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RESEARCH ARTICLE

Prevalence, Enterotoxin Gene and Antimicrobial Resistance of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* from Clinical Healthy Dairy Cows

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ABSTRACT

Coagulase- positive *Staphylococcus aureus* (CPS) is a leading cause of both clinical and subclinical bovine mastitis. Moreover, methicillin- resistant *Staphylococcus aureus* (MRSA) have been identified as an emerging mastitis pathogen in dairy cows. In our study, 121 raw milk samples were collected from individual clinical healthy cows from five commercial farms. 52 samples (42.98%) were positively detected as CPS. Fifty-two strains of CPS were isolated and identified using phenotypic and molecular approaches. Their coagulase gene (*coa* gene) amplification showed a single amplicon of a size between 600 and 1000 bp. Five CPS isolated strains from YZ farm, which was resistant to oxacillin and contained a specific 310-bp for the *mec*A gene, were identified as MRSA. The staphylococcal enterotoxin a (SEa) gene was detected in 18 strains. None of the isolates carried the gene SEb, SEc, SEd and SEe. All isolates including the five MRSA isolates were uniformly susceptible to Cephalothin (KF), Ofloxacin (OFX) and Vancomycin hydrochloride (VA) at minimum inhibitory concentrations (MIC). Besides P and CIP, the resistance of these isolates resistances to Oxacillin (OX), rifampicin (RIF), Cefotaxime (CTX), Azithromycin (AZM) and Clindamycin (DA) were relatively low (<20%). Together, our findings demonstrated that there was high prevalence of CPS and ten MRSA contaminations in raw milk from clinical healthy cows.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the primary pathogens that causes intramammary infections in dairy cows (Jørgensen *et al*., 2005; Hussain *et al*., 2012). *S. aureus* causes chronic and contagious clinical and subclinical bovine mastitis. The presence of *S. aureus* in raw milk is caused by either direct excretion from udders (even from clinically healthy cows) or from contamination from the environment during handling and processing of raw milk. MRSA has been reported in bovine mastitis (Vanderhaeghen *et al*., 2010; Hussain *et al*., 2013) and is increasingly a health risk for both humans and animals (Raygada and Levine, 2009). The mechanism for MRSA resistance is the presence of the *mec*A gene, which encodes an altered penicillin binding protein (PBP2a or PBP20), that has a low binding affinity for all beta-lactam antimicrobials (Deurenberg and Stobberingh, 2008). Coagulase-positive *S. aureus* (CPS) are frequent contaminants of milk and raw milk products (Jørgensen *et al*., 2005). Moreover, there is concern over the presence of CPS in milk because it can lead to the production of toxic *Staphylococcus* enterotoxins (SEs) (Freitas *et al*., 2013; Watanabe *et al*., 2009). Five classical enterotoxins (SEa to SEe) have been described (Seo and Bohach, 2007). The SEs are heat-stable proteins, produced by enterotoxinogenic strains of coagulase-positive staphylococci (mainly *S. aureus*) that cause illness, whereas coagulase-negative staphylococci have never been reported to cause foodborne outbreaks of illness.

Despite significant control measures for *S. aureus*, it is difficult to eradicate the intramammary infections caused by this pathogen and *S. aureus* infections remain a substantial economic problem. Antimicrobial therapy is one of the main measures for controlling *staphylococcal* mastitis. However, antimicrobial resistant *S. aureus* is a major public health problem all over the world. Therefore, obtaining data to characterize the antimicrobial

susceptibility of clinical isolates is required not only for therapy but also for monitoring the spread of resistant strains. Furthermore, susceptibility data for *S. aureus* isolates in a particular area or region will also be useful to veterinarians (Kateete *et al*., 2013).

The aim of this study was to investigate the occurrence of CPS, especially MRSA, in clinically healthy cows in large dairy farms located in Jiangsu province of China. We also determined the prevalence SE gene encoded by these isolates and their antimicrobial susceptibility.

MATERIALS AND METHODS

Sampling of raw milk: Five large dairy farms (denoted as YZ, SF, XG, XZ and HA) were enrolled in our study. The herd size varied from 100 to 1000 Holstein cows. The cows whose somatic cell counts (SCC) found in milk were lower than 3×10^5/ml and did not have any visible symptoms were deemed to be as clinically healthy cows and were the subjects of our study. The method of raw milk sampling was done according to Shuiep *et al*. (2009). In brief, the surfaces of the udders of clinically healthy cows were washed with distilled water thoroughly and dried with single towels. The first two streams of milk were discarded. A milk sample (25mL) was collected aseptically, from each teat of clinically healthy cows, and cooled to 4°C immediately. A total of 121 raw milk samples, of which 56 milk samples were from YZ, 34 milk samples were from SF, 11 milk samples were from XG, 10 milk samples were from XZ and 10 milk samples were from HA, were studied.

Isolation and characterization of CPS strains: All raw milk samples were tested for the presence of *S. aureus*. Milk samples were diluted with 225 ml of buffered peptone water (BPW), and then, seeded onto Baird-Parker (BP) supplemented with Egg Yolk Tellurite Emulsion (Qingdao Hope Bio- Technology Co. Ltd., China) and incubated aerobically at 37°C for 24 and 48 h. Those colonies showing typical aspects of staphylococci were subjected to hemolytic properties test. The β-hemolysis positive isolates were subjected to biochemically tests, such as colony morphology, Gram-staining, aerobic mannitol fermentation, catalase reaction and their ability to coagulate rabbit plasma (tube coagulase test). The *S. aureus* ATCC 25923 was used as a control strain.

PCR analysis: DNA extraction of *S. aureus* isolates was performed using a DNA extraction kit (TIANGEN Co. Ltd., Beijing) according to the manufacturer protocol. Polymerase chain reaction (PCR) was performed for amplification of 16S rRNA genes, the 3'end region of *coa* gene encoding coagulase (Aarestrup *et al*., 1995), the methicillin resistance gene (*mec*A) (Zhang *et al*., 2004), and the SE genes (Linage *et al*., 2012). PCR reaction solution consisted of 2.5 μL 10×Buffer, 1μL template DNA, 200μM dNTP, 1.5mM Mg^{2+}, 5.0U Taq polymerase (Takara), and 10μM of each oligonucleotide primer in a final volume of 25 μL. The primers of every gene are summarized in Table 1. *S. aureus* ATCC 25923 was used as a control strain.

Antimicrobial susceptibility testing: All 52 *S. aureus* isolates were tested for susceptibility to a panel of 10 antimicrobial agents using the minimal inhibitory concentrations (MIC) method, following the guidelines of CLSI standards (CLSI 2012). The antibiotics included Penicillin (P), Oxacillin (OX), rifampicin (RIF), Cephalothin (KF), Cefotaxime (CTX), Azithromycin (AZM), Ciprofloxacin hydrochloride (CIP), Ofloxacin (OFX), Vancomycin hydrochloride (VA), and Clindamycin (DA). The dilutions tested ranged from 0.125 to 1024 μg/mL for all antimicrobial agents. *S. aureus* ATCC 25923 was used as the quality control strain.

RESULTS

Prevalence of CPS: 52 of 121 raw milk samples (42.98%) were positive for CPS. Specifically, 23 positive samples came from YZ farm, 17 positive samples were from SF farm, 3positive samples came from HA farm, 4 positive samples came from XZ farm and 5 were from XG farm. The isolated percentage of CPS was 41.07, 50, 30, 40 and 45.45% for YZ farm, SF farm, HA farm, XZ farm and XG farms, respectively (Table 2).

Table 1: Gene targets, primers, annealing temperature and expected length of amplicons for gene amplification

Gene	Primers	Annealing temperature (°C)	Expected amplicon length (bp)	Reference
16S rRNA	F: 5′-GCGGTCGCCTCCTAAAAG-3′ R: 5′- TCCCGGTCCTCTCGTACTA-3′	55	419	GenBank Accession No. X68417
coa	Coag2: 5'-ACCACAAGGTACTGAATCAACG-3' Coag3: 5'-TGCTTTCGATTGTTCGATGC-3'	55	600-1000	Aarestrup *et al.* (1995)
*mec*A	F: 5'-GTAGAAATGACTGAACGTCCGATAA-3' R: 5'-CCAATTCCACATTGTTTCGGTCTAA-3'	55	310	Zhang *et al*., (2004)
SEa	F: ACGATCAATTTTTACAGC R: TGCATGTTTTCAGAGTTAATC	50	544	Linage *et al*.,(2012)
SEb	F: GAATGATATTAATTCGCATC R: TCTTTGTCGTAAGATAAACTTC	50	416	
SEc	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCCA	50	257	
SEd	F: TTACTAGTTTGGTAATATCTCCTT R: CCACCATAACAATTAATGC	50	334	
SEe	F: ATAGATAAAGTTAAAACAAGCAA R: TAACTTACCGTGGACCC	50	170	

Table 2: Distribution and SE genes detection of CPS and MRSA strains isolated from raw milk samples.

Farms	City	No. CPS/ Raw milk samples (%)	*No. mec*A positive (%)	No. MRSA (%)	No. SEa positive (%)
YZ	Yangzhou	23/56(41.07)	18(34.61)	5(9.61)	8(15.38)
SF	Nanjing	17/34(50)			9(17.31)
XG		5/11(45.45)			
XZ	Xuzhou	4/10(40)			
HA		3/10(30)			1(1.92)
Total		52/121(42.98)	18(34.61)	5(9.61)	18(34.61)

SE= staphylococcal enterotoxin; SEa, SEb, SEc, SEd and SEe genes were five classical SE; No strains were positive for SEb, SEc, SEd and SEe genes.

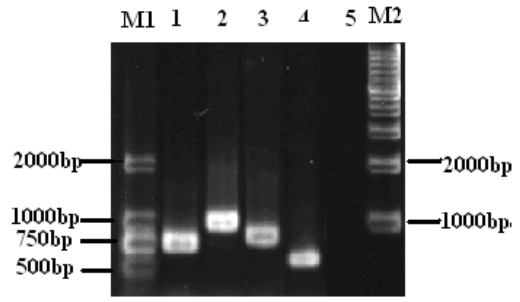

Fig.1: Representation of PCR products of *coa* gene found among all CPS isolates. M1-molecular marker 2000bp; lane1-4-positive CPS isolates; lane 5 – negative control; M2- molecular marker 1kb.

Table 3: Antibiotic resistance of CPS isolates according to the MIC breakpoints defined by CLSI

Antimicrobial agents	MIC Standard (ug/mL)	Resistant	
		No.	%
P	S≤0.12 R≥0.25	49	94.2
OX	R≥4	5	9.6
RIF	S≤1 I=2 R≥4	9	17.3
KF	S≤8 I=16 R≥32	0	0
CTX	S≤8 I=16-32 ≥64	4	7.7
AZM	S≤2 I=4 R≥8	8	15.4
CIP	S≤1 I=2 R≥4	27	51.9
OFX	S≤1 I=2 R≥4	0	0
VA	S≤2 I=4-8 R≥16	0	0
DA	S≤0.5 I=1-2 R≥4	10	19.2

Note: S=sensitive, I=inter, R=Resistance, P= Penicillin, OX=Oxacillin, RIF=rifampicin, KF=Cephalothin, CTX=Cefotaxime, AZM= Azithromycin, CIP=Ciprofloxacin hydrochloride, OFX=Ofloxacin, VA=Vancomycin hydrochloride, DA=Clindamycin.

CPS Characterization: 52 CPS isolates were characterized as CPS. Each CPS strain came from every positive sample. Table 2 shows the characterization of CPS and the detection of genes encoding toxins SEa, SEb, SEc, SEd and SEe. The morphology of these isolates on BP agar plate were round, smooth, moist, black or gray-black, the sizes of which were 1mm to 2mm in diameter, and most colonies surrounded with an opaque halo. Colonies of these 52 CPS isolates were Gram-positive and exhibited typical characteristics of β-hemolysis on blood agar. These strains were able to coagulate rabbit plasma (tube coagulase test). All 52 CPS isolates exhibited an amplified 419-bp band using primers for 16S rRNA (data not shown). Of the 52 CPS isolates, only 18 (34.61%) were positive for SEa gene, while none were positive for SEb, SEc, SEd and SEe genes. Amplification of the *coa* gene with the coag2 and coag3 primer yielded a single fragment of a size between 600 and 1000 bp. The size of *coa* fragments were approximately 1000 bp for 17 CPS isolates (32.69%), 8000bp for 20 CPS isolates (38.46%), 750bp for 11 CPS isolates(21.15%), and 600bp for 4 CPS isolates (7.69%) (Fig. 1). The *coa* gene sizes of ATCC25923 were approximately 1000 bp. No amplification product was obtained from the coagulase-negative *S. aureus*.

MRSA isolates identification: Isolates were characterized as MRSA if it showed the presence of the *mec*A gene and displayed oxacillin resistance at a MIC of ≥4ug/mL. Eighteen isolates were *mec*A gene positive. Only five CPS isolates (9.61%) of 52 CPS isolates were identified as MRSA (Table 2). They were YZ-15, YZ-34, YZ-42, YZ-49 and YZ-56, which were all isolated from the YZ farm. The size of the *coa* gene fragments were all approximately 750 bp.

Antimicrobial susceptibility: The results of the MIC method for the 52 CPS isolates are displayed in Table 3. Of the 52 CPS isolates, 95.2 and 51.9% were resistant to P and CIP, respectively, indicative of high rates of resistance to these antimicrobials. On the other hand, relatively lower rates of resistance were noted for OX, RIF, CTX AZM and DA (9.6, 17.3, 7.7, 15.4 and 19.2%, respectively). Five MRSA isolates were resistant to OX, P, and CIP and MRSA isolates (YZ-34, YZ-42 and YZ-49) were also resistant to RIF. All of the isolates including the 5 MRSA isolates were uniformly susceptible to KF, OFX and VA.

DISCUSSION

Several studies examining clinical or subclinical mastitis have shown that *S. aureus* is one of the most common, and contagious pathogens responsible for development of bovine mastitis in China and other countries (Boynukara *et al*., 2008; Delgado *et al*., 2011; Szweda *et al*., 2012; Khan *et al*., 2013; Szweda *et al*., 2014). MRSA is a prominent health risk for both humans and animals worldwide; however, to the best of our knowledge, there is no published information regarding the occurrence of CPS and MRSA in raw milk from clinical healthy dairy cows in China. Thus, this report is the first survey on CPS and MRSA in raw milk using standardized procedures in east of China. An outstanding characteristic of *S. aureus* isolates is the expression of coagulase (*coa*), which binds to prothrombin and alters the enzyme's active site via the insertion of its N-terminal residues into the activation pocket, thereby promoting the cleavage of fibrinogen to fibrin, thus forming clots when inoculated into human blood (Panizzi *et al*., 2006). In our study, all 52 CPS isolates from 121 raw milk sample (42.98%) were tested positive in the tube test for coagulase production, which was slightly higher than the occurrence of CPS in raw milk in Italy (38.4%) (Normanno *et al*., 2005). However, most *S. aureus* strains in the study of Normanno produced SEc (28.1%), which was different to our findings. In a study by Bianchi *et al*. (2014), they showed that CPS was isolated in 481(39%) of 1245 dairy samples (848 of raw milk and 397 of dairy products), which was a little higher than our data. They also demonstrated that 255 (53%) coagulase-positive

staphylococci (mainly *S. aureus*) were positive for one or more enterotoxins (SEs), which was more prevalent than that seen in our study. Furthermore, Makita *et al*. (2012) observed a significant increase in the occurrence of milk from farms from farms (43.5%) and milk collection centers (72%) that were contaminated with *S. aureus* because of either mammary infection or faulty handling. Therefore, if the animal has direct contact with the reservoirs of pathogens or indirect contact via fomites, the probability of udder infection increases. Raw milk contaminated with CPS can infect the consumer in cases of inefficient pasteurization or when raw milk is used illegally for the production of cheese or other dairy product, thereby adversely effecting human health.

Molecular detection of the *mec*A gene and oxacillin resistance has been adopted as a means for detecting MRSA strains. Our results showed that there is a low prevalence of MRSA (4.13%) in raw milk. In the study by Lee *et al*. (2003), the incidence of MRSA in milk was also low; 265 isolates of *S. aureus* were obtained from 894 milk samples with 12(1.3%) characterized as MRSA. However, because the five MRSA isolates were isolated exclusively from YZ dairy farm, the MRSA rates of the YZ dairy farm was very high (21.74%). Because MRSA can easily spread between animals, this suggests that immediate remedial action is needed when MRSA rates increase on dairy farms.

In this study, the prevalence of CPS isolates derived from clinically healthy cows and their resistance to OX, RIF, CTX, AZM and DA were relatively low (<20%), which is lower than those reported in Korea (29%) and India (35%) (Moon *et al*., 2007; Kumar *et al*., 2010). However, the rate of Persistent CPS isolates reached 94.2%. These data were higher than those from Korea (88.9%) and European countries and the United States (32.4%) (Moon *et al*., 2007). This finding might indicate that β-lactam antibiotics, such as P, have been widely used for treatment of *S. aureus* infections in the sampled area. It was, however, encouraging that all isolates recovered from healthy cows were susceptible to KF, OFX and VA. Thus, these data underline the need for a policy on the judicious use of antimicrobials.

Conclusions: On the basis of phenotypic and molecular approaches, a high prevalence of CPS isolates (42.98%) and five MRSA strains were identified in raw milk from clinically healthy cows. Furthermore, the SEa gene was encoded in 18 strains (34.61%). Although all CPS isolates were sensitive to the antimicrobials KF, OFX, and VA, 94.2 and 51.9% were resistant to P and CIP, respectively. Due to the scarcity of research of CPS and MRSA isolates from clinically healthy cows, continued epidemiological surveillance is essential to gain knowledge on the prevalence, enterotoxin gene and resistance patterns responsible for bovine mastitis, due to its potential implications in both animal and human health.

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Author's contribution: HB conceived and designed study. HB, HZ and YZ executed the experiment. LZ and RW analyzed the data. All authors approved the final version.

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