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The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd

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ABSTRACT

Staphylococci are the leading pathogens of bovine mastitis which is difficult to control. However, the published data on the prevalence of staphylococcal species, virulence and antibiotic resistance genes in bovine mastitis from China are limited. In this study, 104 out of 209 subclinical mastitis milk samples from a single Chinese dairy herd were cultured-positive for staphylococci (49.8%), which were further identified as coagulase-positive staphylococci (CPS) or coagulase-negative staphylococci (CNS). According to the partial *tuf* and/or 16S rRNA gene sequence, the 28 CPS isolates were confirmed to be *Staphylococcus aureus* (26.9%), and 76 CNS isolates were assigned to 13 different species (73.1%) with *Staphylococcus arlettae, Staphylococcus sciuri*, *Staphylococcus xylosus* and *Staphylococcus chromogenes* as the dominant species. In the 28 *S. aureus* isolates, the most prevalent general virulence genes were *coa, Ig* and *eno* (100%), followed by *hla* (96.4%), *hlb* (92.9%), *fib* (92.9%), *clfA* (89.3%), *clfB* (85.7%) and *nuc* (85.7%). Both exotoxin and biofilm-associated genes were significantly less prevalent than the previously reported. Although 19 different virulence gene patterns were found, only one was dominant (32.1%). The prevalence of *blaZ* (82.1%) or *mecA* gene (35.7%) was much higher than the previously reported. In the 76 CNS isolates, the virulence genes were significantly less prevalent than that in the *S. aureus* isolates. Among the 4 main CNS species, *S. chromogenes* (n = 12) was the only species with high percentage (75%) of *blaZ* gene, while *S. sciuri* (n = 12) was the only species with the high percentage (66.7%) of *mecA* gene. The most of antibiotic resistance genes were present as multi-resistance genes, and the antibiotic resistances were attributed by different resistance genes between resistant *S. aureus* and CNS isolates. These data suggest that the prevalence of staphylococcal species, virulence and antibiotic resistance in the mastitis milk from the Chinese dairy herd are different from the previously reported, and that the herd- or farm-based diagnosis of staphylococcal bovine mastitis is required.

1. Introduction

Staphylococci are the bacteria most commonly isolated from bovine mastitis [1]. In mastitis diagnosis, staphylococci can be divided into coagulase-positive (CPS) and coagulase-negative (CNS) based on the ability to coagulate rabbit plasma. The major pathogen *Staphylococcus aureus,* including a broad range of genotypes with distinct pathogenic and epidemiologic characteristics, can cause clinical, but often subclinical mastitis [2]. Although CNS species are traditionally considered as minor mastitis pathogens [3], they have become the dominant pathogens of subclinical or mild clinical infections in many well-managed dairy herds [4,5]. This group of staphylococci consists of more than 40 different species and subspecies of which a dozen are commonly found in milk of dairy cows [6,7]. Although a number of studies have been conducted to identify reservoirs of CNS, the epidemiology CNS mastitis is still unclear, in China in particular [4].

Various virulence factors have been found in *S. aureus* from bovine mastitis, including haemolysins (HLA and HLB), leukocidin, exfoliative toxins (ETA to ETD), staphylococcal enterotoxins (SEs), toxic-shock syndrome toxin-1 (TSST-1), and biofilm formation [8,9]. Except for their pathogenic role in bovine mastitis, some

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toxin-producing *S. aureus* strains pose a risk for humans and animals [10]. These toxin genes are mainly located on the mobile genetic elements, and thus can spread among staphylococcal isolates or species [11,12]. Although these toxin genes were originally identified in *S. aureus* isolates, some of them have also been detected in a variety of CNS species from the mammary glands of cattle and other ruminants [13], which may become a possible reservoir of toxin genes typically identified in *S. aureus*. However, only few studies have so far focused on the virulence factors of CNS isolated from bovine mastitis [3].

S. aureus has the potential to develop resistance to almost all antimicrobial agents [14]. Due to the extensive use of antibiotics as bovine mastitis antibacterial agents, the antimicrobial resistance developed by staphylococci is one of main reasons for low cure rate of mastitis [14,15]. More importantly, the emergence of methicillin-resistant *S. aureus* (MRSA) strains has become a major public health concern [16]. Furthermore, CNS species tend to be more resistant to antimicrobials than *S. aureus*, and easily develop multi-resistance [17]. Therefore, the investigation into antimicrobial resistance in the staphylococci from dairy cows is important not only for bovine mastitis control, but also for public health. However, the published data on the difference in antibiotic resistance genes among CNS species are limited, from China in particular [18,19].

In the light of limited data on the genotypic identification of bovine mastitis staphylococci from China, the objective of this study was to investigate the prevalence of staphylococcal species, virulence and antibiotic resistance in subclinical mastitis milk from a single dairy cow herd.

2. Materials and methods

2.1. Herd and cows

During the period from June 2012 to June 2014, a field study was conducted on a single dairy farm in Jiangsu Province, China. The dairy farm was well-managed and self-contained which had comparable characteristics reflecting the general situation on Chinese middle-sized dairy farms. The herd size was 748 Holstein cows with an average production of 85,000 kg of milk/cow per year. On the dairy farm, cows were housed in freestalls with concrete floors and sawdust bedded cubicles. Sawdust bedding was removed 2 to 3 times a day, and replaced by fresh sawdust from a stock stored indoors. Postmilking teat disinfection was practiced by standard iodine dipping. Dry cow therapy was practiced with ampicillin and cloxacillin enzathine injection. Incidence rates of clinical and subclinical mastitis cases during the past two years were 5% and 18%, respectively. The clinical mastitis cows were treated in rotation with kanamycin, cefazolin and compound Chinese medicine consisting of *Flos lonicerae japonicae*, *Radix scutellariae*, *Taraxacum platypecidum*, *Radix glycyrrhizae*, and *Angelica dahurica*. During the period of this study, 6 cohorts of 35 subclinical mastitis cows were randomly selected for milk sample collection.

2.2. Sample collection

According to the estimation of somatic cell count (SCC) using California mastitis test (CMT), a mammary quarter was considered with subclinical mastitis when the SCC was greater than 250,000/ml in individual quarter foremilk without overt clinical signs [20]. Quarter milk samples were collected aseptically at bimonthly intervals ($n = 6$) from the cohort cows according standard procedures [21]. Samples were transported immediately on ice to the laboratory for bacteriological examination.

2.3. Staphylococcal isolation

Bacteriological culture of milk samples and bacterial identification were done as recommended by the National Mastitis Council [21]. Briefly, 0.1 ml of each milk sample was spread on each sheep blood agar plate and incubated for 24 h at 37 °C. Phenotypic differentiation of bacterial species was done as previously described [22]. Staphylococci were identified presumptively based on colony morphology, Gram's stain, and catalase test. *S. aureus* was differentiated from other *Staphylococcus* spp. based on morphology, pigmentation, hemolysis, Coa tube test and thermonuclase (Nuc) activity. All of non-*S. aureus* staphylococci were a priori considered as CNS. For milk samples yielding at least 3 CNS colonies, 2 colonies were picked and transferred to self-made tryptone soy agar (TSA) for further identification. When more than one type of CNS colony was present, more colonies were picked. The TSA plates were incubated for 18 h at 37 °C. The mammary quarter was considered *S. aureus* and CNS infected when the number of bacterial colonies was ≥50 and 250 cfu/ml, respectively [23].

2.4. Genotyping of staphylococcal isolates

Each colony was picked from TSA plates and grown overnight at 37 °C in LB both. Staphylococcal genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Shanghai, China) according to the manufacturer's instruction. The concentration of DNA was adjusted to 100 ng/μl by addition of deionized water. Staphylococcal isolates were genotypically identified by PCR amplification of *tuf*, *coa* and *nuc* genes as previously described [24]. PCR amplification was performed in 25 μl volumes using 50 ng of DNA template, 2.5 U of rTaq DNA polymerase (TaKaRa, Dalian, China), 1 × Taq buffer, 0.25 mM dNTP mix, 1.5 mM $MgCl_2$, 0.1 μmol of each primer. The primer sequences for amplification of *coa* and *nuc* genes are listed in Table 1. The *tuf* gene segments were amplified using the primer pair of 5′-GCCAGTTGAGGACGTATTCT-3′ and 5′-CCATTTCAGTACCTTCTGGTAA-3′.

2.5. Identification of staphylococcal species

Staphylococcal species were differentiated first by sequencing the PCR products of partial *tuf* gene segments. PCR products were purified using High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instruction, and submitted to forward and reverse sequencing. The generated sequences were searched against staphylococcal *tuf* gene sequences in GenBank, and a sequence identity of ≥98.0% was used as the rule for speciation [24]. For the non-definitive strains, 16S rRNA genes were amplified with primer pair of 5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-CCGTCAATTCMTTTRAGTTT-3′ for sequencing and species discrimination [13].

2.6. Detection of virulence genes

All staphylococcal isolates were tested by PCR for the presence of virulence genes (Table 1). The *coa* gene coding for Coa [25], *spa* gene for protein A [25], *Ig* gene for Ig-binding protein [26], *map* gene for MHC class II analog protein [27] or *bap* gene for biofilm-associated protein [28] was amplified by PCR as previously described. The other virulence genes were amplified by duplex [28–31] or multiplex PCR [32–35] as previously described. The primer sequences for amplification of virulence genes are listed in Table 1.