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REGULAR ARTICLES

Prevalence and pathogens of subclinical mastitis in dairy goats in China

Yanqing Zhao · Hui Liu · Xuanduo Zhao · Yang Gao · Miaotao Zhang · Dekun Chen

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Abstract Subclinical mastitis, a costly disease for the dairy industry, is usually caused by intramammary bacterial infection. The aim of this study was to investigate the prevalence of and pathogens involved in subclinical mastitis in dairy goats in China. A total of 683 dairy goats in the main breeding areas of China were selected, and milk samples were collected. Out of these, 313 (45.82 %) goats were detected distinct or strong positive for subclinical mastitis by using California mastitis test. Among these positive goats, 209 milk samples were used to identify the causing agents by a multiplex PCR assay, and results were listed as follows: coagulase-negative staphylococci (59.52 %), *Staphylococcus aureus* (15.24 %), *Escherichia coli* (11.43 %), and *Streptococcus* spp. (10.95 %). In conclusion, subclinical mastitis is a highly prevalent disease in dairy goats in China, and coagulase-negative staphylococci are the predominant pathogens.

Keywords Subclinical mastitis · Prevalence · Pathogens · Dairy goats

Introduction

Mastitis, characterized as an inflammation in the mammary gland, decreases milk quantity and quality and leads to an annual loss of approximately US$2 billion in the dairy industry of USA and European countries, respectively (Donovan et al. 2005). Based on signs of disease, mastitis can be further classified into two forms: clinical and subclinical. Unlike clinical mastitis, subclinical mastitis can be described as an asymptomatic inflammation in the mammary gland and is one of the most prevalent diseases in the dairy industry. The prevalence of subclinical mastitis in dairy cows has been investigated in many countries across the world, and it has been found to range from 15 to 40 % depending on geographical origins (Plozza et al. 2011; Haftu et al. 2012; Sarker et al. 2013). Interest in dairy goats and goat milk products is increasing with the demand for health food, as goat milk has similar nutritional qualities to human milk and is less allergenic for human consumption than cow milk (Haenlein 2004; Park and Haenlei 2008). Subclinical mastitis in goats has been described in some regions of the world as having a prevalence of 9–50 % (Hall and Rycroft 2007; Min et al. 2007; Marogna et al. 2012); however, there is limited information available for the prevalence of subclinical mastitis in dairy goats in China (Li and Wang 2012), where approximately 6.3 million dairy goats are bred and 1.35 million tons of goat milk are produced (Han et al. 2010; Zhang et al. 2012).

Subclinical mastitis in dairy goats is caused by a number of microorganisms, and the most important bacterial genus is *Staphylococcus*, which accounts for more than 90 % of all the isolated bacteria and is commonly divided into coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* (*S. aureus*) (Koop et al. 2012; Marogna et al. 2012). Although *S. aureus* is the most pathogenic bacterial species and can even cause clinical or gangrenous mastitis, the prevalence of *S. aureus* is less than 15 % in subclinical mastitis which was detected by bacterial culture (Contreras et al. 2003; Hall and Rycroft 2007). Like *S. aureus*, CNS can greatly increase somatic cell count (SCC) in goat milk, but CNS is the most prevalent class of bacteria and occurs at over 50 % in most studies of goat subclinical mastitis (McDougall et al. 2002; Contreras et al. 2007; Bagnicka et al. 2011). After *Staphylococcus*, *Streptococcus* and Gram-negative bacteria are usually considered to be the major pathogens for their severe inflammation, but they are less common in subclinical

Y. Zhao · H. Liu · X. Zhao · Y. Gao · M. Zhang · D. Chen (✉)
College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi Province 712100, People's Republic of China
e-mail: chendekun163@163.com

mastitis in goats (Contreras et al. 2003). In addition, fungal, virus, or *Mycoplasma* infection has occasionally been reported in goat mastitis, especially in clinical mastitis (Ponti et al. 2008; Stuhr and Aulrich 2010; Kumar et al. 2013).

After bacterial infection, somatic cells, including lymphocytes, eosinophils, macrophages, and mainly neutrophils, are greatly increased to defend against and eliminate the invading pathogens in the mammary gland of goats (Bagnicka et al. 2011). A significant correlation between SCC and subclinical mastitis has been shown in previous studies (Haenlein 2002; Souza et al. 2012). As an indirect SCC measurement method, California mastitis test (CMT) has been applied to detect the subclinical mastitis in goats (Persson and Olofsson 2011). The aims of this study were to investigate the prevalence of subclinical mastitis in dairy goats in China with the CMT and to identify the etiological agents in these positive milk samples with the multiplex PCR (mPCR).

Materials and methods

Ethics statement

The animal protocol in this study was reviewed and approved by the Research Ethics Committee of Northwest A&F University according to the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. All the samples were collected from animals with the permission of farm owners.

Farms and animals

In this study, a total of 683 Saanen dairy goats (for milk production) were randomly selected from seven semi-intensive and extensive farms in Shaanxi, Shandong, and Yunnan provinces of China from May to October 2012 (Fig. 1). Only healthy animals without clinical signs of mastitis were included in the study. All selected animals had two to three parities in medium lactation and were milked twice a day by hand.

Milk sample collection

The teat ends were cleaned with 70 % ethyl alcohol, and the foremilk was discharged before sample collection. Approximately 2 ml milk from each udder half was pooled separately into a four-well plastic paddle for CMT determination in the field. For samples showing distinctly positive or strongly positive results in the CMT examination, an additional 10 ml milk was collected from the same animal and stored at −20 °C for mPCR.

California mastitis test

For better evaluating SCC in goat milk, the CMT was performed with a slight modification in detergent (SDS 3.75 %, *w*/*v*; NaOH 1.25 %, *w*/*v*) and pH indicator (bromothymol blue 0.025 %, *w*/*v*) (ZL 200910024340.7, invention patent, China). Briefly, an equal volume of CMT reagent was added to 2 ml of sample milk in a four-well plastic paddle. The paddle was rotated for 20 s. In this procedure, somatic cells were lysed by the detergent, and released DNA could form viscous gel. At the same time, the pH variation, which indicated the level of inflammation, could be evaluated visually with the pH indicator (Hueston et al. 1986). Depending on these reactions, the CMT was scored as 0 (negative), trace, 1 (weak positive), 2 (distinct positive), and 3 (strong positive). A CMT-positive goat was defined as having at least one quarter distinct positive or strong positive.

Development and application of mPCR assay for pathogen detection

Bacterial DNA was extracted directly from milk samples of dairy goats, as described previously (Cremonesi et al. 2006). Briefly, milk samples were centrifuged to pellet the bacteria. After lysis, bacterial DNA was released and bound on silica particles, followed by elution into 100 μl pH 8.0 Tris-HCl buffer (with 1 mM EDTA). DNA samples were measured for quantity and quality using a UV spectrophotometer (Q3000, Quawell, USA).

To identify the pathogens causing subclinical mastitis in dairy goats, four pairs of primers were designed for detection of *Staphylococcus* spp., *Streptococcus* spp., *S. aureus*, and *Escherichia coli* (*E. coli*) (Table 1). Previous studies suggested that in the genus *Staphylococcus*, only *S. aureus* and CNS could infect mammary glands of ruminants and cause subclinical mastitis (Deinhofer and Pernthaner 1995; Taponen and Pyörälä 2009). Therefore, the prevalence of CNS could be indirectly determined by using the formula $L=S-M$, where L is the prevalence of CNS infection, S is the prevalence of infections of *Staphylococcus* spp., and M is the prevalence of *S. aureus* infection. The mPCR conditions were optimized by varying the annealing temperatures and Mg^{2+} concentrations. The optimized mPCR reactions were performed in a total volume of 25 μl, containing 2.5 μl 10×PCR buffer, 25 ng template DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.4 μM of each primer, and 0.05 U *Taq* polymerase (TaKaRa, Dalian, China). Amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 56 °C, 40 s at 72 °C, and a final extension at 72 °C for 10 min. Samples with goat DNA or without genomic DNA were included as controls. The amplified products were analyzed by 2 % agarose gel electrophoresis.

Fig. 1 Location of the selected provinces in China

The specificity and sensitivity of mPCR were evaluated according to Shome et al. (2011) with limited modification. Briefly, the specificity of mPCR was determined by spiking uninfected milk with cultured *Bacillus cereus*, *Enterococcus faecalis*, and *Pasteurella* sp. for use as standards. The sensitivity of mPCR was assessed for standard plate count at 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 colony-forming units (CFU) ml^{-1} of each species. The detection limit was determined as the smallest CFU amount at which a PCR product was still detectable.

After optimization, the mPCR assay was used to analyze 209 milk samples for pathogen identification in subclinical mastitis of dairy goats; these samples were randomly collected from distinct positive or strong positive samples with the CMT from the three studied provinces.

Statistics

Data were analyzed with SPSS (SPSS Inc., v. 16.0, USA). Based on the prevalence of subclinical mastitis, the differences among the selected provinces were analyzed with one-way ANOVA.

Results

The development of mPCR

The mPCR was successfully established to amplify *S. aureus* (*nuc*), *Staphylococcus* spp. (*gap*), *E. coli* (*pho*A), and *Streptococcus* spp. (16S rRNA), with amplicon sizes of 227,

Table 1 Primer sequences and predicted sizes of mPCR products in this study

Organisms	GenBank accession no.	Gene	Oligonucleotide primer (5′-3′)	Amplicon size (bp)
E. coli	FJ546461	*pho*A	F: GGTAACGTTTCTACCGCAGAGTTG R: CAGGGTTGGTACACTGTCATTACG	468
S. aureus	NC_007622	*nuc*	F: TTCGAAAGGGCAATACGCAAAGAGG R: TAACCGTATCACCATCAATCGCTTT	227
Staphylococci spp.	AJ133520	*gap*	F: GCAGCAGCAGAAAACATCATCCCTA R: TTGACGGTCGCCAACTGACATTACA	318
Streptococcus spp.	M58837	16S rRNA	F: ACCAACTGGATGAGCAGCGAACGGG R: TCTCCTCTCCTGCACTCAAGTCTAC	590

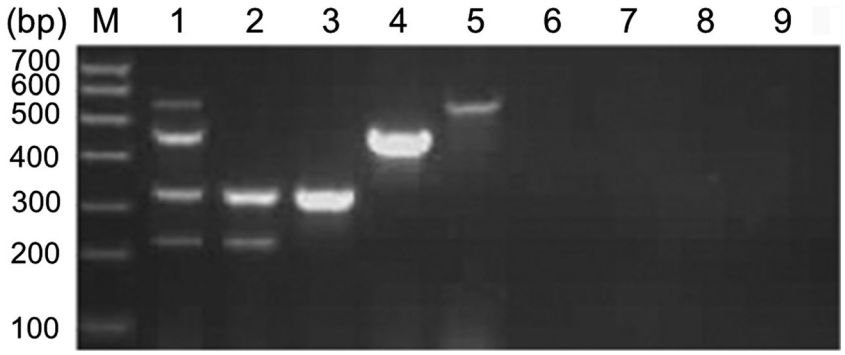

Fig. 2 Specificity of mPCR assay. *Lane M*: 700-bp *Trans* DNA marker I; *lane 1* combination of *E. coli*, *S. aureus*, *S. epidermidis*, *S. chromogenes*, *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*; *lane 2 S. aureus*; *lane 3* combination of *S. epidermidis* and *S. chromogenes*; *lane 4 E. coli*; *lane 5* combination of *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*; *lane 6 Bacillus cereus*; *lane 7 Enterococcus faecalis*; *lane 8 Pasteurella* sp.; *lane 9* negative control

318, 468, and 590 bp, respectively. The specificity of the mPCR assay was confirmed by the absence of amplifications of DNA controls of *B. cereus*, *E. faecalis*, and *Pasteurella* sp. (Fig. 2). The sensitivity of the mPCR assay was 10^2 CFU ml^{-1} for *S. aureus* and *Streptococcus* spp. (Fig. 3a, c) and 10^3 CFU ml^{-1} for *E. coli* and CNS (Fig. 3b, d).

Prevalence of subclinical mastitis in dairy goats in China

Among the collected 683 dairy goats, 313 (45.82 %) goats had a distinct or strong positive CMT reaction in milk samples from unilateral or bilateral udder halves. The positive rate of samples from Yunnan province (36.08 %) was significantly lower than those from Shaanxi and Shandong provinces ($P<0.01$). There was no significant difference in the prevalence of subclinical mastitis between Shaanxi and Shandong provinces (48.92 and 52.67 %, respectively). The prevalence at each farm investigated is listed in Table 2.

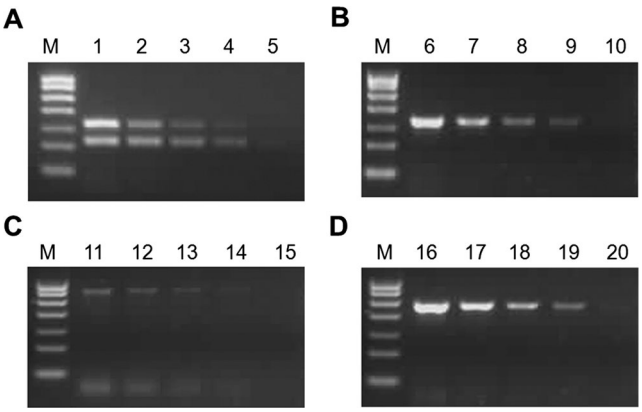

Fig. 3 Sensitivity of mPCR assay. *Lane M* 700-bp *Trans* DNA marker I. **a** *Lanes 1–5*, mPCR products of *S. aureus* at 10^5, 10^4, 10^3, 10^2, and 10^1 CFU ml^{-1}. **b** *Lanes 6–10*, mPCR products of CNS (combination of *S. epidermidis* and *S. chromogenes*) at 10^6, 10^5, 10^4, 10^3, and 10^2 CFU ml^{-1}. **c** *Lanes 11–15*, mPCR products of *Streptococcus* species (combination of *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*) at 10^5, 10^4, 10^3, 10^2, and 10^1 CFU ml^{-1}. **d** *Lanes 16–20*, mPCR products of *E. coli* at 10^6, 10^5, 10^4, 10^3, and 10^2 CFU ml^{-1}

Pathogens causing subclinical mastitis in dairy goats in China

To determine the predominant pathogens causing subclinical mastitis in dairy goats in China, total genomic DNA of 209 milk samples that scored as distinct or strong positive with the CMT were extracted and used for mPCR assays (Table 3). The results from the mPCR indicated that the most prevalent pathogen was CNS (59.52 %), followed by *S. aureus* (15.24 %), *E. coli* (11.43 %), and *Streptococcus* spp. (10.95 %). All of these pathogens could be detected in Shaanxi, Shandong, and Yunnan provinces, and CNS was the most commonly detected pathogen in each province, with prevalence rates of 60.40, 62.82, and 48.39 %, respectively. In addition, a co-infection of both CNS and *E. coli* was observed in one sample from Shaanxi province. In particular, only six milk samples were detected negative with mPCR, and no contaminated sample was found in this study.

Discussion

In this study, a high prevalence of subclinical mastitis in dairy goats in China (45.82 %) was detected with the CMT. Such

Table 2 The prevalence of subclinical mastitis detected by using CMT in dairy goats in China

Province	Location	Farms	No. of samples	No. CMT positive (%)
Shaanxi	Fuping	A	186	94 (50.54 %)
		B	148	70 (47.30 %)
Shandong	Qingdao	C	145	73 (50.34 %)
	Weihai	D	20	11 (55.00 %)
Yunnan	Luxi	E	48	18 (37.50 %)
	Shilin	F	91	30 (32.97 %)
	Kunming	G	45	17 (37.78 %)
Total			683	313 (45.82 %)

Table 3 The prevalent pathogens investigated by using mPCR in this study

Province	Sample size	No. positive pathogens (%)				Total	No. negative pathogens (%)
		Staphylococcus spp.		*E. coli*	*Streptococcus* spp.		
		S. aureus	CNS				
Shandong	78	10 (12.82 %)	49 (62.82 %)	4 (5.13 %)	13 (16.67 %)	76 (97.44 %)	2 (2.56)
Yunnan	31	7 (22.58 %)	15 (48.39 %)	8 (25.81 %)	1 (3.23 %)	31 (100 %)	0 (0.00)
Shaanxi	100	15 (14.85 %)	61 (60.40 %)	12 (11.88 %)	9 (8.91 %)	97 (96.04 %)	4 (3.96)
Total	209	32 (15.24 %)	125 (59.52 %)	24 (11.43 %)	23 (10.95 %)	204 (97.14 %)	6 (2.86)

high prevalence rate might be associated with the detection method employed. Some studies have indicated that CMT is more accurate for excluding mastitis rather than for diagnosing it in goats (Karzis et al. 2007; Petzer et al. 2008). Although CMT is a low sensitive assay, it is significantly correlated with the SCC directly detected by the Fossomatic method, and CMT is still used in most of studies due to its convenience and had been validated in field applications for subclinical mastitis detection in goats (McDougall et al. 2010; Peixoto et al. 2010; Souza et al. 2012). On the other hand, the total positive rate of 45.82 % of subclinical mastitis in dairy goats in China was higher than those previously reported from Sweden and India (18 and 30.2 %, respectively, Persson and Olofsson 2011; Sreeja et al. 2013). The poor milking hygiene and the less prevention awareness of subclinical mastitis might be greatly contributed to this high prevalence. Due to the poor management, the infected goats, including clinical and subclinical mastitis, were usually not separated from the healthy animals, and this contaminative environment and equipment would cause a new infection. The prevalence of subclinical mastitis also varied among the different regions. Yunnan provinces had the lowest prevalence in the investigated provinces in China. A dry and sunny condition, which is unfavorable for the maintenance and transmission of mastitis causing bacteria, could reduce the occurrence of subclinical mastitis in lactating goats (Megersa et al. 2010). This might be a possible explanation for the lowest prevalence in Yunnan province. In this study, only 683 dairy goats with two to three parities in medium lactation in three provinces were selected and detected. To improve the understanding of the situation of subclinical mastitis in China, more samples in most of the areas of China should be collected in the future.

Pathogens have been considered as the main reason for increasing SCC in dairy goat milk (Souza et al. 2012). Accurate identification of pathogens is essential for the control of subclinical mastitis. In addition to bacteriological culture, PCR and mPCR have been explored as rapid and sensitive approaches to identify causing agents for mastitis in dairy animals. Several simplex PCR assays were used to identify *S. aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* in subclinical mastitis in sheep (Guerreiro et al. 2013). And a two-tube mPCR assay was developed to simultaneously detect ten pathogens for subclinical mastitis in cattle, including *S. aureus*, *E. coli*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus sciuri*, *Staphylococcus simulans*, *S. uberis*, *S. dysgalactiae*, and *S. agalactiae* (Shome et al. 2011). Unlike previous reports classifying these pathogens in detail, only four pairs of primers, identified for *S. aureus*, *E. coli*, *Streptococcus* spp., and CNS, were used in this study. Consistent with previous studies, although *S. aureus* has been considered as the most harmful pathogen in mastitis, CNS was the most prevalent species with a prevalence of 59.52 % in our study. *S. epidermidis* and *S. chromogenes* were the dominant species of CNS in subclinical mastitis in dairy goats of the three provinces studied (unpublished data). Given the widespread infection of CNS, a mixed intramammary infection of CNS and *E. coli* observed in our present study is possible, which could be explained by a previous study. Koop et al. suggested that a co-infection caused by a minor and a major pathogen could be considered as the major pathogen infection, e.g., a mixed infection of CNS and *S. aureus* was analyzed as *S. aureus* infection (2011). It might mean that the damage caused by the major pathogen infection is severe enough to neglect the inflammation caused by the minor pathogen infection. It should be noticed that only six milk samples were detected negative with mPCR. Firstly, this result might be associated with the high sensitivity of PCR. By using this technique, a lower concentration of bacteria, the bacteria residing in the immune cells and even the dead bacteria could be detected (Koop et al. 2012). Therefore, more false-positive results would be detected than classical bacteriological culture, which is the gold standard for the diagnosis of subclinical mastitis. Furthermore, this high positive result might be partially explained by the selected samples. Only distinct positive and strong positive milk samples from the CMT were used to bacterial pathogen identification by PCR, and a strong correlation (81.4 %) between bacteriological culture and CMT≥2 had been reported by Peixoto et al. (2010).

In the next step, antibiotic sensitivity assay will be conducted for those bacterial isolates, and antibiotic therapy in goats will be considered. However, McDougall et al. (2010) suggested that antibiotic treatment deed reduce the total bacterial load in the mammary gland, but it cannot improve milk quality and survival rate for goat herds, which results no economic benefits. Therefore, whether to use antibiotics to treat subclinical mastitis in dairy goats in China to improve the economic benefit, animal welfare, and public health is a decision that need be made carefully. In conclusion, subclinical mastitis is a highly prevalent disease in dairy goats in China, and CNS is the predominant pathogen.

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Conflict of interests The authors declare that no conflict of interests exists.

Authors' contributions YQZ, MTZ, and DKC conceived and designed the experiments. YQZ, XDZ, HL, YG, and MTZ performed the experiments. YQZ and HL analyzed the data. DKC contributed reagents/materials/analysis tools. YQZ and HL wrote the manuscript.

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