MOLECULAR DETECTION AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM RAW MILK SOLD IN DIFFERENT MARKETS OF BANGLADESH

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ABSTRACT

The study was intended for molecular detection of *S. aureus* isolated from raw cow's milk. A total of 20 milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh. Milk samples were cultured onto various culture media for the isolation of bacteria. The isolated bacteria were identified by studying cultural properties on different selective media, biochemical tests, and finally by PCR. Out of 20 samples, 15 (75%) milk samples were found to be positive for *S. aureus*. *S. aureus* specific *16S rRNA* gene was amplified from all isolates and identified as *S. aureus*. Antimicrobial sensitivity test was carried out to ascertain the susceptibility of the organism to various antibiotics. Its results showed that the *S. aureus* isolates were resistant to amoxicillin (100%), erythromycin (73.33%) and tetracycline (73.33%) but sensitive to azithromycin (93.33%), ciprofloxacin (93.33%), gentamicin (100%), norfloxacin (86.67%) and streptomycin (86.67%).

Key words: *Staphylococcus aureus, 16s rRNA* gene, raw milk

INTRODUCTION

Milk is considered as one of the high nutritional quality foods. Whole milk is the milk as it comes from the cow and contains about 3.5% milk fat. Research continues to expand the positive role of milk and milk products on individual's health. Evidence goes well beyond bone health to include its effects on immunity, mild hypertension, reducing selected cancers (Giovannucci *et al*., 1998), supporting weight management strategies and increasing satiety in dieters, among other positive effects. However, it is an excellent medium for growth and transmission of different bacterial pathogens to humans (Donkor *et al.*, 2007). *Staphylococcus aureus* is an important food born pathogen and causes a mild skin infection to more severe diseases such as pneumonia and septicemia (Lowy *et al.,* 1998). In Europe, milk and other dairy products are found responsible for 5% of the staphylococcal outbreaks (Bianchi *et al*., 2014). Bacterial contamination of milk usually occurs during the milking process and this depends on the sanitary condition of the environment, utensils used for milking and the milking personnel. It could also result from micro-organisms that enter the udder through the teat opening canal (Smith *et al.,* 2007). Staphylococcal food poisoning is often associated with the ingestion of manually handled foods that contain one or more highly heat stable staphylococcal enterotoxins. The safety of milk with respect to FBD is of great concern around the world. This is especially true in developing countries like where production of milk often takes place under unsanitary conditions and the consumption of raw milk which is typically produced in small dairy farms under unsatisfactory hygienic conditions is a common practice (Lee *et al.,* 2003).Determination of levels of *S. aureus* and an evaluation of the antibiotic-resistant phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize this opportunistic pathogen, which may further limits the risks associated with the consumption of contaminated milk and its products (Wubete, 2004).

Several works have been done throughout the world regarding the milk and microorganisms of contaminated milk (Hassan *et al*., 2014). In Bangladesh, a few works have been done on isolation and molecular characterization of *S. aureus* from raw milk of cattle and buffalo (Islam *et al.*, 2008; Hossain *et al.*, 2011; Hossain, 2015). Moreover very few works have been reported in Bangladesh on molecular detection of *S. aureus* with other pathogenic organisms in cow's raw milk in a specific time period. So, keeping the above facts in mind, the present study was designed with an objective to molecular detection and characterization of bacteria isolated from raw milk samples. We studied the antimicrobial susceptibility patterns of isolated bacteria with the detection of *S. aureus* using *16s rRNA* gene.

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MATERIALS AND METHODS

Sample collection
A total of 20 cow's raw milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh using falcon tube. The samples were collected from July 2015 to December 2015 and investigation was carried out following collection. The collected milk samples were immediately transported on ice to the Microbiology Laboratory at the Department of Microbiology and Hygiene of BAU and District Veterinary Hospital, Sirajgonj for bacteriological analysis.

Isolation and identification of *S. aureus*
The collected milk samples were performed 10 fold dilution using 0.1% peptone water and diluted samples were streaked onto 5% cattle blood agar (HiMedia®, India) incubated at 37°C for overnight. Then the presumptive colonies of *S. aureus* were cultured onto mannitol salt agar (MSA) and then 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, biochemical characterization of the isolates (using sugar fermentation test, indole and MR-VP test), catalase and coagulase 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, then agglutination tests were performed. 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 *16S rRNA* gene.

Bacterial Genomic DNA extraction
The DNA template was prepared by boiling method. In brief for the preparation of DNA template, a single colony of bacteriological culture of *S. aureus* was taken in 250 µl of DW in eppendorf tube, mixed well and then boiled for 10 minutes. After boiling the tubes were immediately placed on ice for cold shock followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected which was further used as DNA template (Engler and Kelley, 2000).

Identification of *S. aureus* by PCR
PCR reaction was performed to detect *S. aureus* specific *16S rRNA* gene from isolated *S. aureus* in a thermal cycler (Astec, Japan). Two different primers pairs were used for this purpose, *16S rRNA* gene (F 5'-CGATTCCUTAGTAGCGGCG-3' and R 5'-CCAATCGCACGCTTCGCC-3') according to the methods described by Swaminathan and Feng (1994). Each 20 µl reaction mixture consists of 3 µl genomic DNA, 10 µl PCR master mixtures (Promega, USA), and 1 µl of each of the two primers with the final volume adjusted to 20 µl with 5µl of nuclease free water. Amplification was done by initial denaturation at 95°C for 5 minutes, followed by denaturation at 94^0C for 1 minute, annealing temperature of primers was 53°C for 1 minute and extension at 72°C for 1 minutes. The final extension was conducted at 72°C for 7 minutes. The total reaction was performed at 35 cycles. The amplified PCR products were resolved by electrophoresis in 2% agarose gel at 100v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator.

Antibiotic Sensitivity Test
All isolates of *Staphylococcus aureus* were tested for antimicrobial drug susceptibility against antimicrobial agents by disc diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007). Sensitivity pattern of the isolates were determined against ciprofloxacin, azithromycin, gentamicin, amoxycillin, streptomycin, erythromycin, tetracycline and norfloxacin. Antimicrobial testing results were recorded as sensitive, intermediate sensitive and resistant according to zone diameter interpretative standards provided by CLSI, 2007.

RESULTS AND DISCUSSION

20 raw milk samples were analyzed in the laboratory by different cultural, biochemical, staining and molecular methods to detect *Staphylococcus aureus* in raw milk. Out of 20 samples 75% (n=15) were contaminated with *S. aureus*. In blood agar the *S. aureus* produced β-hemolysis which is the typical characteristics of *S. aureus* (Figure 1).The growth of *S. aureus* on mannitol salt (MS) agar was confirmed by the fermentation of mannitol with change of color of media and formation of yellow color colonies (Figure 2). In Grams staining, the organism revealed Gram positive, violet colored, cocci shaped and arranged in grapes like cluster under light microscope (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 (Figure 5). The positive result of coagulase test was confirmed by the formation of curd like clotting compared to negative control. The optimized PCR assay was able to successfully amplify the target *16S rRNA* gene (1267 bp fragment) from the DNA templates of all isolated *S. aureus*. Result of PCR for *S. aureus* is shown in Figure 6. The organism was subjected to antimicrobial susceptibility test by disc diffusion method against 8 most commonly used antimicrobial agents. All isolates were found to be 100% resistant to amoxycillin, 11 (73.33%) were resistant to erythromycin and 8 (53.33%) were resistant to tetracycline. Furthermore, all isolates were susceptible to gentamicin, 14 (93.33%) were susceptible to azithromycin, 14 (93.33%) were susceptible to ciprofloxacin and 13 (86.67%) were susceptible to norfloxacin (Figure 7).
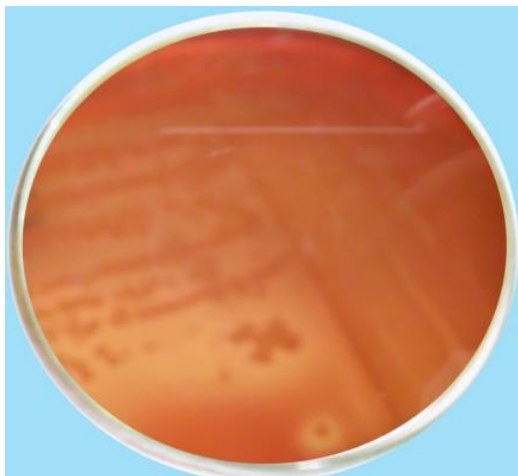

Figure 1. β -hemolysis on 5% cattle blood agar by *S. aureus*

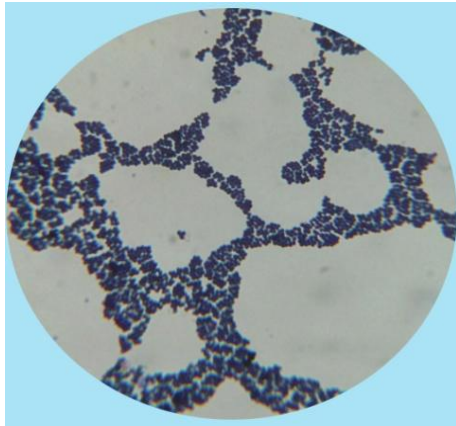Figure 2. Growth of yellowish color colonies on MS agar by *S. aureus*

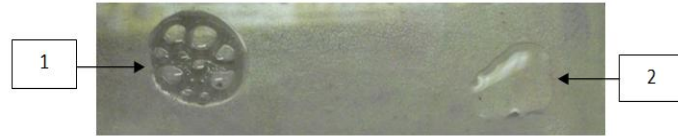Figure 3. Gram positive, cocci, arranged in grapes like cluster of *S. aureus* under light microscope.

Figure 4. Catalase test of *S. aureus*.

1: Catalase positive;
2: Catalase negative

Figure 5. Coagulase test of *S. aureus.*
　　1: Coagulation of plasma by *S. aureus*;
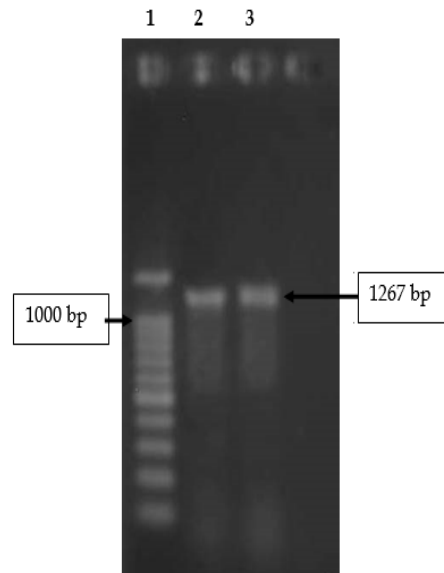　　2*:* Control

Figure 6. *16S rRNA* gene based PCR of *S. aureus.* Lane 1: 100 bp DNA ladder, Lane 2, 3: Tested samples were positive for *16S rRNA* gene.
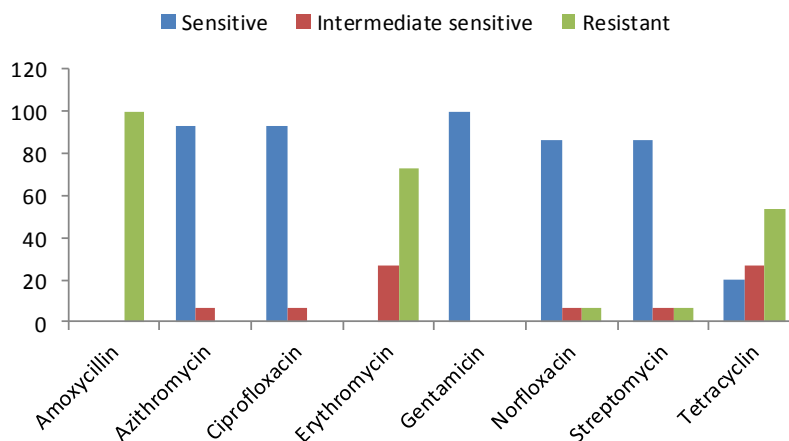
Figure 7. Antibiotic resistance patterns of *S. aureus* against commonly used antibiotics in Bangladesh

   The above study revealed that cow's raw milk contained *Staphylococcus aureus* which can be transmitted to human through milking and consumption of milk. Milk is the best media for the growth of many pathogenic and non pathogenic bacteria. The *S. aureus* might be hazardous if proper boiling of milk is not done during consumption. It causes disease if proper hygienic procedure is not maintained during milking. In Bangladesh, Parveen (2000) characterized *Staphylococcus aureus* isolated from human and animal samples and Das (2012) isolate and identify *Staphylococcus aureus* from laboratory animals and human and also determine antibiogram profile. Jorgensen *et al.* (2005) stated that the presence of strains assigned to this *Staphylococcus* spp. in bulk milk or in raw milk products could reflect human contamination. In most cases, milk containing *S. aureus* were obtained from animals with subclinical mastitis. Zafolon *et al.* (2008) studied at Nova Odesa, São Paulo and 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 Sternesjo, 2004; Gundogan *et al.,* 2006; Aziz *et al*., 2013; Hossain, 2015). 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 an 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; Hossain, 2015)**.** Findings of antimicrobial susceptibility test were slightly correlated to De Oliveira *et al.* (2000), Guerin *et al.* (2003) where they analyzed 119 isolates of *S. aureus* collected between 1998 and 2000 in France from cows with clinical mastitis. Also, these findings were correlated to Hossain (2015), where 60 samples were investigated.

CONCLUSION
   The present research was undertaken with a view to isolating and characterizing *Staphylococcus aureus* present in cow's raw milk. Out of 20 samples, 15 (75%) milk samples were found positive for *S. aureus*. The isolates were identified as *S. aureus* on the basis of Gram staining, colony morphology, biochemical characterization, catalase and coagulase tests, slide agglutination test and PCR. Further the isolates were confirmed by amplification of *S. aureus* specific *16S rRNA* gene. Resistant pattern against broad spectrum antibiotic (e.g., amoxicillin) delimitate an alarming situation which needs special consideration.

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