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Expression of vascular cell adhesion molecule 1 (VCAM-1) in the mammary lymph nodes of cows with subclinical mastitis

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Abstract

Introduction: Vascular cell adhesion molecule 1 (VCAM-1) is a member of Ig superfamily. The aim of this study was to prepare highly specific polyclonal antibodies against bovine VCAM-1 and to evaluate the expression of VCAM-1 in the mammary lymph nodes of cows with subclinical mastitis. **Material and Methods:** The VCAM-1 gene was cloned from bovine Peyer's patches and inserted into the pGEX-4T-1 and pET-28a vectors. The recombinant plasmids pGEX-4T-1/VCAM-1 and pET-28a/VCAM-1 were transferred into *Escherichia coli* BL21 and the recombinant strains were induced by isopropyl-D-thiogalactoside to produce fusion proteins tagged with polyhistidine (His) and glutathione S-transferase (GST), respectively. The expressed fusion proteins His-VCAM-1 and GST-VCAM-1 were identified by SDS-PAGE and Western blot. His-VCAM-1 protein was used as an antigen to immunise Wistar rats and polyclonal antibody serum against VCAM-1 was obtained. **Results:** The serum titre tested by indirect ELISA was 128,000 using GST-VCAM-1 as the well coating antigen. Western blots indicated that the antibody recognised recombinant VCAM-1 protein as well as endogenous VCAM-1. In addition, using qPCR and Western blot, VCAM-1 mRNA and protein expression levels were measured in dairy cows with subclinical mastitis. It was demonstrated that VCAM-1 levels in the mammary lymph nodes of the cows were significantly higher than those from healthy controls ($P < 0.05$). **Conclusion:** These results are to our knowledge the first report that VCAM-1 expression in the mammary lymph nodes is elevated in dairy cows with subclinical mastitis.

Keywords: dairy cows, mammary lymph nodes, subclinical mastitis, VCAM-1, polyclonal antibody.

Introduction

Vascular cell adhesion molecule 1 (VCAM-1) is an extracellular transmembrane glycoprotein predominantly expressed in high endothelial venules during inflammation as a ligand for α4β1/α4β7 integrins (8, 16, 20). The interaction between VCAM-1 and α4β1/α4β7 is important for many pathological and physiological processes, including modulation of inflammation, angiogenesis, T lymphocyte homing and recycling, and regulation of cardiovascular system development, and it is also involved in immune cell responses and autoimmune diseases (5, 10, 14, 17). VCAM-1 is strongly upregulated in the chronic inflammation stage of several diseases (1, 4, 13, 18, 22). Bovine subclinical mastitis is an inflammation of the mammary gland infected with bacteria. This disease results in a considerable economic loss, endangers food safety, and concerns animal welfare in dairy industry (3). The lymphocytes of the mammary gland could resist pathogen invasion, but lymphocyte cell migration is not a random process in cows (9). VCAM-1 expression in mammary gland vessels in mouse during lactation period and its role in lymphocyte cell migration has been reported (21). In ovine species, VCAM-1 was detected on the vascular endothelium of mesenteric lymph nodes and Peyer's patches, but VCAM-1 is weakly expressed in high endothelium venules (HEVs) in tonsilla and peripheral lymph nodes (19). In bovine species, VCAM-1 is expressed in Peyer's patches and uterine endometrium (2), but it is unkown whether VCAM-1 is expressed in mammary gland lymph nodes.

There are two structural forms of the VCAM-1 gene (7). The full-length VCAM-1 7D form contains seven Ig-like domains with integrin binding sites located in domains 1 and 4 (domain 1 or 4 of VCAM-1 is necessary for α4β1/α4β7-dependent adhesion) (15). The VCAM-1 6D alternatively spliced form contains six Ig-like domains and lacks domain 4. VCAM-1 7D is the major form with domains 1–3 similar to domains 4–6 in amino acid sequence (6, 11, 12). It is well known that mouse/human VCAM-1 plays a critical role in T lymphocyte trafficking and homing. However, relatively little is known about the function of VCAM-1 in bovine species, especially the functions of the first and fourth domains of VCAM-1 7D. An effective and specific antibody against bovine VCAM-1 would be a powerful and indispensable tool to study the function of the bovine VCAM-1 protein. Therefore, in this study, a sequence containing domains 1–4 of VCAM-1 7D was expressed in *Escherichia coli* (*E. coli*) with polyhistidine (His) and glutathione S-transferase (GST) tags.

Material and Methods

Experimental animals. Lymph nodes were obtained from three healthy cows and three cows with subclinical mastitis caused by *Staphylococcus* sp. Additionally, small intestine Peyer's patches were isolated from healthy dairy cows. All bovine tissues were stored at –80°C in the super-cold refrigerator. Wistar rats (180–220 g) were provided by the Experimental Animal Center of Jilin University (Changchun, China).

Cloning and identification of the VCAM-1 gene in *E. coli*. Total RNA was isolated from Peyer's patches using RNAiso and immediately mRNA was reverse transcribed into cDNA using an ALV RT Kit (Takara, China) following manufacturer's instructions. PCR primers were designed according to the gene sequence of VCAM-1 (GenBank accession No 174484.1). The forward primer was 5′- GAA TTC TCC CAA ATC GAC ATA TTC CC -3′ and the reverse primer was 5′- CTC GAG TTA TTT CTC TTG AAC AGT TAA TT -3′. The forward and reverse primers contained *Eco*RI and *Xho*I restriction sites, respectively. PCR was performed with the following parameters: pre-denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were analysed by electrophoresis on 1% agarose gels containing ethidium bromide. The gel band containing the amplified product was isolated, from which DNA was recovered and purified using a Gel DNA Purification Kit (Takara), then ligated to the pMD18-T vector (Takara). The recombinant plasmid named pMD-18T/ VCAM-1 was identified by double-enzyme digestion and nucleotide sequencing.

Expression and identification of the recombinant VCAM-1 protein. The recombinant proteins His-VCAM-1 and GST-VCAM -1 were prepared as described below. Briefly, the PCR product (pMD-18T/VCAM-1) was digested with *Eco*RI and *Xho*I and inserted into the His and GST fusion protein sites of the prokaryotic expression vectors pGEX-4T-1 and pET-28a (Takara) respectively, to create the recombinant plasmids pGEX-4T-1/VCAM-1 and pET-28a/VCAM-1. In order to achieve fusion protein expression, the recombinant plasmids were transformed into *E. coli* BL21 (DE3) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. The recombinant *E. coli* cells were harvested by high speed centrifugation after IPTG induction.

Analysis of rVCAM-1 protein solubility. The achieved *E. coli* pellet was collected as described above, then the pellet was sonicated by ultrasonic system CPX- 600 (Cole-Parmer, USA). Firstly, the cell pellet was resuspended in PBS and cooled on ice for 10 min. Then, cell suspension was sonicated with 10 short bursts of 10 s followed by interval of 30 s for cooling. Finally, the lysate was ultracentrifuged at 4ºC for 10 min at 12,000 rpm. For confirmation of rVCAM-1 expression, the supernatant and the precipitates were analysed by SDS-PAGE.

Purification of the recombinant VCAM-1 protein. Recombinant VCAM-1 protein with the His tag was purified using His GraviTrap (GE Biosciences, Sweden) and recombinant VCAM-1 protein with the GST tag was purified using Gluthathione-Sepharose 4B (GE Healthcare, USA), following kit protocols for both purifications. The recombinant proteins His-VCAM-1 and GST-VCAM-1 were analysed by 12% SDS-PAGE and Western blot using monoclonal antibodies against the His and GST tags (Sigma, USA) respectively. The protein concentrations of both purified recombinant proteins were determined using a BCA protein kit (Bio-Rad, USA).

Preparation of polyclonal antibodies against recombinant bovine His-VCAM-1 protein in rats. Wistar rats were immunised with 50 μg of purified recombinant bovine His-VCAM-1 protein emulsified in complete Freund's adjuvant (Sigma, USA) on day 1. Intramuscular booster injections in the same dose were administered on days 14, 28, 35, and 42. The rats were euthanised and blood was collected on day 7 after the last immunisation. Polyclonal antibodies against bovine VCAM-1 were prepared and stored at −20°C until use.

Detection of anti-VCAM-1 polyclonal antibody titre by ELISA. ELISA plates were coated with 2 μg/mL of recombinant His-VCAM-1 protein in PBS and incubated overnight at 4°C. After washing five times with PBS-0.05% Tween 20 (PBS-T), nonspecific binding sites were blocked with PBS containing 3% bovine serum albumin for 1 h at 37°C. Wells were then incubated with 50 μL of serum samples at different dilutions for 1 h at 37°C. The microplate was washed five times in PBS-T, incubated with phosphatase-

labelled goat anti-rat IgG (Sigma, USA) for 1 h at room temperature, and then washed five times with PBS-T. Finally, the reaction was developed by adding disodium 4-nitrophenyl phosphate substrate (Sigma), and the absorbance was measured at 405 nm in a microplate reader (Bio-TEK, USA). All serum samples were tested in triplicate on each plate.

Detection of recombinant VCAM-1 protein using anti-bovine VCAM-1 polyclonal antibody by Western blot. After recombinant VCAM-1 protein was identified by 12% SDS-PAGE, the gel was transferred to nitrocellulose membrane. The membrane was incubated with anti-bovine VCAM-1 polyclonal antibody and goat anti-rat IgG. The experimental method of SDS-PAGE and Western blot was performed as previously described.

Primers of VCAM-1 for real-time PCR. A pair of primers was designed based on the VCAM-1 gene sequence (GenBank accession no. 174484.1; amplicon length 80 bp) with PrimerExpress 3.0 (ABI, USA). The primer sequences were as follows: forward primer – 5′-TGA CGA TGA CGT GTG CCA GT-3′; reverse primer – 5′-GCT GTC GGT TCC CAT TGT CT-3′. The primers were synthesised by Sangon Biotech (China).

Primers of β-actin for real-time PCR. A pair of primers was designed based on the β-actin gene sequence (GenBank AY141970; amplicon length 222bp) with PrimerExpress 3.0. The primer sequences were as follows: forward primer – 5′- GTG GGC CGC CCT AGG CAC CA-3’; reverse primer – 5′- GGG CCT CGG TCA GCA GCA C-3’. The primers were synthesised by Sangon Biotech.

Two-step quantitative RT-PCR assay. Two-step qPCR assays were performed to quantify the levels of VCAM-1 mRNA in mammary lymph nodes. The qPCR was performed using a two-step qPCR kit (Takara) according to manufacturer’s instructions. Briefly, VCAM-1 gene sequence was amplified with a two-step qPCR kit. Total RNA was extracted by routine methods. The cDNA was synthesised using the qPCR kit. PCR assays in the ABI 7500 Real Time PCR System (Applied Biosystems, USA) used optimised 20 μL reaction mixtures containing 10 μL of SYBR Premix ExTaqTM, 0.5 μL of each primer, 0.5 μL of ROX Reference Dye, 2.5 μL of cDNA sample, and 6.0 μL of double-distilled water. The thermocycling protocol called for initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 58°C for 45 s. Each sample was measured in triplicate. Melting curve analyses were performed for the PCR products of VCAM-1 and β-actin gene amplifications to evaluate primer specificity. The relative abundance of VCAM-1 mRNA was evaluated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and was normalised to β-actin mRNA levels.

Detection of VCAM-1 protein levels in mammary lymph nodes using anti-bovine VCAM-1 polyclonal antibody by Western blot. Membrane protein was analysed by SDS-PAGE. Briefly, 15 μL samples were homogenised in SDS-PAGE sample loading buffer and electrophoresed. Protein bands were stained using Coomassie brilliant blue. In addition, Western blot analysis was applied for further identification of VCAM-1. Proteins were transferred to nitrocellulose membranes. The membranes were blocked with blocking buffer for 1 h at room temperature and incubated with anti-bovine VCAM-1 polyclonal antibody（1:2,000 dilution). After five washes with PBS-T, the membranes were incubated with goat anti-rat IgG (1:20,000 dilution). Proteins were visualised by incubation with enhanced chemiluminescence detection system reagents (Amersham Pharmacia Biotech, USA) and the amount of target protein was calculated by gray scanning with Quantify One software (Bio-Rad). The level of VCAM-1 protein expression was expressed as the ratio of VCAM-1 to β-actin expression.

Statistical analysis. All data analyses were performed with IBM SPSS10.0 software (Armonk, USA). Data were expressed as means ± standard deviation (SD). One-way ANOVA was used for comparisons between subclinical mastitis and healthy groups (Peyer’s patches were used as positive control). In all data analysis, $P < 0.05$ was regarded as significant.

Results

Identification of the expressed product. The gene fragment encoding VCAM-1 was amplified by PCR (Fig. 1), and the resulting products were cloned into the pGEX-4T-1 and pET-28a expression vectors. The successful incorporation of the PCR products into plasmids pGEX-4T-VCAM-1 and pET-28a-VCAM-1 was confirmed by restriction enzyme digestion (Figs 2, 3).

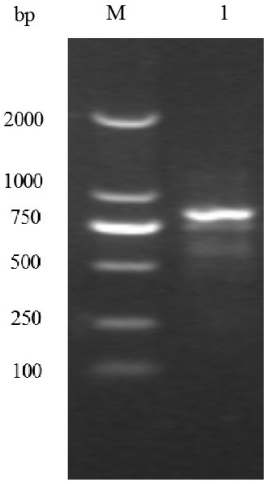

Fig. 1. The PCR product of VCAM-1 gene. M – DL2000 Marker; 1 – M gene

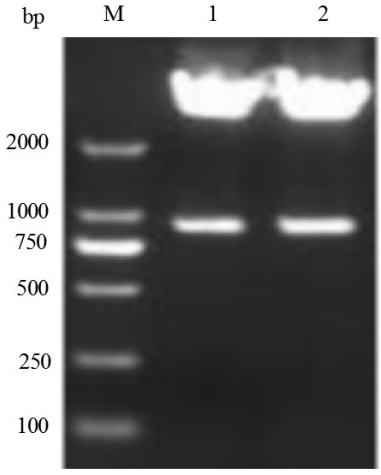

Fig. 2. Enzyme digestion profile of pGEX-4T-1-VCAM-1. M – DL2000 Marker; 1, 2 – Identification of pGEX-4T-1-VCAM-1 with *Eco*R I and *Xho* I digestion

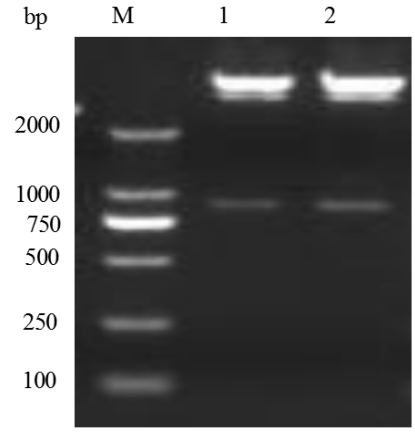

Fig. 3. Enzyme digestion profile of pET-28a-VCAM-1. M – DL2000 Marker; 1, 2 – Identification of pET-28a-VCAM-1 with with *Eco*R I and *Xho* I digestion

Identification of recombinant VCAM-1 protein by SDS-PAGE and Western blot. The recombinant VCAM-1 protein with GST tag was expressed and purified, and its molecular mass was determined to be 56 kDa (Fig. 4). The recombinant His-tagged VCAM-1 protein was also expressed and purified and its molecular mass was 37 kDa (Fig. 5). The recombinant GST-VCAM-1 and His-VCAM-1 proteins were identified by Western blot using anti-GST and anti-His monoclonal antibodies, respectively (Figs 6, 7).

Determination of anti-bovine VCAM-1 antibody titres by ELISA. The serum antibody titres of rats immunised with the recombinant His-VCAM-1 protein were tested by ELISA using the purified recombinant GST-VCAM-1 protein as coating antigen. The titres were calculated at 1:128, 000 dilution, indicating that high titres of bovine polyclonal antibodies were generated.

Identification of recombinant GST-VCAM-1 protein by Western blot. The expressed recombinant GST-VCAM-1 protein was identified by Western blot using the rat anti-bovine VCAM-1 antibody (Fig. 8).

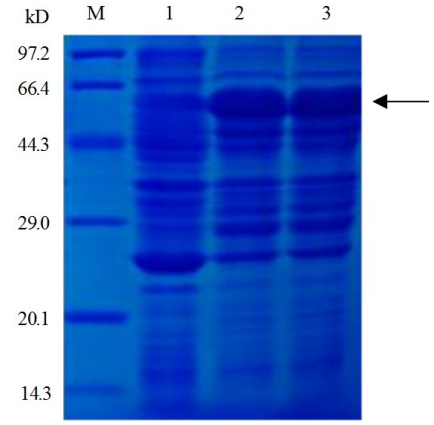

Fig. 4. Expression of GST-VCAM-1 analysed by 12% SDS–PAGE. Lane1 – whole lysate of cells harbouring pGEX-4T-1-VCAM-1 without IPTG induction; Lanes 2 and 3 – whole lysate of cells harbouring pGEX-4T-1-VCAM-1 after IPTG induction

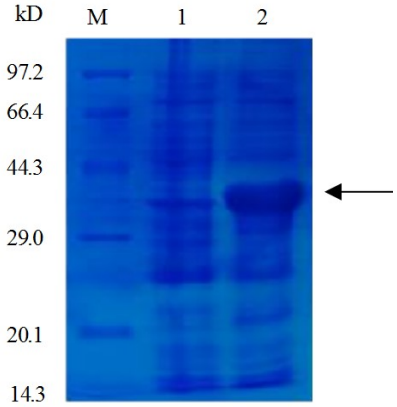

Fig. 5. Expression of His-VCAM-1 analysed by 12% SDS–PAGE. Lane 1 – whole lysate of cells harbouring pET-28a-VCAM-1 without IPTG induction; Lane 2 – whole lysate of cells harbouring pET-28a-VCAM-1 after IPTG induction

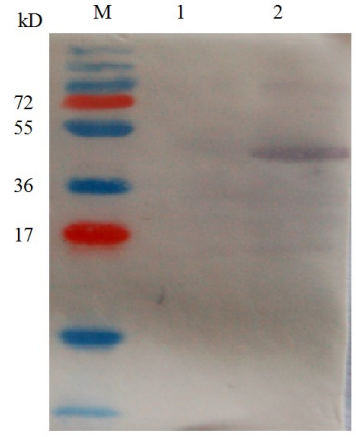

Fig. 6. Western blot analysis of recombinant VCAM-1-GST protein. M – prestained protein marker; 2 – anti-GST antibody as the first antibody

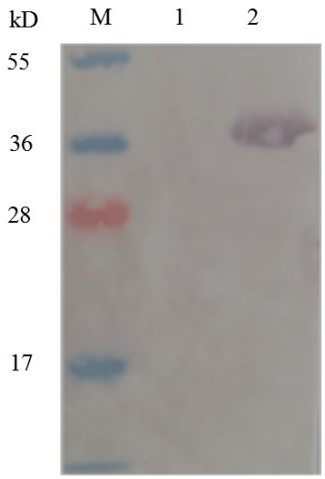

Fig. 7. Western blot analysis of recombinant VCAM-1-His protein. M – prestained protein marker; 2 – anti-His antibody as the first antibody

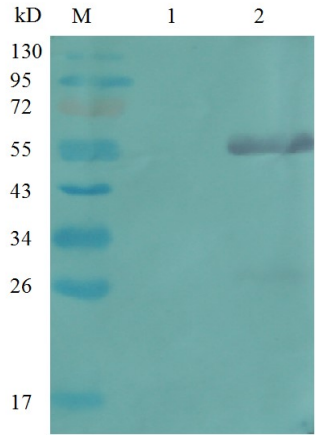

Fig. 8. Western blot analysis of VCAM-1-His Polyclonal antibody to identify recombinant VCAM-1-GST protein. M – prestained protein marker; 1 – GST; 2 – VCAM-1-GST

VCAM-1 mRNA levels in cows with subclinical mastitis by qPCR. The qPCR showed that VCAM-1 mRNA was expressed in both cows with subclinical mastitis and healthy control animals (Peyer's patches were used as positive control). Compared with the healthy controls, VCAM-1 mRNA levels in cows with subclinical mastitis were significantly increased ($P < 0.05$, Fig. 9).

VCAM-1 protein levels in cows with subclinical mastitis by Western blot. Similarly to the qPCR results, Western blots showed that VCAM-1 protein was expressed in cows with subclinical mastitis and healthy controls (Peyer's patches were used as positive control). Compared with the healthy controls, VCAM-1 protein levels in cows with subclinical mastitis were significantly increased ($P < 0.05$) (Fig. 10).

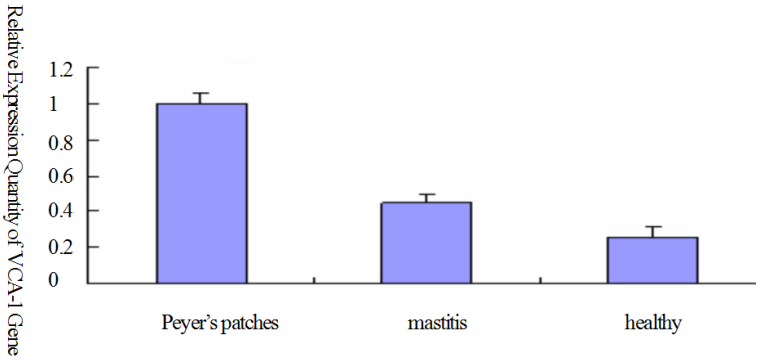

Fig. 9. Detection of VCAM-1 gene transcription differences in lymphoid tissues from subclinical mastitis and healthy dairy cows by real time-PCR

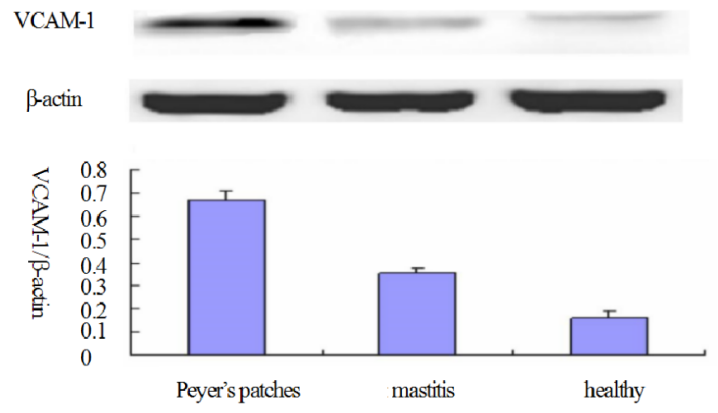

Fig. 10. Detection of VCAM-1/ MAdCAM-1 gene expression differences in lymphoid tissues from subclinical mastitis and healthy cows by Western blot

Discussion

Our previous research found that peripheral lymphocytes express integrin α4β7 in dairy cows with subclinical mastitis. Because the interaction between VCAM-1 and integrin α4β7 plays an important role in inflammation, we further investigated the functions of VCAM-1 protein in dairy cows with subclinical mastitis. In this study, we successfully expressed VCAM-1 as a fusion protein with either a His or GST tag in an *E. coli* expression system. Wistar rats were immunised using purified recombinant His-VCAM-1 protein to obtain immunopositive serum, and indirect ELISA and Western blotting were used to confirm the immunogenicity of the target protein using GST-VCAM-1 as coating antigen. Detecting the specificity of bovine VCAM-1 polyclonal antibody by the construction and application of prokaryotic expression plasmids containing GST and His tags may prevent false-positive interactions of the antibody with the tag.

Our previous study showed that α4β7 (VCAM-1 receptor) was expressed on the surface of lymphocytes in the peripheral blood of dairy cows with subclinical mastitis. The current results indicated that the VCAM-1 gene is expressed in lymph nodes from dairy cows with subclinical mastitis. Taken together, our experiments showed that the interaction between VCAM-1 and integrin α4β7 plays an important role in dairy cows with subclinical mastitis. We successfully cloned a bovine VCAM-1 gene fragment, constructed the prokaryotic expression vectors pGEX-4T-1/VCAM-1 and pET-28a/VCAM-1, expressed the fusion protein and obtained a high-titer specific anti-bovine VCAM-1 polyclonal antibody. The antibody not only recognises the expressed recombinant bovine VCAM-1 protein, but endogenous bovine VCAM-1 as well. The preparation of this bovine polyclonal antibody may offer a useful tool for studying the properties and function of bovine VCAM-1. In the future, in order to explore the potential effect of VCAM-1 in the pathological mechanism of dairy cows with subclinical mastitis, we intend to use vascular endothelial cells from bovine mammary glands to determine, using our anti-bovine VCAM-1 antibody, whether exogenous VCAM-1 protein blocks or enhances the expression of VCAM-1 in cells.

In conclusion, we generated polyclonal antibodies against bovine VCAM-1, a member of the Ig family, which has been regarded as a possible regulator of inflammation. We found that mammary lymph node VCAM-1 levels in dairy cows with subclinical mastitis were significantly higher than those in healthy cows. To our knowledge this is the first report showing that VCAM-1 levels in the mammary lymph nodes were elevated in dairy cows with subclinical mastitis. We suggest that VCAM-1 may play an important role in the mechanisms regulating infection in dairy cows.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: This study was approved by the Committee on the Ethics of Animal Experiments of Heilongjiang Bayi Agricultural University.

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