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Research Article

Title:	Isolation and Identification of Slime Forming *Staphylococcus aureus* from Raw Cow Milk and their Antibiotic Susceptibility Pattern
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ABSTRACT

Staphylococcus aureus is an emerging pathogen from dairy animals that can form biofilms in the udder of dairy cows affected by mastitis. The use of antibiotics in dairy farms leads to a decrease in bacteria, finally making healthy food. The aim of this study was to determine the occurrence, antibiotic susceptibility, and biofilm forming ability of *S. aureus* isolated from cow milk. A total of fifteen raw milk samples were collected from Mohammadpur, Narayangonj, Savar, Bosila, Siddheshwari and Norshingdi dairy farms for isolation of *S. aureus*. All the samples were highly loaded with *Staphylococcus* and the total staphylococcal count ranged from 10^5 to 10^6 cfu ml^{-1}. Biochemical tests (TSI, catalase, oxidase, motility, MR/VP etc.) were performed for the confirmation of the Staphylococcal isolates. The 100% resistance was observed against oxacillin, erythromycin, cotrimoxazole, amoxicillin, cefixime and vancomycin. In contrast to these results, the isolates showed 100% sensitivity to gentamicin and ciprofloxacin. The sensitivity against kanamycin and tetracycline was observed to be variable. The present study reveals the presence of multidrug resistant *S. aureus* in milk. Therefore, appropriate control measures like proper sanitation of the place where the milking cows live, maintenance of hygiene before and after milking, using proper mechanical methods for milking with strict maintenance of sanitation etc. are needed to be ensured.

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INTRODUCTION

Milk is a highly nutritious and a good source of proteins, fats, carbohydrates, vitamins and minerals. A variety of animals such as cows, goats, sheep and buffalo provides milk for human consumption. Milk serves as a growth medium for many microorganisms due to its high nutrient content, near neutral pH and high water activity (Doyle et al., 2020).

Although milk is virtually sterile when secreted from healthy udder (Tolle, 1980), but it can be contaminated by microorganisms from myriad sources e.g. dirty exterior of the udder of the animals, milk handlers with no hygienic practices, storage and other equipment's being in the contact with milk, farm environment, contaminated soil, feaces, water, feed etc. (Torkar and Teger, 2008). The common bacteria found in milk and dairy products are *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Micrococcus* spp., and coliforms etc. (Torkar and Teger, 2008). In cattle, bacteria can cause mastitis, an inflammation of mammary glands and mastitis causing bacteria are predominant source of raw milk contamination (Hennekinne et al., 2012). It also causes decreased milk production, and necessitates treatment costs imposing huge economic burden to the dairy community. The microbial composition of milk has direct impacts on sensory, texture, flavor, quality, shelf life and organoleptic properties of resultant dairy products (Wouters et al., 2002; Hantsis-Zacharov and Halpern, 2007). The consumption of raw milk, contaminated with pathogens can lead to, in some cases, the severe illness of human (De Oliveira et al., 2009). To reduce contamination incidences and to provide milk of longer shelf life, pasteurization and ultra-high temperature treatment (UHT), aseptic packing has become standard practices (Ammara et al., 2009).

S. aureus is a gram positive bacterium. It is considered as one of the most causative agents of food poisoning linked to consumption of raw milk and milk products such as milk, cheese, yoghurt and other dairy products. These products have been implicated as potential ways for the transmission of this pathogen to human (Spanu et al., 2012; Normanno et al., 2007).

Moreover, contaminated foods transmit antibiotic resistant strains (Angulo et al., 2004; Phillips et al., 2004). Extensive use of antimicrobials for therapeutic purposes or as growth promoters in animal feed production, have led to the development of antibiotic resistance which has now become an important health problem worldwide (Rogues et al., 2007; Cosgrove, 2006). The development of antibiotic resistant especially methicillin resistant *S. aureus* (MRSA) has drawn considerable attention of the scientific community (Doyle et al., 2012). This study aimed to investigate *S. aureus* contamination in milk obtained from different dairy farms. For this purpose, we analyzed the occurrence of *S. aureus* in raw cow milk samples to assess the antibiotic resistance and potential to form biofilm.

MATERIALS AND METHODS

Collection and preparation of milk samples

A total of fifteen milk samples (approximately 10 ml from each cow) from apparently different healthy cows were collected from the dairy farms located in Mohammadpur (2 samples- S1, S2), Narayangonj (3 samples- S3, S4, S5), Savar (3 samples- S6, S7, S8), Bosila (2 samples- S9, S10), Siddheshwari (2 sample- S11, S12) and Norshingdi (3 samples- S13, S14, S15). The investigation was done with replicates with a view to minimizing data uncertainty. Three replications of each of the samples were collected from the same farms just like the original sampling. The samples were collected from 1st October to 31st October, 2017. Samples were collected in sterile tubes by maintaining aseptic conditions during the milking time. Milking was carried out manually by the specified person involved in the farm. After collection, samples were kept at approximately 4°C, transported in an icebox to laboratory. All samples were immediately tested within three hours of their collection. The sampling design in the research was developed by the procedure of American Public Health Association (APHA, 1998).

Microbiological analyses

All samples were serially diluted up to 10^{-8} in the distilled water. Nutrient agar (NA) (Oxoid-UK, Sigma) plates were inoculated by droplet method as described by Miles et al. in 1938. The three replicates of each sample (from highest dilution 10^{-6} to 10^{-8}) were inoculated using three drops, each of 20 μL. After incubation at 37°C for 24 hrs, the plates were observed and colonies were counted. Mannitol salt agar (MSA) (Oxoid-UK, Sigma) was used as a selective and differential medium for the isolation of *S. aureus*. The appearance of yellow colonies on MSA indicated the presence of *Staphyloccus* spp. The suspected colonies were streaked onto NA medium which were subjected to further biochemical confirmatory tests. Some control plates were used for MSA with inoculation of milk samples to determine whether the *Staphylococcus* spp. was found due to contamination.

Morphological, cultural and biochemical tests for the identification of the isolates

The suspected isolates of *S. aureus* on MSA plates were identified by standard microbiological procedures including colony morphology on NA and MSA agar medium, gram's staining and a series of biochemical tests (Catalase test, oxidase reaction, carbohydrate fermentation, indole production and motility test) (Cappucino and Sherman, 2005).

Determination of beta-hemolytic activity

Hemolytic activities of the isolates were tested by using blood agar (BA) medium (HiMedia®, India). On BA plates, colonies of *S. aureus* were frequently surrounded by zones of clear beta-hemolysis. Methicillin-resistant strains of *S. aureus* (i.e., MRSA) often have only weak or no beta-hemolysis and special cultivation media with oxacillin, mannitol and NaCl for their isolation were used. During the study, all Staphylococcal isolates collected from raw cow milk were used to screen out the beta hemolytic isolates depending on their ability to produce clear zone around their growth on BA medium.

Antibiotic susceptibility test

Fifteen isolates of *S. aureus* were subjected to antibiotic susceptibility assay against different groups of antibiotics in vitro by the Kirby-Bauer method (Bauer et al., 1968). The suspensions of the test organisms were prepared using Muller-Hinton broth agar (MHB) (Oxoid-UK, Sigma) by adjusting the turbidity of the broth to match the equivalent turbidity of McFarland 0.5 and standards 0.5. MHB agar plates were inoculated by sterile cotton swabs dipped into the suspensions. A number of antibiotic discs (Salubris Inc., Massachusetts, USA) were placed aseptically on the surface of the inoculated plates (Ahmed et al., 2013). Drug resistance was observed against amoxicillin (30 μg), cotrimoxazole (25 μg), cefixime (5 μg), kanamycin (30 μg), gentamicin (10 μg), vancomycin (5 μg), tetracycline (30 μg), erythromycin (15 μg), ciprofloxacin (5μg), imipenem (10 μg) and oxacillin (1 μg). After 24 hrs of incubation at 37°C, all the plates were observed for clear zones (zones of inhibition) around the colonies. If present, the zones of inhibition were measured and interpreted as susceptible, intermediate and resistant categories by referring the recommended interpretative standards (NCCLS, 2000).

Phenotypic characterization of biofilm production in congo red agar (CRA) medium

The CRA medium was composed of 37.0 gL^{-1} brain heart infusion broth (HiMedia, India), 5.0 gL^{-1} sucrose, 10 gL^{-1} agar number 1 (Oxoid) and 0.8 gL^{-1} CRA agar (BDH Ltd.). The medium, congo red stain (HiMedia®, India) was prepared as a concentrated aqueous solution and sterilized separately (autoclaved at 121°C for 15 minutes) (Bose et al., 2009). The sterilized agar medium was cooled down to 55°C followed by addition of congo red stain. After proper mixing the medium was poured

into sterilized petri plates and solidified for further experimentation. After inoculation, the plates were incubated for 24 hrs at 37°C. The production of rough black colonies by slime producing strains were used to differentiate them from non-slime producing *S. aureus* strains (red smooth colonies) (Freeman et al., 1989). *S. aureus* ATCC 35556 was used as positive control for slime production and *S. aureus* ATCC 25923 was used as negative control. These ATCC cultures were collected from the repository of the Microbiology laboratory of the Department of Microbiology, University of Dhaka.

RESULTS AND DISCUSSION

In the study, fifteen cow milk samples were screened for *S. aureus*. The total viable bacterial count was done on NA for enumeration of *Staphylococcus* spp. (Table 1). The total viable bacteria were found between the ranges of 1.6×10^9 cfu ml^{-1} to 2.6×10^{11} cfu ml^{-1}. The staphylococcal count was found in all of the samples ranged from 1.2×10^3 cfu ml^{-1} to 6.4×10^4 cfu ml^{-1}. In the control plates of MSA, sometimes we found 4/5 suspected Staphylococcal colonies and we subtracted the colony numbers from the original plates while calculating the cfu. The results indicated that the milk samples were unacceptable due to the presence of high number of bacteria.

All the milk samples (100%) were contaminated with *S. aureus*. Previous report showed the total bacterial count in milk ranged from 1.3×10^7 to 5.2×10^8 cfu ml^{-1} (Banik et al., 2014). According to the Food Safety Authority of Ireland, 10^5 - <10^7 cfu g^{-1} is the borderline of the acceptable level of bacterial load in non-fermented dairy

products including milk (FSAI, 2016). The samples used in the study exceeded this limit (20,000 cfu ml^{-1}) recommended by the United States Public Health Service (USPHS) commission (Tekinsen et al., 2007). The huge bacterial load might be responsible for rapid spoilage of milk, very poor shelf life without any treatments and also can cause disease to its consumers. Total staphylococcal count in these milk samples was similar to that reported by Marjan et al. (2014). When the udder is infected, *S. aureus* may be excreted through milk in variable numbers up to 10^8 cfu ml^{-1} (Asperger and Zhangeri, 2003). The high incidence of *S. aureus* indicates poor hygienic practice during production, handling and distribution (Zakary et al., 2011). There is a risk of enterotoxin production when higher concentration (≥10^5 cfu ml^{-1}) of *S. aureus* is present in milk (Hamama et al., 2002). Approximately 30%-40% of all mastitis cases are associated with this bacterium (Asperger and Zhangeri, 2003). Therefore, presence of mastitis infection in the dairy cows included in this study and the potential risk associated with them cannot be excluded. Isolation of *Staphylococcus* spp. following 24 hrs of incubation at 37°C, yellow colonies were observed on MSA agar indicating the presence of *S. aureus*. The suspected yellow colonies were sub-cultured on NA agar medium. The colonies on NA medium were moderate to large in size, circular, convex, smooth, and golden yellow in color. All of the fifteen isolates were beta-hemolytic. Clear zones surrounding the colonies were observed on BA plates and further confirmation of *S. aureus* was done following gram's staining, microscopy as well as biochemical tests. All the isolates were gram positive, cocci in shape and mostly arranged in clusters. The cells were non motile, catalase positive, alkaline butt/slant and beta hemolytic.

Table 1. Total viable bacterial count and total Staphylococcal count

Area of sampling	Sample No.	Total viable bacteria		*Staphylococcus* spp.	
		Dilution	Cfu ml^{-1}	Dilution	Cfu ml^{-1}
Mohammadpur	S1	10^{-8}	1.5×10^{11}	10^{-2}	1.2×10^3
	S2	10^{-8}	4.5×10^{10}	10^{-2}	4.3×10^4
Narayangonj	S3	10^{-8}	1.1×10^{10}	10^{-2}	6.0×10^3
	S4	10^{-8}	2.4×10^{11}	10^{-2}	2.8×10^3
	S5	10^{-8}	2.6×10^{11}	10^{-2}	4.2×10^3
Savar	S6	10^{-8}	1.6×10^9	10^{-2}	2.4×10^3
	S7	10^{-8}	1.8×10^{11}	10^{-2}	5.3×10^4
	S8	10^{-8}	1.4×10^{11}	10^{-2}	3.6×10^3
Bosila	S9	10^{-8}	2.5×10^9	10^{-2}	2.1×10^4
	S10	10^{-8}	1.2×10^{10}	10^{-2}	3.4×10^3
Siddheshwari	S11	10^{-8}	2.3×10^{19}	10^{-2}	1.3×10^5
	S12	10^{-8}	3.4×10^9	10^{-2}	4.1×10^4
Norshingdi	S13	10^{-8}	1.3×10^{11}	10^{-2}	5.2×10^4
	S14	10^{-8}	2.1×10^{10}	10^{-2}	6.4×10^4
	S15	10^{-8}	1.7×10^{10}	10^{-2}	3.0×10^3

**data were collected from single plate instead of doing average of replicates

In the study, the *S. aureus* isolates have been found resistant to most of the antibiotics tested including oxacillin and amoxicillin which indicates their intrinsic resistance to beta-lactam antibiotics. However, it is necessary to confirm the resistance phenotype by different methods such as oxacillin disk diffusion test,

agar plate screen, the micro broth dilution and the E-tests (Araj et al., 1998). Isolates that were sensitive to some antibiotics produced clear zones of inhibition whereas no such zone was observed against some antibiotic disc (Figure 1).
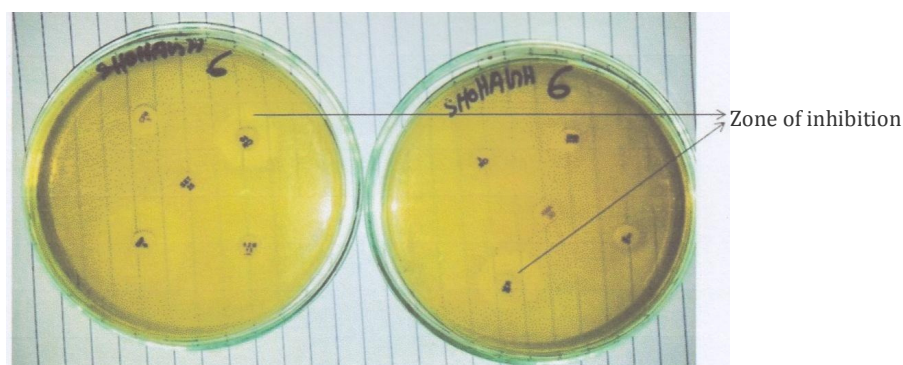
Figure 1. Antibiotic susceptibility test of *S. aureus* isolates. Clear zones around the bacterial colony indicate sensitivity towards a specific antibiotic

According to the antibiogram (Figure 2), 100% resistance was observed against oxacillin, erythromycin, cotrimoxazole, amoxicillin, cefixime and vancomycin. In contrast, the isolates showed 100% sensitivity to gentamicin and ciprofloxacin. Variable sensitivity was observed against kanamycin and tetracycline.

In this study the *S. aureus* isolates showed 100% sensitivity to gentamicin and >70% sensitivity to kanamycin. Marjan et al. (2014) reported 87% resistance against penicillin, 71.6% resistance to ampicillin and 67% resistance to vancomycin among the *S. aureus* strains isolated from cow milk; 90% sensitivity of the isolates to kanamycin and erythromycin and 87.7% sensitivity to trimethoprim-sulfamethoxazole were also reported. In another study, staphylococcal isolates showed moderate to high sensitivity to gentamicin, ciprofloxacin, chloramphenicol, enrofloxacin and oxytetracycline whereas less to moderate sensitivity was obtained with erythromycin (Kaszanyitzky et al., 2003). The results from the current study are similar to a recent report of Akindolire et al. (2015). They isolated more than 200 *S. aureus* isolates from both raw and pasteurized cow milk samples and found a large proportion (60%-100%) of the isolates showing resistance to penicillin G, ampicillin, oxacillin, vancomycin. On the contrary low level of resistance (8.3%-40%) was observed for gentamicin, kanamycin and sulphamethoxazole tests (Akindolire et al., 2015). Regarding the biofilm formation by *S. aureus*, all the isolates produced gummy, black colony on CRA medium indicating the production of slime or extracellular matrix of biofilm (Figure 3).

Biofilms are structured commodity unity of bacterial cells enclosed in a self-produced polymeric matrix and are capable of adhering to an inert or living surface (Costerton et al., 1999). One of the virulence factors of staphylococci is extracellular polysaccharides or slime factor which is required for biofilm formation (Drewry et al., 1990). Residual antibiotics, left from incomplete metabolism are excreted in feces and urine of the animals. These excreta contaminate the environment and help to develop antibiotic resistance. Presence of antibiotic residues in food for human consumption produces potential threat to direct toxicity, development of cancer, allergic reactions etc. These may lead to alteration of microflora and the possible development of resistance (Ahaduzzaman et al., 2014; Hassan et al., 2014).
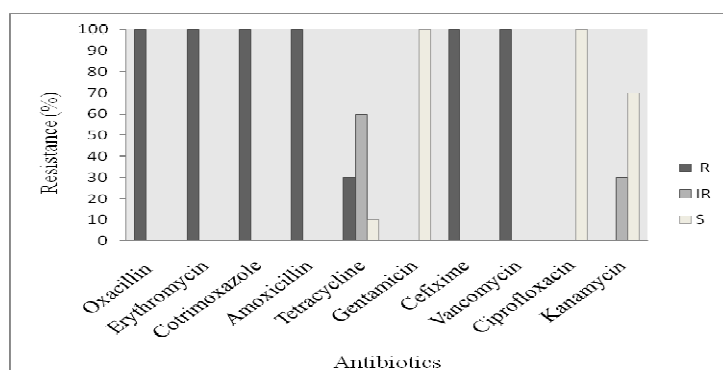

Figure 2. Antibiogram of *S. aureus* with resistance (R), intermediate resistance (IR) and sensitive (S)

Production of slime provides the protection against antibiotics possibly due to the decreased metabolic activity of bacteria and decreased diffusion of antibiotics through the biofilm matrix (Amorena et al., 1999). Gordon and Lowy (2008) reported a correlation between slime production and antibiotic resistances where the resistance was increased for biofilm producers. On the contrary, another study did not observe the same type of correlation i.e. there was no relation of the biofilm formation with antibiotic resistance (Ciftci et al., 2009). However, it is apparent that the microbial quality and safety of the raw milk samples used in this study were unsatisfactory. To know the bacterial scenario in the dairy farms in Bangladesh,

such study should be carried out with a large number of milk samples. Presence of multi drug resistant *S. aureus* possesses a serious threat to public health, thus strict regulations on dairy farm management are essential.
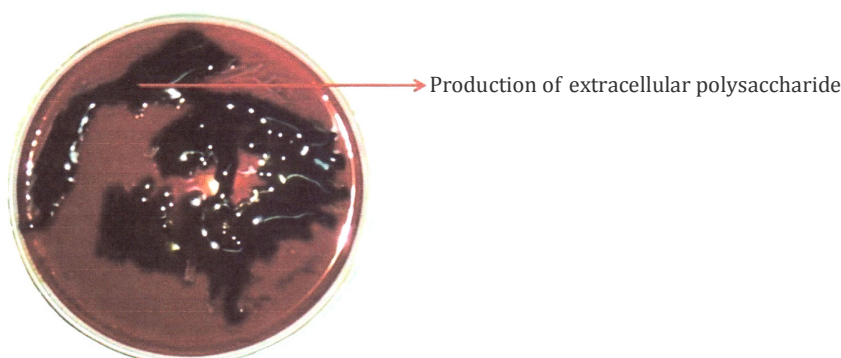

Figure 3. Extracellular polysaccharide (slime) formation by *S. aureus* on CRA medium

CONCLUSIONS

All the raw milk samples were heavily loaded with bacteria from which *S. aureus* was isolated. The isolates were multidrug resistant and produced slime. The study pointed out serious threats to public health due to the presence of multidrug resistant *S. aureus* in milk samples. Going forward, more detailed studies are necessary to fill in specific data gaps. Infected people with these multidrug resistant bacteria might face difficulties with antibiotic treatments. Some basic approaches could be applied to lower multidrug resistant bacteria in public health. Such as cow health must be monitored and proper treatments should be practiced; without diagnosing the appropriate disease conditions the cows should not be given any drugs for their betterment of health. As and when necessary, the antibiotic courses prescribed for the infected cows must be fulfilled. Diseased and healthy animals should be kept separated at all times. Milking machine and the personnel's personal hygiene should be maintained strictly. The environmental conditions where the milking cows stay should be kept clean always. By following these ways, it would be possible to reduce the incidents of disease caused by resistant *Staphylococcus* spp.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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