International Journal of Veterinary Science and Medicine xxx (xxxx) xxx–xxx

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Contents lists available at ScienceDirect

International Journal of Veterinary Science and Medicine

journal homepage: www.elsevier.com/locate/ijvsm

Full Length Article

# Molecular characterization of *Staphylococcus aureus* strains in bovine mastitis milk in Bangladesh

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ARTICLE INFO

*Keywords:*
Bangladesh
Bovine mastitis
Characterization
Multidrug resistant
Plasmids
*Staphylococcus aureus*

ABSTRACT

*Staphylococcus aureus* is a common causative agent of bovine mastitis in dairy herds worldwide. This study was designed to assess the prevalence of mastitis in cows through screening tests and molecular characterization of *Staphylococcus aureus* strains. Out of 175 randomly screened cows, mastitis was detected in 50 cows by California Mastitis Test (CMT), and from those mastitic cows, 200 quarter milk samples were collected for subsequent culture and PCR based identification. The herd, cow and quarter level prevalence of mastitis was 73.3, 28.6 and 29.5% respectively, and subclinical mastitis (SCM) was the predominant type in all cases. According to bacteriology the overall prevalence of herd, cow and quarter level *Staphylococcus aureus* mastitis was 72.7, 74.0 and 62.0%, respectively, and the pathogen was mostly associated with clinical mastitis (CM). Cows breed, parity, daily milk yield, regular teat dipping, and dry cow therapy were significantly associated ($P < 0.05$) risk factors for mastitis onset. This study identifies 145 *Staphylococcus aureus* isolates which varied greatly with the categories of mastitis (higher in CM), udder quarter location (highest in right rear quarters), and to a lesser extent in the study areas ($P < 0.05$). Antimicrobial susceptibility testing revealed that 79.3% *Staphylococcus aureus* strains were resistant to at least one antimicrobial, 49.0% to two or more antimicrobials, and clinical isolates showed more resistance to all tested antibiotics. The highest resistance rate was found to oxytetracyclin, and no resistance to ceftriaxone and azithromycin. Seven enterotoxin gene profile were detected in the tested isolates, and *mecA* was found in 20.0% isolates indicating the emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA). The isolates were carrying genes in combination, and were found higher in SCM cases. In this study, plasmids (> 23 kb to 2.9 kb) were detected in 70.3% strains, and 54.9% plasmid bearing strains were multiple drug resistant (MDR). Thus, the high prevalence of *Staphylococcus aureus* mastitis is an important concern for diary industry of Bangladesh since the strains of this pathogen is becoming more resistant to commercially available antimicrobials, and this is an alarming concern for both animal and public health.

## 1. Introduction

Mastitis has a profound impact on dairy production, milk quality, animal health and welfare, and causes considerable economic losses to the dairy holders [1]. *Staphylococcus aureus* is most frequent cause of mastitis in dairy animals, which is often difficult to cure and is prone to resurgence [2,3]. This pathogen is an increasingly recognized and most frequently isolated etiology of bovine mastitis in most countries [4]. Most of the dairy animal researchers consider this organism as the true mastitis pathogens with important virulence factors [3], a high level of antimicrobial resistance [5], and the ability to cause chronic infections [4,6]. Intramammary infections (IMIs) caused by this bacterium are highly transmittable, especially during milking [7]. Once established, this fearsome pathogen usually does not respond to antibiotic treatment, and in most cases treatment is associated with poor success leading to a relatively high culling rate [8]. Furthermore, the treatment efficacy against this organism is usually disappointing since it causes great damages in the glandular tissues of udder, and thus, most of the antimicrobials are not able to penetrate all infected sites [9,10]. This bacterium also suppresses phagocytosis and cell mediated immunity, and produces an enzyme that inactivates most penicillin based treatments [11]. In recent years, the emergence and spread of antimicrobials resistant *Staphylococcus aureus* strains, especially multidrug resistant (MDR) strains have become a major public health concern [12].

Peer review under responsibility of Faculty of Veterinary Medicine, Cairo University.
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https://doi.org/10.1016/j.ijvsm.2018.03.008
Received 24 February 2018; Received in revised form 29 March 2018; Accepted 29 March 2018
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Please cite this article as: Hoque, M., International Journal of Veterinary Science and Medicine (2018), https://doi.org/10.1016/j.ijvsm.2018.03.008

Studies from Asian countries also reported *Staphylococcus aureus* as the chief etiologic agent of mastitis in cattle and buffalo [13]. Cumbersome prevention and control of mastitis caused by this bacterium can be achieved through proper isolation, and characterization of the strains, segregation of the infected animals, dry cow therapy, treatment of clinical cases during lactation and culling program. Thus, steadfast and speedy methods for detection of *Staphylococcus aureus* in mastitic milk samples are crucial for the control of this disease, and economically sound udder health management [14]. However, phenotypic characterization of *Staphylococcus aureus* is no longer beneficial in controlling mastitis caused by this organism since inter-strain variations exist in terms of virulence potential [1,14]. Molecular diagnostic methods like DNA-based mastitis diagnostic system have already been introduced for routine use in the dairy herds [15]. Recently, PCR has become a very popular molecular technique, especially for the detection and identification of bacteria in mastitic milk by targeting their specific genes in the DNAs [16,17]. Therefore, the present study was designed to estimate the prevalence of bovine mastitis at herd-and cow level, and characterize the strains of *Staphylococcus aureus* in milk from cows having mastitis through conventional bacteriology and molecular approaches.

## 2. Materials and methods

### 2.1. Ethical approval

All the procedures of the study were performed under the approval of Bangabandhu Sheikh Mujibur Rahman Agricultural University's Animal Experimentation Ethics Committee.

### 2.2. Study area

The present research work was conducted in three districts (Chittagong, Mymensingh and Gazipur) of Bangladesh during July 2015 to June 2016. The geographic position of the study area is Latitude: 20°45′–26°40′ N, Longitude: 88°05′–92°40′ E. The average annual rainfall is 3,450 mm. The day temperature ranges from 7 to 20 °C in the cool months (November to February), and in the other months it varies between 23 and 32 °C.

### 2.3. Study population and farm management

A total of 45 small-holding dairy farms were selected from the study areas which had previous history of mastitis, and the mean farm size was 12 (range; 5–26). In total, 175 lactating cows were randomly screened for mastitis, of which 50 were mastitis positive, and were included to this study. Most of them (74.2%) were cross-breeds (Holstein × Zebu, Sahiwal × Zebu) whilst the rest (25.8%) were local breeds (Zebu and Red Chittagong breeds). With regard to management, 14 (31.1%) of the farms were managed intensively while 31 (68.9%) farms were semi-intensive. The intensively managed cattle were kept indoors, and received concentrate feeds in addition to hay, green grass and crop residues (such as corn stalks, wheat/barley straw and other leftovers from grain threshing). On the other hand, the semi-intensively managed cattle grazed freely on pasture, but received supplementary feeds in the morning and evening when they were milked. The parity of the selected cows ranged from 1 to 5 with an average milk production 8.5 L per cow per day (range 2.0–17.0 L). The cows gave birth randomly throughout the year (no particular control breeding), were milked once daily with their calves used for stimulating milk let-down. Calves survived on residual milk after the hand milking. Control weaning was not practiced. The cows were milked manually, and the milkers did not wear gloves during the milking procedure. Pre- and post milking teat disinfection and dry cow therapy were not practiced in the study farms. Cows were housed in open shed with brick made floor, and most of the floors were wet and soiled with feces.

### 2.4. California mastitis test (CMT) and sample collection

CMT was used as a screening test for mastitis. It was carried out according to the procedure described by Hoque et al. [18]. The CMT results were scored as 0(negative), trace, 1(weak positive), 2(distinct positive) and 3(strong positive) based on gel formation. The CMT score of 0 was considered as negative while CMT scores of 1 and 2 were considered indicators of subclinical mastitis, and 3 for clinical mastitis. Positive cows were defined as having at least one quarter with CMT score of > 1. In total, 200 quarter milk (80 clinical and 120 subclinical) samples from 50 CMT positive cows were collected aseptically into the sterile plastic tubes (10–15 mL/sample). Sampling was done from all quarters of CMT positive cows, and was transported to the laboratory using ice-box.

### 2.5. Isolation and identification of Staphylococcus aureus

To identify the chief etiology, milk samples were collected from cows assuming that the causative organisms within a herd are similar, and also to reduce the time, labor and cost burdens. In case where only one mastitis case found in a farm, that positive cow was directly sampled. Bacteriological examination was performed within 24 h of sampling following the method described previously by [3,12] with some modifications. In brief, 25 mL of collected milk sample were placed into a sterile glass flask containing 225 mL of buffered peptone water (BPW, Difco, Cockeysville, MD). The solution was incubated at 37 °C in a water bath with shaking at 100 rpm for 24 h. After pre-enrichment, a 5 mL aliquot was transferred to 50 mL of trypticase soy broth (TSB, Beijing LB Technology Ltd., China) containing 7.5% NaCl. After 18–24 h incubation at 35 °C, a loopful of the culture was inoculated onto Baird-Parker agar (BPA, Beijing LB Technology Ltd., China) plates with 5.0% egg yolk and tellurite. Following incubation at 35 °C for 24 h, one or two presumptive coagulase-positive colonies per sample (black colonies surrounded by 2–5 mm clear zones) were transferred to trypticase soy agar (TSA, Beijing LB Technology Ltd., China) plates with 0.6% yeast extract for further purification. Colonies suspected of being *Staphylococci* were initially identified by their colony morphology and Gram staining. Catalase activity and coagulase tests were performed to distinguish catalase-negative *Streptococcus* spp. from catalase-positive, coagulase production by coagulase-positive *Staphylococci* was examined using the tube coagulation method. Then, all initially identified isolates were further confirmed as *Staphylococcus* by multiplex PCR detection (at least two times confirmation) using genus specific oligoneucleotide primers as previously described by Wang et al. [12]. Colonies were confirmed as *Staphylococcus aureus* by PCR detection of the thermonuclease gene (*nuc*; *Staphylococcus aureus* specific gene). Finally, all isolates were stored in brain heart infusion broth with 15.0% glycerol at −80 °C until further use.

### 2.6. Antimicrobial susceptibility testing

The susceptibility of the isolates to various commonly used antimicrobials was performed by using both disk diffusion and agar dilution methods according to the guidelines of the Clinical Laboratory Standards Institute [19]. The disk diffusion test was performed following the procedure described by [10]. The agar dilution method was used to measure the MICs of penicillin, erythromicin, oxytetracycline, trimethoprim/sulfamethoxazole, ciprofloxacin, gentamicin, amoxicillin, oxacillin, ceftriaxone and azithromycin [10,12]. The breakpoints of CSLI for the tested antimicrobials (for both disk diffusion and agar dilution) were used to determine the susceptibility profiles. All antimicrobial susceptibility testing assays were repeated at least 3 times. *E. coli* ATCC 25,922 and *Staphylococcus aureus* ATCC 29,213 were included as quality control strains in each run [12].

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