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Characterization of *Staphylococcus aureus* from Milk and Dairy Products Sold in Some Local Markets of Mymensingh District of Bangladesh

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Abstract

This study was designed to isolate and characterize the *Staphylococcus aureus* from raw cow's milk and some other dairy products sold in the local markets of Mymensingh district of Bangladesh by using conventional methods and molecular techniques. Raw cow's milk, pasteurized milk, yogurt, roshmalai, cheese, lassi, matha, milk-shake, custard, faluda, pudding and borhani sampled from different retail shops and renowned restaurants of the local markets of Mymensingh. Out of 72 samples tested, all the samples revealed presence of *Staphylococcus* spp. and 57 isolates found coagulase positive *S. aureus*. The antimicrobial susceptibility pattern of the 57 pathogenic isolates was determined by using 10 commercially available antimicrobial drugs by disk diffusion assay. It exposed that majority of the isolates (79.16%) showed resistant to more than three antimicrobial agents. Among 57 isolates, 14 (24.56%) showed resistance against both methicillin and oxacillin, also intermediately resistant against vancomycin. Molecular detection of *mecA* and *mecC* gene in the 14 methicillin and oxacillin resistant isolates for the identification of methicillin resistant *Staphylococcus aureus* (MRSA) strains revealed 8 isolates (57%) from raw milk, yogurt, roshmalai, borhani and cheese to be positive for *mecA* gene while it was not detected in any other of the samples. None of the tested samples found *mecC* positive. Our findings revealed that the milk and dairy food products sold at local markets of Mymensingh are contaminated with multidrug resistant *S. aureus* elucidating a possible risk of MRSA infection which is alarming for both human and animal health.

Keywords: Pathogenic; *S. aureus*; Multidrug resistant; MRSA

Introduction

Food is one of the most fundamental needs of human being. Among various food items milk and dairy products are frequently consumed by people as an ideal source of nutrients. Food is also a good territory for colonizing and proliferating many disease causing pathogenic microorganisms [1-6]. On the other hand microorganisms are also essential for production of food such as yogurt cheese, bread, wine, beer and other fermented food. So it is very usual that there are so many chances of having diseases arising from food. Therefore, the contamination of these food products with the pathogens and its persistence, growth, multiplication and/or toxin production is of great concern for public health. A number of pathogens has been responsible for food-borne diseases and among them *Staphylococcus* spp., *Escherichia coli* and *Salmonella* spp. tops the list. *Staphylococcus aureus* is an important foodborne pathogen, found in a good extent in milk as it is a nutrient enriched medium for their rapid growth and because of that milk and dairy products get frequently contaminated by this pathogen. Several researchers have reported the high prevalence of *Staphylococcus aureus* in the raw milk sample even in pasteurized milk as they can produce heat-stable enterotoxin [7-9]. However, in Bangladesh, the overall sanitary maintenances of the production, processing, packaging and distribution of milk and hygiene policies have unfortunately been reported not to be within the required standard [10,11]. So *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA) related infections are parting as a heavier public health problem in both hospital and community setting, provoking a wide spectrum of diseases. The ubiquitous use of antimicrobial drugs in animal husbandry and other agricultural activities along with veterinary and human medicine has contributed to antimicrobial resistance setting-up. So beside proliferation of bacteria in milk and other milk products, an additional important concern is the increased antibiotic resistance, possessing a major clinical impediment in treatment especially in developing countries like Bangladesh [2,3,12-16]. Multidrug resistant bacteria can transmit to human from farm animals not only by direct contact with animals but also through contact with, or ingestion of those animal originated foods. So, the rise and spread of MRSA infections, associated with health care as well as community has raised an objection in infection containment intervention and treatment. In Bangladesh, a limited research has been the presence of *S. aureus* in raw milk and cheese [17-19] and still no systematic study has been conducted to isolate and identify this organism from entirely the milk and dairy products. Reports are very scanty on the microbial status of different milk and dairy products. A recent study report by International Centre for Diarrhoeal Disease Research, Bangladesh also revealed that 75 percent of pasteurized milk is unsafe for direct consumption due to heavy bacterial load. It confers more study should be conducted focusing the microbial status and their risk arising from animal originated food products in aspect of Bangladesh. Considering these facts, present study attempted to isolate and characterize *S. aureus* in milk and dairy products available in and around Mymensingh town of Bangladesh which may exhibit a good significance in the field of food.

Materials and Methods

Sample collection

A total of 72 samples of 12 different types of milk and dairy products were collected from different shops and restaurants available

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in and around Bangladesh Agricultural University and Mymensingh City Corporation during the period from January 2017 through December 2017 aseptically using sterile eppendorf tube, falcon tube and polyethylene bag and then immediately transferred to the laboratory at fresh condition.

Bacteria isolation and identification

An aliquot of each sample inoculated into nutrient broth containing test tube and incubated for 6 hours for enrichment. Samples were then spreaded and streaked respectively onto the Staphylococcus Medium No. 110 agar (Oxoid, Egland) and Mannitol Salt agar (HiMedia®, India), specific media for *Staphylococcus* spp. growth and then incubated at 37°C for 48 hours. All the isolates were then subjected to sub-culture on Mannitol Salt agar for obtaining pure colony, Gram staining and culture on blood agar for morphological confirmation. Catalase and coagulase test were done for biochemical confirmation.

Antimicrobial susceptibility test

All the coagulase positive isolates were subjected to antimicrobial susceptibility test by following the disk diffusion method described by Kirby-Bauer [20]. In this study ten commonly available antibiotics such as norfloxacin (30 µg), ciprofloxacin (5 µg), nalidixic acid (5 µg), methicillin (5 µg), oxacillin (1 µg), tetracycline (30 µg), gentamycin (10 µg), erythromycin (15 µg), vancomycin (30 µg) and penicillin (10 µg) of Oxoid and Himedia were used. The zones of complete inhibition were observed and diameters were measured and interpreted as susceptible, or resistant according to the CLSI given standard (Clinical Laboratory Standards Institute) [21].

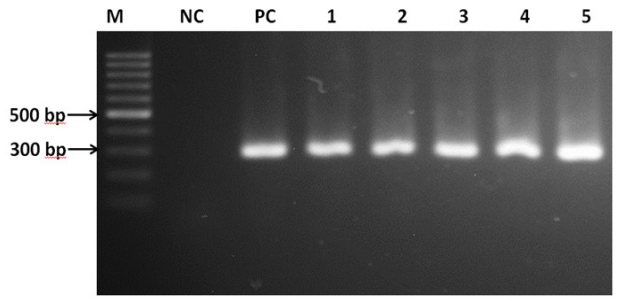

Figure 1: PCR amplification of *nuc* gene of *Staphylococcus aureus* M=100 bp size DNA marker (Tracit, Invitrogen, USA), NC=Negative Control, PC=Positive Control, Lane 1-5=Test Samples (Lane 1= DNA extracted from bacteria isolated from raw milk, Lane 2= DNA from yogurt, Lane 3= DNA from roshmalai, Lane 4= DNA from borhani, Lane 5= DNA from cheese).

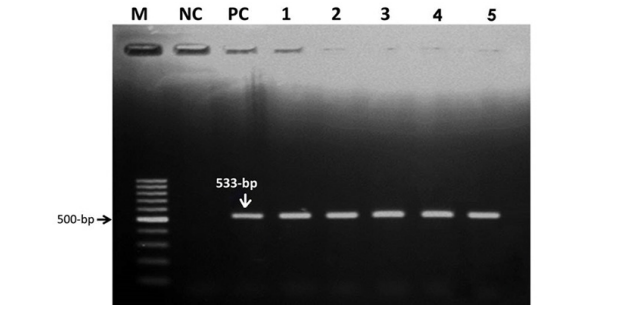

Figure 2: PCR amplification of *mecA* gene of *Staphylococcus aureus* M=100 bp size DNA marker (Tracit, Invitrogen, USA), NC=Negative Control, PC=Positive control Lane 1-5=Test Samples (Lane 1= DNA from raw milk, Lane 2= DNA from yogurt, Lane 3= DNA from roshmalai, Lane 4= DNA from borhani, Lane 5= DNA from cheese).

Molecular characterization of *S. aureus*

Molecular identification and characterization of isolated Methicillin and Oxacillin resistant *S. aureus* strains were carried out by the detection of *nuc* [22], *mec*A [23] and *mec*C [24] gene using three different primer pairs. Polymerase Chain Reaction (PCR) were done following the method described by Kalorey et al., Lee et al. and Stegger et al. (Table 1) [22-24].

Results and Discussion

Out of 72 samples tested, all the samples were found positive for *Staphylococcus* spp. that was confirmed by observing the cultural and morphological characteristics of the isolates grown on specific culture media (Table 2). The appearance of *Staphylococcus* spp. on the Staphylococcus Medium No. 110 agar found deep orange colored pigmented colony which was similarly reported in Bacteriological Analytical Manual, 5th edition, AOAC, Washington DC [25]. According to Kabir et al. [19] *S. aureus* growth on mannitol salt (MS) agar was confirmed by the changed color of media and formation of yellow color colonies due to fermentation of mannitol and in Gram's staining, the organism was Gram positive, violet color, cocci shaped and grapes like cluster under light microscope.

To confirm the initial pathogenicity of the isolated *S. aureus*, catalase and coagulase test were performed as biochemical test that showed 57 isolates were coagulase positive out of 72 and then those were subjected to culture on blood agar (Table 3). Catalase and coagulase positive *S. aureus* were identified by observing the characteristics of bubble formation and formation of curd like clotting accordingly [19]. 12 (25.53%) *S. aureus* was isolated from 47 raw milk sample on the basis of cultural and biochemical properties where all positive isolates showed β-hemolysis on 5% sheep blood containing blood agar media in a study reported by Jahan et al. [17], but this study revealed 52 β-hemolysis producing isolates out of 57 coagulase positive isolates.

The coagulase positive isolates further subjected to antibiotic susceptibility test by using 10 antimicrobials among which the highest resistance was observed against penicillin and lowest against vancomycin. The resistance pattern of coagulase positive *S. aureus* isolates in this study revealed that the most of the isolates (79.16%) were resistant to more than three antimicrobial agents (Table 4). Some isolates (24.56%) showed resistance against both methicillin and oxacillin and intermediately resistant against vancomycin. The pathogenic isolates found in milk and cheese samples exhibited the multi-drug resistance trait against six commonly used antibiotics among which coagulase positive *S. aureus* was found to be highly resistant against oxacillin (100%) and methicillin (58.82%) as reported by Nusrat et al. [18], which were significantly higher than the present study. Based on antibiotic sensitivity test, about 20-25% *S. aureus* isolates were found multidrug resistant and 100% were resistant to penicillin which are very similar to the findings by Jahan et al. [17].

For molecular characterization by PCR among the 14 isolates of methicillin and oxacillin resistant all were *nuc* positive (Figure 1) and 8 isolates from raw milk, yogurt, roshmalai, borhani and cheese were found to be *mecA* positive (Figure 2) which shows 57% was confirmed as MRSA (Table 5) where none of them showed positive result for *mecC* gene (Figure 3) but no isolates were *mecA* positive in a similar study reported by Jahan et al. [17] and yet no report is available on *S. aureus*

expressing *mecC* gene in Bangladesh. Characterization of MRSA by PCR through *mecA* gene demonstration has established as a gold proof method [26,27]. Indicating resistance against β-lactamase drug group the *mecC* is a very emerging gene. The emergence of this *mecC* gene is not yet understood sufficiently. It was described first in samples from bulk tank milk of southwest England region, also have been found in other type of samples at 13 European countries from 14 distinct wild and domestic animal varieties [27].

A number of studies reported that pesticides application in agricultural commodities pose human health risk and cause detrimental effects to the environment. In addition, indiscriminate use of antimicrobial agents in human and animals also affects the human, animal and environment health. However, our study was limited to staphylococcus contaminations of milk and dairy products and their

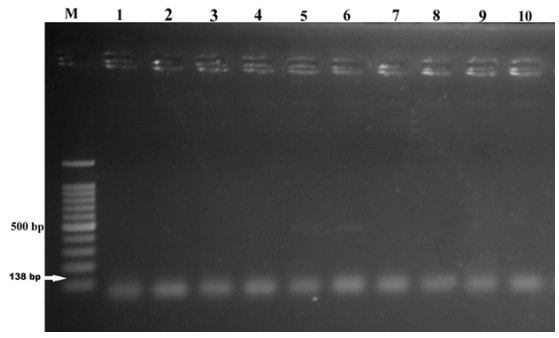

Figure 3: PCR amplification of *mecC* gene of *Staphylococcus aureus* M=100 bp size DNA marker (Tracit, Invitrogen, USA), Lane 1-5=Test Samples (Lane 1,2= DNA from raw milk, Lane 3,4= DNA from yogurt, Lane 5,6= DNA from roshmalai, Lane 7,8= DNA from borhani, Lane 9, 10= DNA from cheese).

Primer name	Gene targeted	Primer sequence (5´-3´)	Amplicon size (bp)	Reference
nuc F	*nuc*	5´GCGATTGATGGTGATACGGT 3´	279	Kalorey et al. [22]
nuc R		5´AGCCAAGCCTTGACGAACTAAAGC 3´		
mecA F	*mecA*	5´AAAATCGATGGTAAAGGTTGG 3´	533	Lee et al. [23]
mecA R		5´AGTTCTGGCACTACCGGATTTTGC3´		
mecC-P1	*mecC*	5'-GAAAAAAAGGCTTAGAACGCCTC-3'	138	Stegger et al. [24]
mecC-P2		5'-GAAGATCTTTTCCGTTTTCAGC-3'		

F=Forward; R=Reverse; bp= Base pair

Table 1: Primers used in this study with sequences.

Total no. of sample	No. of *S. aureus* positive isolates	No. of catalase positive isolates	No. of coagulase positive isolates	No. of isolates showing hemolysis on blood agar
72	72	70	57	52

Table 2: Biochemical characterization of the isolated *S. aureus*.

Cultural characteristics				Morphological and Staining characteristics
Nutrient broth	Staphylococcus Medium No. 110	Mannitol salt agar	Blood agar	Gram positive, purple colored, small cocci shaped organisms arranged in grape like structure.
After overnight incubation the organisms showed turbidity.	Smooth, circular Orange colony.	Smooth, circular, yellowish to orange colonies with changing color of the media due to fermentation of mannitol.	Small round colonies with beta hemolysis.	

Table 3: Cultural, morphological and staining characteristics of the isolated *S. aureus*.

Antimicrobial agents	No. of isolates with their antibiotic susceptibility pattern (%)		
	Resistant	Intermediate	Sensitive
Penicillin	57 (100%)	-	-
Erythromycin	42 (74%)	7 (12%)	8 (14%)
Tetracycline	43 (75%)	9 (16%)	5 (9%)
Gentamycin	21 (37%)	9 (16%)	27 (47%)
Nalidixic Acid	44 (78%)	2 (3%)	11 (19%)
Ciprofloxacin	29 (51%)	5 (9%)	23 (40%)
Norfloxacin	17 (29%)	3 (5%)	37 (66%)
Methicillin	19 (33%)	15 (26%)	23 (41%)
Oxacillin	16 (28%)	6 (11%)	35 (61%)
Vancomycin	-	14 (24%)	43 (76%)

Table 4: Results of antibiotic susceptibility test.

No. of total sample	No. of coagulase positive sample	No. isolates resistant to Methicillin and Oxacillin in antibiotic sensitivity test	No. of isolates used for PCR targeting *nuc*, *mecA* and *mecC* gene	No. of positive isolates in PCR		
				nuc gene	*mecA* gene	*mecC* gene
72	57 (79.16%)	14 (24.56%)	14 (24.56%)	14 (100%)	8 (57%)	0

Table 5: Molecular characterization of *S. aureus*.

antimicrobial susceptibility to common available and antibiotics. It was our limitation and we did not consider the pesticides and antibiotic residues in this research which are also very important to manage and control public health.

To the best of our knowledge, this is the first study investigating *mecC* gene in *S. aureus* isolates from milk and dairy food consumed by people in Bangladesh. Detection of *S. aureus* isolate carrying *mecC* gene will indicate a potential public health problem for our country and may highlight the importance for surveillance and monitoring program of reservoirs related to animal and environment for the existence and evaluation of *mecC* gene harboring *S. aureus* strains.

Conclusion

Foodborne pathogens like toxigenic *S. aureus* can easily be transmitted through the food produced from raw milk under neglected hygienic conditions. Animal-associated strains of MRSA isolation from different dairy products like raw milk or a raw-milk cheese has reported worldwide. This study find out the status of the MRSA present in milk and dairy products available in Mymensingh city of Bangladesh. Considering the outcome of this study a control strategy may be developed to prevent and control the harmful effects of MRSA on public health in aspect of our country. In conclusion, our findings revealed that the milk and dairy product sold in and around Mymensingh town are contaminated with multidrug resistant *S. aureus* which are very alarming for both human and animal health.

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