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Clinical Mastitis Caused by Methicillin-resistant *Staphylococcus aureus* in a Heifer

Fei Teng[1], Xin Wang[1], Ting Ting Li[1], Shi Yu Liu[1], Chun Yun Wan[1*], Xiao Shan Li[1*] and Feng Li Yang[1*]

[1]College of Animal Science, Yangtze University, Jingzhou, People's Republic of China.

Authors' contributions

This work was carried out in collaboration between all authors. Author FLY designed the study. Author FT wrote the first draft of the manuscript. Authors XW and TTL practically conducted the study. Author SYL helped in data collection and managed the literature searches. Authors CYW and XSL were jointly supervised the whole research work from the beginning to finish and also check the final draft. All authors were read, check and finally approved the final manuscript.

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Short Communication

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ABSTRACT

Emergence of methicillin-resistant *Staphylococcus aureus* among bovine mastitis is the major cause of frequent therapeutic failure and a cause of concern for veterinary practitioners. The current study describes a case of heifer mastitis caused by the isolates of the methicillin-resistant *S. aureus*. On the 24th day of postpartum, the right hind quarter of the heifer was shown clinical mastitis, isolate and cultivate the pathogens in milk, and confirmed the bacterial was methicillin-resistant *Staphylococcus aureus* by PCR the 23S rRNA and mecA gene and antimicrobial susceptibility tests. The MRSA were resistant to penicillin, ampicillin, cefazolin, gentamicin, erythromycin, and trimethoprim/sulfamethoxazole. The heifer was treated with Chinese medicine Ruyanxiao twice a day for 5 days, and the symptoms disappeared and CMT score was 0. This article is the first report of the occurrence of mastitis caused by methicillin-resistant *Staphylococcus aureus* in Holstein heifer.

**Corresponding author: Email: wanchunyun@yangtzeu.edu.cn, tearfirs@qq.com, fengli168@qq.com, yangfengli@yangtzeu.edu.cn;*

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1. INTRODUCTION

Staphylococcus aureus is one of the most contagious pathogens causing bovine mastitis in many countries [1]. Mastitis caused by *S. aureus* is characterized by subclinical mastitis and long-term persistent infection, and the curative effect of antimicrobial therapy is low. Therefore, mastitis due to *S. aureus* spreads insidiously and is very difficult to prevent and cure [2]. Bovine mastitis due to methicillin-resistant *S. aureus* (MRSA), well known as a typical multidrug-resistant organism, has been reported in all parts of the world and has attracted a great deal of attention [3-4]. Notably, MRSA possesses the mecA gene encoding PBP2a, which is a modified penicillin-binding protein that plays a role in methicillin resistance. Moreover, methicillin resistance, could complicate the treatment of bovine mastitis caused by *S. aureus* [5]. Indeed, this mechanism also confers resistance to almost all types of beta-lactam antibiotics still commonly used in mastitis treatment.

De Vliegher et al. reported that *S. aureus* is isolated less frequently from heifers than CNS (coagulase-negative staphylococci) [6]. Mastitis in heifers poses a potential threat to milk production and udder health since development of the milk secretory tissue occurs mainly during first pregnancy, affecting future lactations [6]. Heifer mastitis has been extensively studied over the past decades [7], but it still remains unclear how heifers are infected with the etiological agents of mastitis. Infection may be caused by bacteria that are part of the normal teat skin flora and oral cavity of heifer calves that suck on each other, or by microorganisms present in the environment of these animals (bedding, manure) that are transmitted by flies. Another hypothesis is the transmission of these pathogens from older cows kept together with heifers [8].

This paper describes a case of heifer mastitis caused by the isolates of the methicillin-resistant *S. aureus*. Details that include clinical symptom of the heifer, the Isolation and identification of pathogen, the antimicrobial susceptibility tests, and treatment measures.

2. PRESENTATION OF CASE

2.1 History and Clinical Examination

A 2-year old Holstein heifer gave birth to a clinical healthy female calf in Yangtze dairy farm, which located in Hubei province, China. The heifer was machine-milked and milked two times a day during the postpartum, and the diets consisted of hay, straw, and silage. On the 24th day of postpartum, the right hind quarter was enlarged and pain on palpation of the teat. The milk secretion from the right hind quarter becomes watery, and California Mastitis Test (CMT) score was done as the method described by Yang et al. [9] and the CMT score was 3+. The heifer was having normal feed and water intake. Rectal temperature was 38.8℃ which was found within normal physiological range. No other abnormalities were detected during clinical examination. Based on these observations, the heifer was suspected to be infected with mastitis.

2.2 Isolation and Identification of Pathogen

The right hind teat was cleaned and wiped with 70% alcohol solution before taking the sample. The first three to five squirts of milk were discarded and approximately 10 mL of milk was collected in a sterile tube. And then the sample was kept in ice box during transport to the laboratory. For the bacteriological examination, about 0.01 mL of milk was plated onto a blood agar medium containing 5% ovine blood and MacConkey agar, and the sample was applied using the streaking technique. The plate was then incubated under aerobic conditions at 37℃ and observed after 24, 48, and 72 h of incubation.

The isolate was identified based on morphological characteristics (coloring, size, hemolysis), Gram staining, and biochemical tests (production of coagulase, catalase, and DNase; fermentation of glucose, maltose, mannitol; and hydrolysis of esculin). To further confirm the identification of *S. aureus*, we used the primer 23S rRNA, which was previously described by Yang et al. [10]. Staphylococcus DNA was extracted using the bacteria genomic DNA purification kit (Tiangen BioTech, Beijing) according to the manufacturer's instructions. A pair of primers was designed (primer-1, 5'-ACG GAG TTA CAA AGG ACG AC-3'; primer-2, 5'-AGC TCA GCC TTA ACG AGT AC-3'). The PCR program contained initial denaturation at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 30s, annealing at 57℃ for 30s, and primer extension at 72℃ for 1 min; and a final extension step at

72℃ for 5 min. The PCR product was analyzed using electrophoresis gel with 1.5 % agarose, stained with ethidium bromide solution, and viewed under ultraviolet light. The agarose gel shows a 1250 base pair (bp) fragment, and confirmed that the strain isolated from milk sample was *S. aureus*.

2.3 Confirmation of MRSA

Methicillin Resistant *Staphylococcus aureus* was tested for presence of mecA gene by PCR. The gene target was amplified with primers mecA1 (5'-AAA ATC GAT GGT AAA GGT TGG-3') and mecA2 (5'-AGT TCT GCA GTA CCG GAT TTG-3'), described by Murakami et al. [11]. The PCR program contained initial denaturation at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 30s, annealing at 55℃ for 30s, and primer extension at 72℃ for 1 min; and a final extension step at 72℃ for 5 min. The PCR product was analyzed using electrophoresis gel with 1.5 % agarose, stained with ethidium bromide solution, and viewed under ultraviolet light. The agarose gel shows a 533 base pair (bp) fragment, and confirmed that the *S. aureus* contain mecA gene and which was MRSA.

2.4 Antimicrobial Susceptibility Tests

Disc diffusion test was used to test the susceptibility of the *S. aureus* isolate according to the guidelines of Clinical and Laboratory Standards Institute [12]. Nine commonly used antibiotics including penicillin (10 µg/disk), ampicillin (10 µg/disk), cefazoline (30 µg/disk), amikacin (30 µg/disk), gentamicin (10 µg/disk), erythromycin (15 µg/disk), ciprofloxacin (5 µg/disk), trimethoprim / sulfamethoxazole (1.25/23.75 µg/disk) and chloramphenicol (30 µg/disk) (Hangzhou Microbial Reagent Co., Ltd, China). The results of the antibiotic resistance showed that the MRSA were resistant to penicillin, ampicillin, cefazolin, gentamicin, erythromycin, and trimethoprim/ sulfamethoxazole. While, it was susceptible to amikacin, and intermediate to ciprofloxacin and chloramphenicol.

2.5 Treatment

The milk from the right hind quarter of the heifer was drained out completely and then treated with Chinese medicine Ruyanxiao (dosed at 15 ml intra-mammary injection and slightly massaging for 1 min) at 12 hourly intervals for 5 days. The main ingredient of Ruyanxiao is the extract of Tokyo violet herb, honeysuckle flower, Angelica sinesis, Angelica Dahurica, and white wax [13]. Post-treatment assessment was carried out after 5 days based on milk SSC by CMT and bacterial isolation.

3. DISCUSSION

Bovine mastitis is considered as the most common, economically unbearable and complicated disease in dairy animals worldwide [14]. *S. aureus* is one of the leading pathogens causing intramammary infections in dairy cows, and inducing clinical and subclinical mastitis [15]. In previous reports, *S. aureus* was initially identified on the basis of phenotypic characteristics including morphological features (coloring, size, hemolysis), Gram staining, and biochemical tests (production of coagulase, catalase, and DNase; fermentation of glucose, maltose, mannitol; and hydrolysis of esculin) [16]. Recently identification of isolates used to be confirmed by molecular techniques, such as the PCR of 23S rRNA gene of *S. aureus* [10].

Multi-drugs resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [17]. Considering the multiple antibiotic resistance, the rapid and correct detection of MRSA strains must be performed to select appropriate antibiotic regimens. However selected methods detecting MRSA strains must be useful, reliable, simple and rapid. Several phenotypic methods such as oxacillin disk diffusion test, agar plate screen, the microbroth dilution and the E-tests have been used to detect the MRSA isolates [18]. Phenotypic-based identification and susceptibility testing methods are time consuming and most have inherent limitations [19]. However, PCR-based methods have shown to be a rapid and reliable approach for the identification and genotypic characterization of these organisms. mecA-based PCR methods has accepted as "gold standard" [18].

β-Lactam antibiotics are commonly used in cattle mastitis treatment. The highest penicillin resistance was reported to be in Ireland (71.4%) and England (67.3%) within the European countries followed by 50% in USA [20]. It is extremely important to determine the antibiotic sensitivity of the *S. aureus* isolates from bovine mastitis, which can be helpful in choosing the most suitable drug for the treatment of the disease.

It is well reported that emergence of drug resistance is the consequence of the improper use of antimicrobials [21-22]. Such as lack of post-milking teat dipping and udder towels were used on more than one cow. In addition, the indiscriminate use of antibiotics without proper identification of the causative pathogen and drug sensitivity testing in mastitis cases, the emergence of multidrug resistant pathogens has accelerated [23].

Bovine mastitis due to MRSA, well known as a typical multidrug-resistant organism, has been reported in all parts of the world and has attracted a great deal of attention [24]. However, There are few reports of MRSA mastitis in dairy heifers. The multidrug-resistant phenotype of MRSA strains and their intrinsic β-lactam resistance make them difficult and costly to treat [25]. To effectively control contagious MRSA-associated mastitis in dairy herds, the general recommendation is to implement procedures to prevent the spread of bacteria mainly at the time of milking, including good udder hygiene and pre- and postmilking teat disinfection with an effective teat antiseptic. In addition, all quarters should be infused with antibiotics approved for use in nonlactating cows after the last milking of the lactation period to eliminate existing infections and prevent new infections during the early dry period, when the udder is highly susceptible to new infection.

4. CONCLUSION

On the 24th day of postpartum, the right hind quarter of the heifer was shown clinical mastitis, isolate and cultivate the pathogens in milk, and confirmed the bacterial was methicillin-resistant *Staphylococcus aureus* by PCR the 23S rRNA and mecA gene and antimicrobial susceptibility tests. The heifer was treated with Chinese medicine Ruyanxiao twice a day for 5 days, and the symptoms disappeared and CMT score was 0.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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