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Characterization of Bovine Subclinical Mastitis Caused by *Staphylococcus aureus* in Southern Bangladesh by Bacteriological and Molecular Approaches

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ABSTRACT

The disease mastitis caused by different microorganisms can lead to significant yield losses of milk and its quality. The detection of disease at subclinical stage is much more effective to prevent the occurrence of the disease than the detection of the disease at later stages. For determining the prevalence of *Staphylococcus* sp. causing Subclinical Mastitis (SCM) in dairy cows in Chittagong, a Southern district of Bangladesh, milk samples were collected from 4 different dairy farms under Chittagong City Corporation. After initial screening by California mastitis test to identify milk samples infected with SCM pathogens, *Staphylococcus* sp. were isolated and identified by culturing CMT-positive milk samples on selective Mannitol Salt Agar (MSA) medium which was followed by biochemical characterization. PCR was employed to detect *S. aureus* from *Staphylococcus* isolates using species-specific primers. Culture Sensitivity (CS) test was done to determine the antibiotic sensitivity pattern of *Staphylococcus* isolates against 8 commercially available antibiotic discs (ampicillin, amoxycillin, cephalexin, ciprofloxacin, erythromycin, gentamycin, doxycycline hydrochloride and oxytetracycline). To develop plasmid profiles, further extraction of plasmids from *Staphylococcus* isolates were performed. The results of this research showed that the prevalence of SCM was 74.49%. The 26.71% of milk samples were infected with *Staphylococcus* sp., 11.64% were contaminated with coagulase positive *Staphylococcus* and 15.07% with coagulase negative *Staphylococcus*. Molecular study using PCR revealed that the prevalence of *S. aureus* causing SCM was 5.48%. CS test of *Staphylococcus* isolates showed that a high percentage (88.89%) of *Staphylococcus* isolates were resistant to oxytetracycline while most (94.44%) of the *Staphylococcus* isolates were susceptible to ciprofloxacin. Plasmid profile analysis revealed that 72.22% of *Staphylococcus* isolates carried 1 or more plasmids, while further analyses indicate that the multi drug resistance properties may or may not be associated with their presence.

Key words: Mastitis, *Staphylococcus*, PCR, plasmid, Bangladesh

INTRODUCTION

Subclinical mastitis is an important constraint that accounts for high economic losses in dairy farms across the world. Several authors have reported the prevalence and distribution of mastitis

in dairy cows in Bangladesh and most of these dairy farms are confronted with problems of clinical and subclinical mastitis (Rahman *et al*., 1968, 1997, 2010). In both clinical and subclinical forms, mastitis is a frustrating, costly and extremely complex disease that results in a marked reduction in both quality and quantity of milk (Harmon, 1994). The contaminated milk obtained from the affected cow is unfit for human consumption and provide a mechanism to spread of diseases like tuberculosis, sore-throat, brucellosis, leptospirosis etc. (Sharif *et al*., 2009). Subclinical mastitis is more common than clinical mastitis and is responsible for great economic losses in dairy herds (Jasper *et al*., 1982). If subclinical mastitis is not detected early, it may lead to clinical mastitis which is irreversible in most cases. SCM also represents a constant risk of infection for the whole stock of ruminants. So, it is of great importance to diagnose subclinical mastitis at an early stage in order to reduce the economic losses caused by this disease in dairy industry and to protect the human consumers from the diseases caused by consumption of contaminated milk from mastitic milch cows. The primary cause of mastitis is a wide spectrum of bacterial strains, although, incidences of viral, algal and fungal-related mastitis were also reported (Pyorala, 2003). More than 200 infectious causes of bovine mastitis are known to date and the commonest pathogens in large animals are *Staphylococcus aureus*, *Streptococcus agalactiae,* other *Streptococcus* and Coliforms (Kader *et al*., 2002; Sudhan *et al*., 2005; Chahar *et al*., 2008; Sharma, 2008; Yong *et al*., 2009; Sharma and Maiti, 2010). Although, a number of bacteria can cause mastitis, *S. aureus* has emerged as one of the most prevalent pathogens which, once established in the mammary gland is difficult to eradicate (Nickerson *et al*., 1995). Studies from Asian countries also report *S. aureus* as the chief etiologic agent of mastitis in cattle and buffalo (Kang-Hee *et al*., 2001; Sharma *et al*., 2007; Abdel-Rady and Sayed, 2009; Rahman *et al*., 2010; Sharma and Maiti, 2010).

The need for reliable and rapid methods for identification of *S. aureus* is crucial for the control of disease and for economically sound udder health management (Hameid *et al*., 2004). Molecular methods provide accurate confirmation of the identity of microorganism isolated from a sample. In most laboratories of Bangladesh, this is done by identification of phenotypic traits of cultured bacteria, which sometimes provide confusing results. Again it needs sufficient time for bacterial growth in culture medium. On the contrary, the PCR-based approach is a rapid, efficient and cost-effective tools for accurate characterization of the causal agents. Worldwide a number of reports are available describing the use of PCR in identification and characterization of Staphylococcal isolates (Geha *et al*., 1994; Canvin *et al*., 1997; Gribaldo *et al*., 1997; Hameid *et al*., 2004). The objective of this present work was; (1) To estimate the prevalence of *Staphylococcus aureus* causing SCM in selected areas, (2) To detect *Staphylococcus* sp., in milk sample by routine culture and biochemical tests, (3) To optimize modern molecular tools like PCR as a confirmatory test for diagnosis of *S. aureus* from mastitic milk samples, (4) To perform CS test to reveal antibiotic sensitivity of *Staphylococcus* isolates against some commercially available antibiotic discs and (5) To develop plasmid profiles from bacterial isolates with a view to detect their significance for drug resistance patterns of different isolates.

MATERIALS AND METHODS

Collection of milk samples: A total of 196 milk samples were collected from 4 selected local dairy farms located in urban and periurban areas of Chittagong metropolitan area of Bangladesh. Before sampling, the teat end was scrubbed with cotton soaked in 70% ethanol. During sampling, the first squirt of milk was discarded since their cells and bacterial counts were likely to reflect the situation within the teat rather than that of the udder as a whole (Radositis *et al*., 2000). In the

field, the California Mastitis Test (CMT) was performed immediately to confirm positive mastitic samples. All CMT positive milk samples (collected in sterile containers from infected teats) were placed on ice and transported to PRTC (Poultry Research and Training Centre) lab of Chittagong Veterinary and Animal Sciences University (CVASU) for further analysis.

Bacteriological approaches: One loopful of milk from each sample was streaked on each mannitol salt agar plate. The plate was incubated at 37°C and examined after 24-48 h for growth and change in the color of the medium. White or yellow (golden yellow) colonies with yellow halo or cream or pink colonies grown on MSA were *Staphylococcus* sp. All *Staphylococcus* sp. identified were grown on nutrient agar slant and subjected to gram staining, catalase test and coagulase test.

Molecular approaches

DNA extraction: DNA was extracted from all the *Staphylococcus* sp. isolated from milk samples according to the method published earlier (Salehi *et al.*, 2005). Briefly, pure bacterial culture from nutrient agar slant was subcultured in nutrient broth medium. Each mililitre broth culture was taken in separate eppendorf tube and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and any remaining liquid was removed by soaking (with wipes). The pellet was collected and replenished with 200 µL autoclaved deionized water followed by finger shaking to dissolve the pellet. The cap of the eppendorf tube was pierced by sterile needle before placing it in a water bath at 100°C for 10 min. Immediately after boiling, the eppendorf tube was kept in ice for 10 min followed by centrifugation at 10,000 rpm for 10 min. Finally, around 100-150 µL supernatant containing bacterial chromosomal DNA was collected and stored at -20°C.

PCR amplification: Following DNA extraction, PCR analysis was done based on 16S-23S ribosomal RNA intergenic spacer region as described before (Ghorbanpour *et al.*, 2007; Forsman *et al.*, 1997): Forward primer: 5'- TCTTCAGAAGATGCGGAATA -3' Reverse primer: 5'-TAAGTCAAACGTTAACATACG -3'. All the PCR reaction materials (Go Taq Green Master Mix (2X) (Promega), forward and reverse primers, template and nuclease free water) were mixed in a PCR tube and the run condition was primary denaturation at 94°C 2 min, denaturation for 30 sec, annealing at 55°C for 30 sec, extension for 30 sec and final extension at 72°C was maintained for 5 min. The total reaction cycle was 30. Along with each set of PCR reaction, a positive control with known *S. aureus* DNA template and a negative control (water instead of extracted DNA) were used as known standards.

PCR products were electrophoresed in a 1.5% agarose gel containing 500 µg mL^{-1} of ethidium bromide and the gel was visualized by UV transilluminator (Biometra GmBH, Germany).

Culture Sensitivity (CS) test of isolated *Staphylococcus* sp.: The isolated bacterial isolates were subjected to CS test. Each of the isolates was first inoculated in 3 mL of nutrient broth in a test tube separately. The test tubes were then incubated at 37°C overnight. On the following day with the help of a cotton bud, each of the samples was spread on Mueller-Hinton agar plate separately. Commercially available antibiotic discs were placed on the edge of the plate and a control disc soaked with autoclaved distilled water was placed in the center of the plate. Then, the plate was incubated at 37°C for 24 h. After incubation, diameters of zones of inhibition were measured with respect to the data available from National Committee for Clinical Laboratory Standards (NCCLS, USA). Then, the tested organism was reported as 'sensitive', 'intermediate' or

'resistant'. The antibiotic discs used were ampicillin, amoxycillin, cefalexin, ciprofloxacin, erythromycin, gentamycin, doxycycline hydrochloride and oxytetracycline.

Plasmid extraction from *Staphylococcus* isolates: Plasmid was extracted from *Staphylococcus* isolates according to the method based on Sambrook and Russell (2001).

RESULTS

Bacteriological analysis: The prevalence of subclinical mastitis in dairy cows of Chittagong region was found to be 74.49% (146 of 196 samples) by CMT screening. All the colonies isolated using mannitol salt agar as a selective medium were found to be gram positive and catalase positive. The 17 of the isolated 39 colonies were coagulase positive. Overall, 26.71% of CMT-positive milk samples were found to be infected with *Staphylococcus* sp. from this research work. Only 11.64% samples were found to be infected with coagulase-positive *Staphylococci* and 15.07% with coagulase-negative *Staphylococci* (Table 1).

Molecular analyses

Optimization of DNA template concentration: The bacterial DNA was extracted from the culture derived colonies by classical heat-thaw method and subsequently used for PCR analysis. To optimize the reaction condition, PCR was performed with different concentrations of template DNA (2, 4, 6, 8 and 10 µL) isolated from known *S. aureus* samples. Strong specific band was found at concentration of 2 µL (Fig. 1). Less bright band was found at other concentrations and no band was found at concentration of 10 µL. For all subsequent PCR, 2 µL DNA template concentration was used during this study.

Prevalence of *Staphylococcus aureus*: Through PCR analyses, 41.18% (7 out of 17) of coagulase positive *Staphylococci* samples were confirmed as *Staphylococcus aureus*. Overall, *S. aureus* was obtained in 5.48% of all CMT positive samples. The *S. aureus* strain was identified on the basis of 420 bp PCR product corresponding to the 16S to 23S rRNA intergenic spacer region on 1.5% agarose gel (Ghorbanpour *et al*., 2007). The results of PCR identification of *S. aureus* have been presented in Fig. 2.

Antibiotic susceptibility pattern: The antibiotic susceptibility pattern was examined for 36 *Staphylococcus* isolates by antibiotic disc diffusion method. Results of culture sensitivity test have been displayed in the Table 2. According to this result, 88.89 and 83.33% *Staphylococcus* isolated from CMT positive milk samples were resistant to oxytetracycline and doxycycline hydrochloride, respectively, while cephalexin showed resistance (55.56%), erythromycin (19.44%), ampicillin and amoxicillin (11.11%) and least resistance (5.56%) was found to ciprofloxacin and gentamycin. The

Table 1: Results of different (biochemical and bacteriological) tests of milk samples

Total milk samples	CMT test (positive)	Mannitol salt test (positive)	Gram staining (positive)	Catalase test (positive)	Coagulase test (positive)	
					Slide coagulase test	Tube coagulase test
196	146	39	39	39	13	4
					Total coagulase positive = 17	

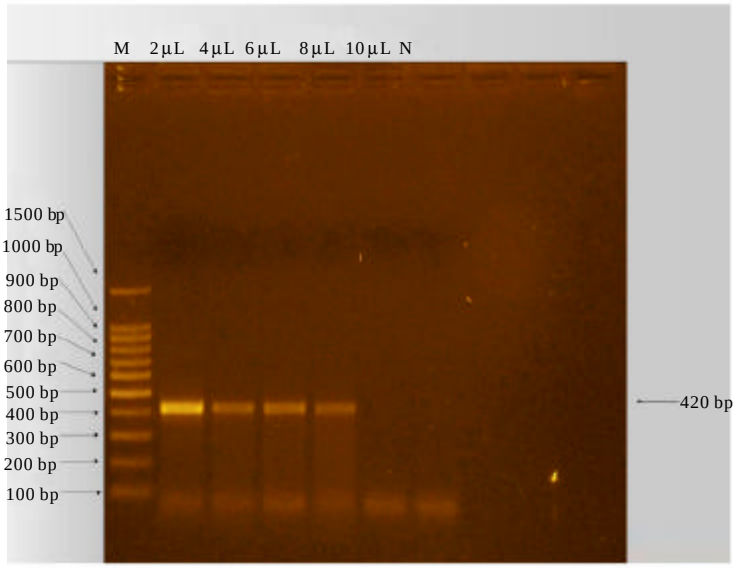

Fig. 1: Optimization of DNA template concentration. Strong band was found at 2 µL of template concentration. No band was found at 10 µL of template concentration, M: Marker DNA, N: Negative control

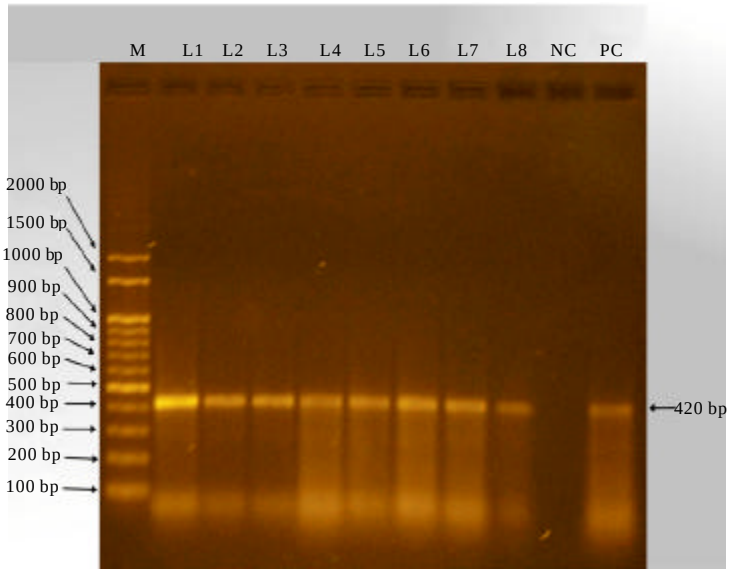

Fig. 2: Agarose electrophoresis (1.5%) of the product of PCR that has amplified 420 bp gene fragment of *S. aureus*. M: Marker DNA, L1: Positive sample 16, L2: Positive sample 53(o), L3: Positive sample 57(o), L4: Positive sample 63(o), L5: Positive sample 64, L6: Positive sample 65(o), L7: Positive sample 76(o), L8: Positive sample 78(o), NC: Negative control, PC: Positive control, Here, the sample (o) means the colony which was found to be orange colored on mannitol salt agar plate and the rest were cream colored colony

Table 2: Susceptibility of *Staphylococcus* isolates to 8 different antibiotics

Antibiotic (μg disc^{-1})	Resistant (R)	%	Intermediate (I)	%	Susceptible (S)	%
AMP* (25)	4	11.11	14	38.89	18	50.00
CN 30	20	55.56	1	2.78	15	41.67
CIP 5	2	5.56	0	0.00	34	94.44
AMX 30	4	11.11	6	16.67	26	72.22
E 15	7	19.44	3	8.33	26	72.22
DO 30	30	83.33	4	11.11	2	5.56
OT 30	32	88.89	2	5.56	2	5.56
GEN 10	2	5.56	16	44.44	18	50.00

*AMP: Ampicillin, CN: Cephalexin, CIP: Ciprofloxacin, AMX: Amoxicillin, E: Erythromycin, DO: Doxycycline hydrochloride, OT: Oxytetracycline, GEN: Gentamycin

highest susceptibility of *Staphylococcus* (94.44%) was found toward ciprofloxacin. The 72.22% of *Staphylococcus* was susceptible to amoxicillin and erythromycin, 50% to ampicillin and gentamycin, 41.67% to cephalexin and 5.56% to doxycycline hydrochloride and oxytetracycline. *Staphylococcus* sp. showing moderate susceptibility to gentamycin and ampicillin were 44.44 and 38.89%, respectively.

Plasmid profile analyses: Plasmid profiles of these 36 *Staphylococci* isolates were examined during this study with a view to identify their relationship with drug resistance pattern of different isolates. Plasmids were extracted from 36 *Staphylococcus* isolates. Plasmid profile analysis revealed that 72.22% (26 out of 36) of the *Staphylococcus* isolates were having one or more plasmids. From the result of CS test, it has been found that 21 out of these 26 plasmid bearing isolates are Multiple Drug Resistant (MDR). One of the rest 5 isolates was found as susceptible to all of the tested antibiotics. The remaining 4 plasmid-bearing isolates were resistant to 1 drug. Plasmids with 2 different sizes were observed. The size of the plasmids was found to be >10 kb. No plasmid DNA was found in 10 of 36 (27.78%) *Staphylococcus* isolates. Among these 10 isolates, 9 were multiple drug resistant and 1 was single drug resistant. Images of gels with extracted plasmid DNA have been shown in the Fig. 3a, b.

DISCUSSION

The California Mastitis Test (CMT) is a widely used and user-friendly test to detect subclinical mastitis. During this study the prevalence of Subclinical Mastitis (SCM) determined by CMT was found to be 74.49% in selected farms. This observation was similar with that of Verma (1978), Singh and Baxi (1980), Motie *et al.* (1985) who reported 36.1-61%, 54.0 and 54-81.5% SCM with indirect tests in dairy cattle, respectively. Karimuribo *et al.* (2008) reported 75.9% prevalence of subclinical mastitis with CMT in dairy cows. However, several early reports in the country reported fewer cases of mastitis as determined through CMT. For example, Prodhan *et al.* (1996) reported 15.8% incidence of SCM with CMT, Sen *et al.* (1996) reported 14.4% incidence, Islam *et al.* (2012) reported 29.5% and Khakpoor *et al.* (2011) reported 30.76% incidence of SCM with CMT in dairy cows. However, as there are lots of risk factors associated with the incidence and prevalence of mastitis in any particular area, further analysis of those factors can describe the high rate of infection as found in present study.

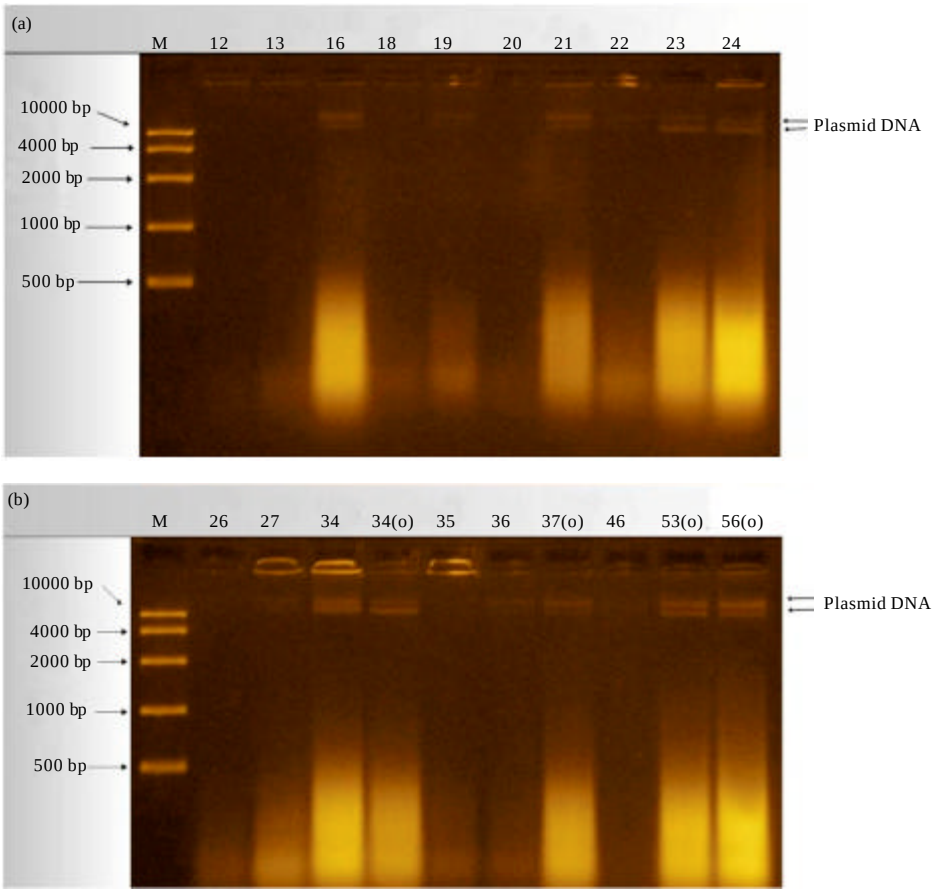

Fig. 3(a-b): Plasmid profile of 20 *Staphylococcus* isolates, M: Marker DNA. Here, the sample (o) Colony which was found to be orange colored on Mannitol Salt Agar (MSA) plate and the rest were cream colored colony

A number of bacterial culture techniques are available for *Staphylococcus* sp. During this study, all 146 CMT positive milk samples were cultured on Mannitol Salt Agar (MSA) plate. MSA is a selective medium for *Staphylococcus* due to the high concentration of sodium chloride that the agar contains (Leboffe and Pierce, 2002). Several biochemical tests are also in place for confirmation of bacterial isolates in any selective media. During this study, all the colonies grown on MSA plate were subjected to gram staining and catalase test. Gram staining of colonial isolates showed that the bacteria were gram positive, cocci and arranged in irregular, grapelike clusters, which is characteristic of *Staphylococcal* species (Holt *et al*., 1994). All the colonies were also catalase positive. All gram and catalase positive bacterial colonies were subjected to coagulase test. Ultimately the results of these colony properties and biochemical tests were complemented with that of molecular PCR-based analysis.

Overall, 26.71% of milk samples were found to be contaminated with *Staphylococcus* sp. by bacteriological analysis during this work. Notably, this rate might vary from country to country. Sindhu *et al*. (2010) isolated *Staphylococcus* sp. from 21.25% milk samples of cows in India. Chu *et al*. (2012) found 37.9% prevalence of *Staphylococcus* sp. in goat milk in Taiwan,

Addis *et al.* (2011) reported 39.5% raw bovine milk to be contaminated with *Staphylococcus* sp. in Debre Zeit in Ethiopia. In separate studies, Ndegwa *et al.* (2007) and Taufik *et al.* (2008) reported 60.3 and 78.7% *Staphylococcus* sp. infection in goat milk in Kenya and Indonesia, respectively. Rahman *et al.* (1968) found 42.68% (35 out of 82) cow milk samples to be contaminated with *Staphylococcus* sp., in Mymensingh in Bangladesh. The prevalence of *Staphylococcus* sp., causing subclinical mastitis in dairy cows was reported to be 31% in Sylhet region of Bangladesh by Rahman *et al.* (2010).

In this study, Coagulase Positive *Staphylococci* (CPS) and Coagulase Negative *Staphylococci* (CNS) were isolated from 11.64 and 15.05% CMT positive milk samples, respectively. The coagulase positive *Staphylococci* may include *Staphylococcus aureus*, *Staphylococcus intermedius* and *Staphylococcus hyicus* and coagulase negative *Staphylococci* may include *S. epidermidis*, *S. chromogenes*, *S. haemoliticus*, *S. warneri* and *S. saprophiticus*. These results were similar to that obtained by Baranski *et al.* (2008) and Addis *et al.* (2011) in similar studies earlier. Further species specific PCR analysis can elucidate the extent of infection by each different type of *Staphylococci*.

PCR is a powerful tool, where DNA template and specific primers are used for molecular identification of any specific bacteria. Optimization of PCR is an important issue for any successful PCR run. During this study, the concentration of template DNA was optimized by running PCR with known positive samples. Five different concentrations of template (2, 4, 6, 8 and 10μL of DNA in a 25 μL reaction) were tested where strong single band was found at concentration of 2 μL. For all subsequent reactions, similar template proportion was used. According to PCR results, 41.18% (7/17) of coagulase positive *Staphylococci* samples were *Staphylococcus aureus*. This result is in accordance with the result reported by Khakpoor *et al.* (2011) who detected 52.63% (10/19) of coagulase positive *Staphylococci* samples as *S. aureus* using PCR. In case of one isolate, it was found that although biochemical test confirmed it as a coagulase negative *Staphylococcus*, however, PCR analysis revealed it as *S. aureus*. This might indicate the limitations of coagulase test. Again, this coagulase negative *Staphylococcus* may probably be MRSA (Methicillin Resistant *S. aureus*) isolates which are reported to react weakly or negatively with tube coagulase test or they may be rare *S. aureus* which are reported to be coagulase negative (Kateete *et al.*, 2010; Koneman *et al.*, 1997). During this study, only 5.48% (8 out of 146 samples) of CMT positive milk samples were found to be infected with *S. aureus* through PCR. This is almost similar to other previous reports in other countries. For instance, Fagundes *et al.* (2010) reported 7.3% prevalence of *S. aureus* in raw milk following molecular investigation in Sao Paulo state of Brazil. Baranski *et al.* (2008) reported 1.92% incidence of *S. aureus* at quarter level of dairy cows in North-East Poland. Chu *et al.* (2012) found 2.9% prevalence of *S. aureus* in goat milk in Taiwan. However, as mentioned before, this may vary from country to country and region to region and a different prevalence rate of *S. aureus* is reported by other investigators working with mastitis pathogens. For instance, Krystyna *et al.* (2003) obtained *S. aureus* in 72.5% of milk samples in Poland. *S. aureus* was isolated from 28.33% of milk samples in Turkey by Kirkan *et al.* (2005). In Ethiopia, Bitew *et al.* (2010) reported 20.3% prevalence of *S. aureus* in milk samples. This variability between different reports could be attributed to differences in farm management practices or to differences in study methods and instruments employed by different researchers. The antibiotic sensitivity tests are routinely done to select best drug to treat mastitis. During this study, a total of 36 *Staphylococcus* isolates were subjected to CS test. The activities of antibiotics against *Staphylococcus* showed the varying levels of multiple antibiotic resistance.

The results of CS test obtained by some researchers correspond with the results found in this study. For instance, Rajala-Schultz *et al.* (2004) found 12.2% resistance of CNS against ampicillin. Baranski *et al.* (2008) reported 9.3% CNS and 12.5% *S. aureus* to be resistant to erythromycin. 21.56% of *Staphylococci* isolated from cow milk were resistant to erythromycin according to Celik and Solmaz (2010). 100% of *S. aureus* strains isolated from bovine milk were sensitive to ciprofloxacin reported by Kirkan *et al.* (2005). Due to the observed high resistance seen in cows with SCM against certain antibiotics in this study and due to the variations in the result of CS test obtained by different researchers indicate that the effectiveness of antibiotic treatment administered without carrying out antibiogram tests will be compromised. However, according to the present study, *Staphylococcus* sp. was the most susceptible to ciprofloxacin, amoxicillin and erythromycin which could be the best choice of treatment in case of *Staphylococcus* infection. Gentamycin and ampicillin are also a good choice to treat *Staphylococcus* infection.

Plasmid profiles of these samples were examined to correlate the presence of plasmid with the resistance to single or multiple drugs. Plasmids were extracted from 36 *Staphylococcus* isolates found during this study. Plasmid profile analysis revealed that 72.22% (26 out of 36) of the *Staphylococcus* isolates were plasmid bearing. From the result of CS test it has been found that 21 of these 26 plasmid bearing samples are Multi-Drug Resistant (MDR). However, one of the rest 5 samples is susceptible to all of the tested antibiotics. So, no genes resistant to these tested antibiotics are present in the plasmid of this *Staphylococcus* isolate. The rest 4 plasmid bearing isolates are single drug resistant. Plasmids with two different sizes were observed. The size of the plasmids was found to be >10 kb. Further analysis using drug resistant gene-specific primers can increase our understanding of the MDR properties of different *S. aureus* isolates. Again, no plasmid DNA was found in 10 of 36 (27.78%) *Staphylococcus* isolates. Among these 10 samples, 9 were multi-drug resistant and 1 was single drug resistant.

The fact that 58.33% (21 out of 36) of *Staphylococcus* isolates carrying plasmids showed MDR properties indicate that plasmids have considerable importance in developing resistance through horizontal gene transfer. However, the absence of plasmids in some isolates (25%, 9 of 36) with MDR properties proves that antibiotic resistance is not always dependent on the presence of plasmid. Further analysis is warranted to this analysis which will help us elucidate mechanism of drug resistance in mastitic organisms.

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