئین سطحی ایمنی‌زای قوی حفاظتی *استرپتوکوکوس آگالاکتیه* با استفاده از واکنش زنجیره‌ای پلیمراز تکثیر و در حامل بیان پروکاریوتی pET32a کلون شده و به صورت یک پروتئین نوترکیب در *E. coli* BL21 (DE3) بیان گردید. یک روش سنجش ایمونوسوربنت متصل به آنزیم (الایزا) غیر مستقیم با استفاده از پروتئین خالص شده Sip به عنوان آنتی ژن جهت کوتینگ توسعه یافت که می‌توانست آنتی بادی اختصاصی *استرپتوکوکوس آگالاکتیه* را در سرم‌ها شناسایی کند. آنتی ژن جهت کوتینگ در غلظت ۳/۱۲۵ µg/ml، سرم رقیق شده به نسبت ۱:۱۶۰، و آنتی بادی ثانویه کنژوگه شده با HPR در غلظت ۱:۴۰۰ موثرترین بودند که نتیجه مثبتی را به دنبال داشتند. الایزا ویژگی بالایی برای *استرپتوکوکوس آگالاکتیه* نشان داد که ممکن است برای ردیابی پاتوژن در موارد ورم پستان، برای مطالعات اپیدمیولوژیکی و نظارتی استفاده گردد.

واژه‌های کلیدی: ورم پستان گاوی، الایزا، *استرپتوکوکوس آگالاکتیه*