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Isolation and characterization of *Staphylococcus aureus* from raw cow milk in Bangladesh

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ABSTRACT

The study was intended for identification and characterization of *Staphylococcus aureus* isolated from raw cow milk. A total of 47 milk samples were collected from Sheshmore, Shutiakhali and Bangladesh Agricultural University Dairy Farm, Mymensingh. Using bacteriological, biochemical and PCR-based identification schemes, 12 (25.53%) isolates were confirmed as *S. aureus*. All the isolates showed β-hemolysis on 5% sheep blood agar. *S. aureus* specific *nuc* gene (target size 279-bp) was amplified in the cases of all isolates. The isolates were found as resistant to Penicillin (100%), Erythromycin (75%) and Amoxicillin (100%). On the other hand, the isolates were sensitive to Ciprofloxacin (83.33%), Oxacillin (100%), Cloxacillin (100%) and Neomycin (100%). The isolated *S. aureus* showed increased resistance to broad spectrum antibiotic (e.g., Ciprofloxacin). As many people have a tendency to drink raw milk and raw milk products, there is high risk of *S. aureus* infection in human.

Keywords

Staphylococcus aureus, Methicillin resistance, *nuc* gene, *mecA* gene

ARTICLE HISTORY

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INTRODUCTION

Milk is a highly valuable food, but raw milk contains and favors growth of many microorganisms (Helena et al., 2010). Milk and its derivates are considered as a major source of *Staphylococcus aureus* infection in man (Zecconi and Piccinini, 2000). In Europe, milk and other dairy products are found responsible for 5% of the staphylococcal outbreaks (Bianchi et al., 2014). *S. aureus* causes a variety of diseases in human and animals; the infections vary greatly in severity. There may be a mild skin infection to severe pneumonia and septicemia (Lowy et al., 1998). The bacterium is frequently found associated with subclinical mastitis in dairy cattle (Adesiyun et al., 1998) and may be present in milk and other dairy products (Capurro et al., 2010).

Staphylococcus aureus is the most prevalent and economically significant pathogen causing inflammatory infections in dairy ruminants (Akineden et al., 2001; Cabral et al., 2004; Katsuda et al., 2005). Approximately 30%-40% of all mastitis cases are associated with the bacterium (Asperger et al., 2003). *S. aureus* can get access to milk either by direct excretion from udders with clinical or subclinical *Staphylococcal* mastitis or by contamination from the environment during handling of raw milk (Scherrer et al., 2004; Jorgensen et al., 2005). When the udder is infected, *S. aureus* may be excreted through milk in variable numbers up to 10^8 CFU/mL (Asperger et al., 2003).

The *S. aureus* is Gram positive and produce smooth, circular colonies, convex and lustrous; size of the colony may be 0.5-1.5 µm in diameter. Under microscope, it appears like irregular three dimensional bunch of grapes-like cluster of cells. The colony pigmentation may vary from grey, grey-white, grey-

white with yellowish to orange shades and in blood agar typical β-hemolysis may be produced; depending on the growth condition (Deresse et al., 2012; Sushma et al., 2012).

S aureus may be pathogenic or non-pathogenic and the pathogenic strains are usually coagulase-positive and cause disease in their hosts. The infection may manifest as abscesses or mastitis to a severe toxic shock syndrome. The bacteria may contaminate the milk during milking and the contamination depends on the sanitary condition of the plant, milking utensils and milking personals. Contamination may also result from the micro-organisms entering the udder through teat canal (Smith et al., 2007).

The hygienic standard ensured in a milk plant may be estimated based on the level of contamination with *S. aureus* and the antibiotic-resistant phenotype assessment may serve as a tool to observe the hygienic standard during milking. Antibiotic sensitivity pattern of the isolates are also useful to categorize these opportunistic pathogens, this categorization may minimize the risks of infection through milk and milk products (Wubete, 2009). Considering the aforementioned points, our investigation was conducted with an objective to isolate and characterize *S. aureus* from raw milk samples. We studied the antibiotic resistance and sensitivity pattern of the isolates along with the intended detection of *mecA* gene in the organism that is responsible for the Mithicillin resistance.

MATERIALS AND METHODS

Sample collection: A total of 47 raw milk samples (approximately 10 mL from each) of apparently healthy cow were collected from Bangladesh Agricultural University (BAU) Dairy Farm; Sheshmore area and Shutiakhali area of Mymensingh, Bangladesh using sterile test tube. The samples were collected from December 2012 to May 2013 and investigation was carried out following collection. The laboratory works were accomplished in the laboratories of the Department of Microbiology and Hygiene, Bangladesh Agricultural University.

Isolation and identification of *S. aureus*: The collected milk samples (0.01 mL) were streaked onto 5% sheep blood agar (HiMedia®, India) incubated at 37°C for overnight. The presumptive colonies of *S. aureus* were further cultured onto mannitol salt agar (MSA) and repeatedly sub-cultured to get pure culture. These isolates were preserved for further bacterial identification. The isolates were identified as *S. aureus* on the basis of Gram staining, colony morphology on mannitol salt agar (MSA) (HiMedia®, India), beta-hemolytic patterns on blood agar enriched with 5% (v/v) sheep blood, catalase and coagulase tests. To perform agglutination tests, the pure colony of *S. aureus* were placed on the clean glass slide using sterile inoculation loop and a drop of respective reagents were added and mixed with the loop. For catalase and coagulase tests 3% hydrogen peroxide and fresh rabbit plasma were used respectively. Further the isolates were confirmed by amplification of *S. aureus* specific *nuc* gene.

Extraction of bacterial Genomic DNA: The genomic DNA was extracted by boiling method. In brief for the extraction of genomic DNA, a single colony of *S. aureus* was taken in 100 µL of distilled water, mixed well and boiled for 10 min. After boiling the tubes were immediately placed on ice for cooling followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant containing DNA was collected which was further used as template DNA.

Identification of *S. aureus* by PCR: Multiplex PCR reaction was performed using *S. aureus* specific *nuc* gene and methicillin resistant gene *mecA* for the identification of Methicillin Resistant *S. aureus* (MRSA). Two different primers pairs were used for this purpose, *nuc* gene (F 5'- CGCG ATT GAT GGT GAT ACG GTT-3' and R 5'- ACG CAA GCC TTG ACG AAC TAA AGC-3') and for *mecA* gene (F 5'-AAA ATC GAT GGT AAA GGT TGG-3' and R 5'- AGT TCT GCA CTA CCG GAT TTG C -3') according to the methods described by Dewanand et al. (2007) and Brakstad et al. (2009). Each 25 µL of reaction mixture consisted of 3 µL genomic DNA, 12.5 µL 2X PCR Master Mix (Promega®, USA), 1 µL of each of the two primers and final volume adjusted to 25 µL by adding remaining volume of nuclease free water. Amplification was proceeded with thermocycler (Mastercycler personal®- Eppendorf® Germany) with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, anneling temperature of primers was 55°C for 45 seconds and extension at 72°C for 1 min. The amplified PCR products were resolved by electrophoresis in 1.8% agarose gel at 100 V for 30 min, stained with ethidium bromide and finally visualized and documented under UV trans-illuminator (UVsolo TS® Imaging System, Biometra®, Germany).

Antibiotic Susceptibility Assay: All the *S. aureus* isolates were subjected to antibiotic sensitivity testing by standard disc diffusion method on Muller-Hinton agar (Merck®, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Sensitivity pattern of the isolates to Penicillin, Erythromycin, Cloxacillin, Amoxicillin, Oxicillin, Neomycin, Ciprofloxacin were determined. Isolates were divided into three groups based on the zone of inhibition produced by the antibiotic disc; susceptible, intermediately susceptible and resistant according to the Clinical and Laboratory Standards Institute (CLSI) guideline; Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2007).

RESULTS AND DISCUSSION

A total of 47 raw milk samples were tested and *S. aureus* was isolated from 12 (25.53%) samples based on cultural and biochemical properties. All the 12 isolates showed β-hemolysis on blood agar media enriched with 5% sheep blood (**Figure 1**). Gram-stained smears of the pure cultures exhibited clusters of Gram-positive cocci (**Figure 2**). The isolates also fermented mannitol with the color change of MSA (Mannitol Salt Agar) and production of small yellow colonies (**Figure 3**). These isolates were positive for catalase and coagulase test. In catalase test; Hydrogen peroxide was broken-down into water and oxygen. Production of oxygen was indicated by bubble formation, whereas the negative control did not produce any bubble (**Figure 4**). The isolates were identified as *S. aureus* by coagulase test. The positive result of coagulase test was confirmed by the formation of curd like clotting compared to negative control. The prevalence of *S. aureus* in raw milk and dairy product was found to be 56% in Turkey by Gundogan and Avci (2014), and 75% in Bangladesh by Begum et al. (2007), which were significantly higher than the present study (25.53%).

The *S. aureus* specific *nuc gene*; amplicon size 279-bp was successfully amplified from the genomic DNA of all isolated *S. aureus* (**Figure 5**), whereas the isolates were negative for *mecA* which revealed these *S. aureus* were sensitive to Methicillin; the finding agree with Hummerjohann et al. (2014). Hummerjohann et al. (2014) reported only one of the 623 isolates was Methicillin-resistant from Swiss raw milk cheese.

Based on antibiotic sensitivity test, about 20-25% *S. aureus* isolates were found multidrug resistant. The isolates were found 100% resistant to Penicillin and Amoxicillin, 75% resistant to Erythromycin and 16.67% to Ciprofloxacin. All the isolates found 100% sensitive to Oxacillin. On the other hand several isolates were found sensitive to Neomycin, Cloxacillin and Ciprofloxacin (**Figure 6**).

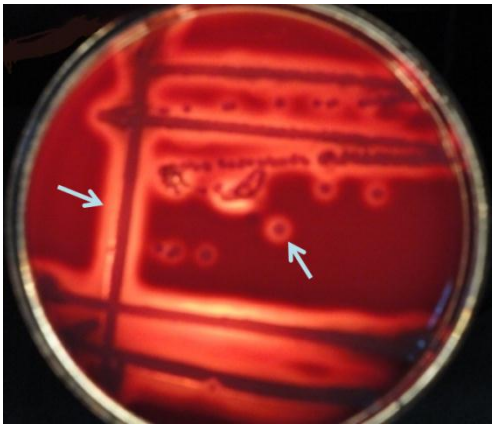

Figure 1: β-hemolysis on 5% sheep blood agar by *S. aureus*. Formation of β-hemolytic colony (white arrow).

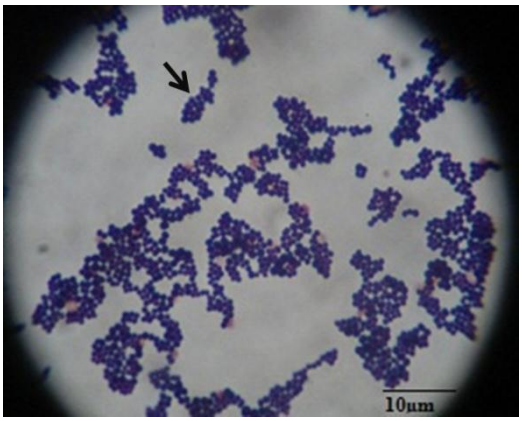

Figure 2: Gram's staining of *S. aureus* (100X). Grapes like (black arrow) Gram positive cocci.

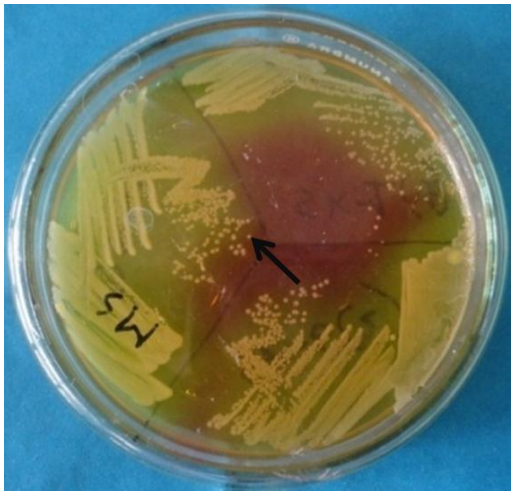

Figure 3: Fermentation of mannitol salt agar by *S. aureus*. Formation of yellow color colony (black arrow).

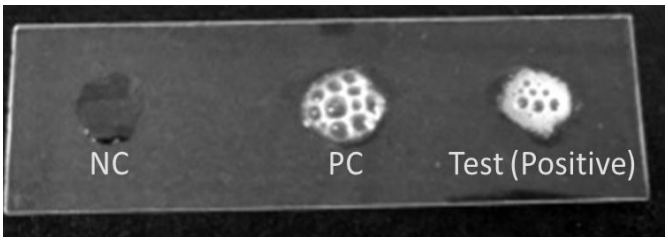

Figure 4: Catalase test of *S. aureus (***NC**, negative control; **PC**, positive control; **Right**, positive test sample).

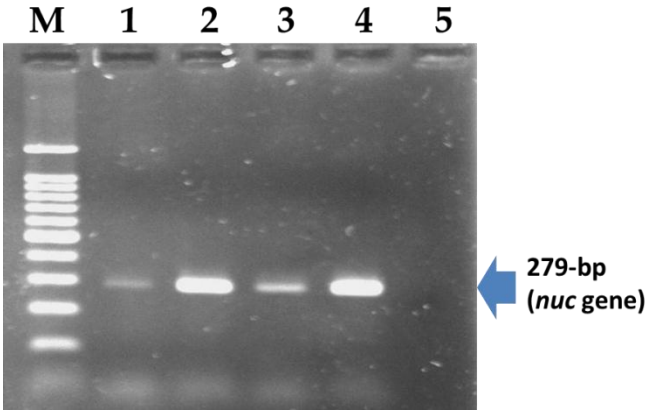

Figure 5: Amplification of *nuc* gene from *S. aureus*. **M**, 100-bp ladder; **Lane 1-4**, amplified *nuc* gene of *S aureus*; **Lane 5**, negative control.

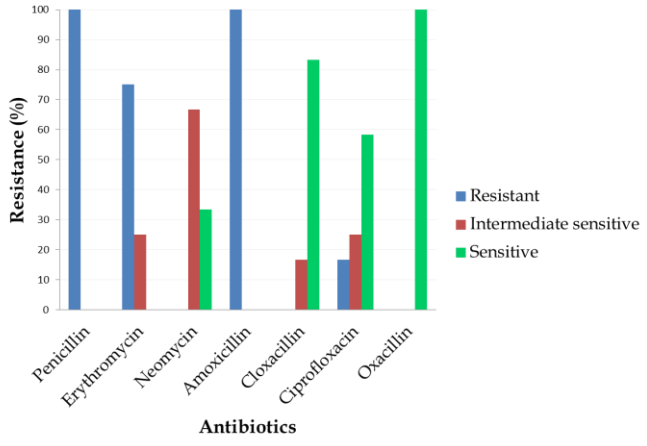

Figure 6: Antibiotic resistance patterns of *S. aureus* against commonly used antibiotics in Bangladesh.

The present study was designed for the isolation, identification and characterization of *S. aureus* from raw milk samples from dairy cattle. Our results indicated that 12 samples were positive for *S. aureus* that means prevalence rate is 25.53% (n=12/47). Various studies have been conducted to evaluate the degree of contamination of milk with *S. aureus;* obtained from communal and commercial farms. In most cases, milk containing *S. aureus* were obtained from animals with subclinical mastitis. Zafolon et al. (2008) studied at Nova Odesa, São Paulo; showed that the prevalence of *S. aureus* was 54.4%. The results obtained in our study are likewise higher when compared to those formerly documented (Shitandi and Sternesjö, 2004; Gündoğan et al., 2006). Based on observations made throughout the collection of samples, we concluded that the improper hygiene practice and poor management before and during milking may have contributed to the contamination of milk with *S. aureus,* and the communal farms are more vulnerable in this case. The *S. aureus* incidence at a considerable high percentage indicates the alarming situation both for dairy farming and for public health. The presence of *S. aureus* in the milk sample is a appealing as well as an important finding of this study. *S. aureus* was resistant to multiple antibiotics which can cause serious health problems (Tenover, 2006).

Investigation in other countries reviled similar results obtained in this study. Farhan and Salk (2007) studied on 130 milk samples in Palestine and found 48 (36.9%) samples were contining *S. aureus.* Ekici et al. (2004) found 18.18% of the milk samples positive for S aureus while studying 66 samples in Turkey. In Morocco, Bendahou et al. (2008) studied 27 samples and found 40% of the milk samples were containing *S. aureus;* while in India, 61.7% of the raw milk samples were found positive out of 60 samples studied (Lingathurai and Vellathurai, 2010).

The high incidence of *S. aureus* is indicative of poor hygienic measures during production, handling and distribution, stated in the findings of Zakary et al. (2011). The proper heat treatment followed by the refrigeration can minimize the chance of contamination with *S. aureus*. In our country it is commonly noticed that during heat treatment of milk, the temperature don't rise up to the boiling point many a time or even if it reaches, consumers do not boil it enough. El-Malt et al. (2013) stated that the difference in the prevalence rates of *S. aureus* between the examined products may originate from the method of manufacture, storage and handling or use of unhygienic utensils. The lowest prevalence rate of *S. aureus* was recorded in yogurt might be attributed to the effect of heating and then freezing during manufacture which inhibits the multiplication of organism.

The isolated *S. aureus* were highly resistant to Penicillin, Erythromycin and Amoxicillin and sensitive to Neomycin, Cloxacillin, Ciprofloxacillin and

Oxacillin. These findings are slightly correlated to De Oliveira et al. (2000) and Guerin et al. (2003), where they analyzed 119 isolates of *S. aureus* collected between 1998 and 2000 in France from cows with clinical mastitis. In an another study in Bangladesh conducted by Begum et al. (2007) revealed that *S. aureus* was 82.86% and 37.14% resistant to Penicillin-G and Amoxicillin, respectively; however, in our study, we noticed that both the antibiotics were 100% resistant to *S. aureus*, indicating increasing resistance of the organism against Penicillin and Amoxicillin. Similar types of resistance pattern also reported by Islam et al. (2007a, 2007b). In our study, no Methicillin Resistant *S. aureus* (MRSA) was found. Usually Methicillin is not widely used in livestock, for that reason may be; the isolates were found to be sensitive to Methicillin.

The study was intended for isolation and characterization of *S. aureus* from raw milk. We isolated and confirmed the isolation of 12 *S. aureus* isolates from 47 samples; the antibiotic sensitivity pattern of the isolates were significantly interesting and alarming for livestock and public health sector in Bangladesh; but our attempts were unable to identify any Mithicillin resistant *S. aureus* in the study.

CONCLUSION

Raw milk samples from different farms at Mymensingh, Bangladesh were tested. Overall, 25.53% (n=12/47) isolates were confirmed as *S. aureus* by PCR targeting *nuc* gene. The isolates were resistant to Penicillin (100%), Amoxicillin (100%), and Erythromycin (75%). On the other hand, the isolates were found to be sensitive against Ciprofloxacin (83.33%), Oxacillin (100%), and Neomycin (100%). Resistance pattern against broad spectrum antibiotic (e.g., Ciprofloxacin) depicts an alarming situation, which needs special attention.

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