Identification, Molecular Detection and Antibiogram Profile of Bacteria Isolated from California Mastitis Test Positive Milk Samples of Crossbred Cows of Satkhira District in Bangladesh

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***Abstract*—Present study was conducted for the isolation, identification, molecular detection and antibiotic sensitivity patterns of the bacteria present in the California Mastitis Test (CMT) positive milk samples of clinical and sub-clinical mastitic crossbred (Friesian cross and Sahiwal cross) cows of Satkhira district. A total number of 598 quarters milk samples from 150 cows were tested by CMT kit, among them 82 quarters from 52 cows were positive for CMT. The bacteria isolated from 52 CMT positive milk samples were coagulase positive *Staphylococcus aureus* 27 (49.09%) followed by *Escherichia coli* 15 (27.27%), coagulase negative *Staphylococcus* (CNS) spp. 10 (18.18%) and *Bacillus* spp. 3 (5.45%) respectively. In this present study only 13 (48.15%) positive *Staphylococcus aureus* were positive against nuc gene by PCR. All the isolates of *E.coli* were further confirmed by PCR with species specific 16srRNA PCR. None of the isolates of *E.coli* revealed positivity in PCR by stx1 and stx2 genes. All the coagulase positive *Staphylococcus aureus* isolates were found sensitive to 6 antibiotics and resistant to 5 antibiotics. The Coagulase negative *Staphylococcus* spp. was also sensitive to 7 antibiotics and acquired resistant properties against 3 antibiotics. Most *E.coli* isolates were found resistant against 9 antibiotics already possessed multidrug resistant properties and sensitive to only 3 antibiotics. *Bacillus* spp. isolated from milk samples was highly sensitive against 5 antibiotics compare to other antibiotics of the panel. Antibiogram profile of this study revealed that, all the isolated bacteria were sensitive to Ciprofloxacin only.**

***Keywords*—CMT, PCR, nuc gene, stx1, stx2, Antibiogram.**

Manuscript received January 24, 2014. This work was supported in part by the Department of Microbiology and Hygiene, Bangladesh Agricultural University and Bangladesh Agricultural Research Council.

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I. Introduction

Bangladesh has a good number of both the pure and cross-bred cattle population (21.5 million) with about 3.33 million of dairy cows [1]. Around 20,582 mini dairy farms each with five or more high yielding cross-bred cows have been established in private sector in Bangladesh [2] and most of these dairy farms are confronted with problems of clinical and sub-clinical mastitis [3]. The clinical mastitis can be diagnosed on the basis of history and clinical findings but laboratory examinations are required to ascertain the sub-clinical mastitis. It is now a well known fact that the Sub clinical Mastitis (SCM) is more serious and is responsible for greater loss to the dairy industry in Bangladesh and the annual economic losses incurred due to reduced milk production alone by SCM in Bangladesh have been estimated to Taka 122.6 million (US $ 2.11million) [4]. Besides causing huge economic losses of milk production, the SCM remain as a continuous source of infection to other herd mates. If the infection persists for longer periods, then it may form a fibrous tissue barrier between the organisms and the antibiotics therapy, thus, limiting their efficacy. It is, therefore, important to know the prevalence of SCM in dairy herds and delineate the important factors responsible for it. The SCM can be known only after laboratory examination, as there are no gross inflammatory changes in the udder tissue. The mastitis causing organism, *Staphylococci*, the chief udder pathogen, has been isolated from almost all the body site examined and environment but *Streptococci* from fewer body sites, whereas the prevalence of *Escherichia coli* has been reported to be widespread [5], [6]. The indiscriminate use of antibiotics for the treatment of bacterial mastitis makes the udder more susceptible for the development of resistant bacteria. It is therefore also important to study the sensitivity pattern of different bacteria isolated time to time from mastitic cows in different geographical zones of the country in order to formulae appropriate therapeutic measures with suitable antibiotics. Considering the above facts, this research work was undertaken with the objectives of isolation and identification of important bacterial population from CMT positive milk

samples of the crossbred mastitic cows of Satkhira district and to evaluate the degree of sensitivity of the antibiotics panel to the bacterial isolates in this study.

II. Materials and Methods

A. Study Area, Period and Population

The study was conducted on two kinds of cross-bred lactating cows in four Upazila of Satkhira districts namely Sadar Upazila, Assasuni Upazila, Debhata Upazila and Tala Upazila. The study was carried out during the period of November 2012 to April 2013.The study was carried out on 68 dairy farms in Satkhira district. The complete herd size (including calves and young stock) varied between 2-25 animals and the number of lactating animals ranged 1 to 12 on the visited farms. The majority of animals were cross-breed Friesian and Sahiwal. All cows of the farm areas were milked by hand. A total of 150 lactating cows at different stages of lactation, parity and rate of milk production were included in the study. Of the total 150 cows selected for this study 115 were Friesian cross and 35 were Sahiwal.

B. Isolation and Identification of Bacteria

CMT positive milk samples were transported into the Bacteriology laboratory of the department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh for isolation, identification and molecular detection with antibiogram studies of the bacterial isolates. CMT positive milk samples were first inoculated into the Nutrient broth and incubated at 37°C for 24 hours. To determine the cultural characteristics of each bacterial isolate the primary cultured broth was further inoculated onto different media (BA, NA, EMB, SSA, MSA, MCA and SMA) and incubated at 37°C for overnight. The morphological characteristics of each isolate of bacteria were determined by Gram's staining technique and the motility by hanging drop preparation. Various biochemical tests such as catalase test, coagulase test, sugar fermentation test, MR-VP test and Indole test were conducted to identify the bacterial isolates in this study.

C. Molecular Detection

1) DNA extraction: Isolated bacteria were subcultered on blood agar and DNA was extracted from colonies by simple boiling method. After incubation period, fresh colonies were suspended in 500 µl of DEPC-treated water (DNase-RNase free). The suspension was held in a 100ºC of water bath for 10 min. After centrifugation at 10000 rpm for 5 min, the supernatant containing bacterial DNA was used as a template for subsequent PCR mixture [7].

2) Polymerase Chain Reaction (PCR): PCR was carried out to reconfirm the biochemically characterized *Staphylococcus aureus* and *E.coli* by using gene specific primers against nuc gene for *Staphylococcus aureus* and 16srRNA gene specific for *E.coli* respectively. Genetically confirm each isolate of *E.coli* was further subjected to stx1 and stx2 gene specific PCR to know the prevalence of shiga toxin producing *E.coli* in the CMT positive milk samples. For the detection of *nuc* (*S. aureus* specific) gene 5'-GCG ATT GAT GGT GAT ACG GTT-3' was used as forward primer and 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3' [8] was used as reverse primer. For the detection of 16S rRNA (*E.coli* specific) 5'-GAC CTC GGT TTA GTT CAC AGA-3' was used as the forward primer and 5'-CAC ACG CTG ACG CTG ACC A-3' [9] was used as the reverse primer. Two primers, Forward: 5'- AAA TCG CCA TTC GTT GAC TAC TTC T-3'and Reverse: 5'- TGC CAT TCT GGC AAC TCG CGA TGC A-3' [10] were used to detect *stx1* (Shiga toxin 1 producing) gene and Forward: 5'- CGA TCG TCA CTC ACT GGT TTC ATC A -3'and Reverse: 5'- GGA TAT TCT CCC CAC TCT GAC ACC-3' [10] were used to detect *stx2* (Shiga toxin 2 producing) gene. Firstly, the required number of 0.6 µl of PCR tubes were labeled and kept on ice. Then 20 µl [Master mix (12.5 µl), Forward Primer (1·0 µl), Reverse Primer(1·0 µl) and DEPC-treated water (5·5 µl)] of reaction mixture was dispensed into each of the PCR tubes and 5 µl of extracted DNA from each sample was added to the respective tube and mixed properly with the help of the spinning machine. Then the tubes were placed in the wells of thermocycler (Eppendrof mastercycler personal, Germany). Total cycles and thermal profile for every PCR program was set in the thermocycler according to the thermal profile mentioned below. The amplification of DNA was performed as follows: For nuc gene 95ºC for 5 min of initial denaturation; 30 cycles of 95ºC for 60 s, 55ºC for 45 s and 72ºC for 90 s; and a final extantion at 72ºC for 10 min. For EC16SrRNA gene 95ºC for 3 min of initial denaturation; 30 cycles of 94ºC for 45 s, 58ºC for 45 s and 72ºC for 60 s; and a final extantion at 72ºC for 3 min. For stx1 and stx2 gene 94ºC for 5 min of initial denaturation; 30 cycles of 94ºC for 60 s, 62ºC (stx1), 58ºC (stx2), 60 s and 72ºC for 2 min; and a final extantion at 72ºC for 5 min. After completion of PCR, PCR products were loaded onto 1.5% Agarose Gel containing 1 µg/ml ethidium bromide. The 279-bp (*nuc*) and 585-bp (16SrRNA) amplified DNA fragments were seperated by agarose gel electrophoresis and visualized under UV-light.

D. Antimicrobial Resistance Pattern Test

Antimicrobial susceptibility test was conducted on randomly selected isolates were isolated during the study. The isolates were tested for 9 antimicrobials using the Kirby-Bauer disk diffusion method according to performance standards of CLSI [11]. The following antimicrobial disks (Oxoid, Basing Stoke, UK) with their corresponding concentration were used: Ampicillin (10 µg), amoxacillin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), oxytetracycline (30 µg), sulfamethoxazole-trimethoprim (25 µg) and tetracycline (30 µg). The inhibition zone was reported as the diameter of the zone surrounding the individual disk in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible are according to the guide lines of the NCCLS.

III. Results

1) CMT Result: Milk samples of 150 crossbreed dairy cows from Satkhira district were tested by CMT kit. Out of 150 tested cows, only 52 (34.66%) samples were positive in

CMT test while the rest 98 (65.34%) cows were negative. The result of CMT was recorded into four categories i.e. trace, weak, distinct and strong based on the intensity of gel formation. Around 600 quarters of 150 cows were examined for any abnormalities only 2 quarters were found blind whereas the other 598 quarters were more or less healthy and functioning normal. Out of the total 598 quarters milk samples of 82 (13.71%) quarters were positive in CMT test. Out of 82 positive quarters for CMT, milk of 44 (14.66%) front quarters and 38 (12.75%) of the rear quarters were found positive.

2) Isolation and Identification of Bacteria: The most common bacteria isolated in this study was coagulase positive *Staphylococcus aureus* 27 (49.09%) followed by *Escherichia coli* 15 (27.27%), coagulase negative *Staphylococcus* (CNS) 10 (18.18%) and *Bacillus* spp. 3 (5.45%) in CMT positive milk samples.

3) PCR Result: In this present study 13(48.15%) coagulase positive *Staphylococcus aureus* among 27 isolates were gave positive result against nuc gene (Fig.1). All isolated *E.coli* (100%) were confirmed by PCR with *E.coli* specific 16s rRNA gene (Fig.2) in current study but negative in case of stx1 and stx2 gene.

4) Antimicrobial Susceptibility Test Result: The antibiotic sensitivity test revealed that coagulase positive *Staphylococcus aureus* isolates were found sensitive to 6 antibiotics and resistant to 5 antibiotics. All coagulase positive *Staphylococcus aureus* were sensitive to Ciprofloxacin (100%) and the least number of *S. aureus* were sensitive to Tetracycline (40%). Gentamicin (80%) and

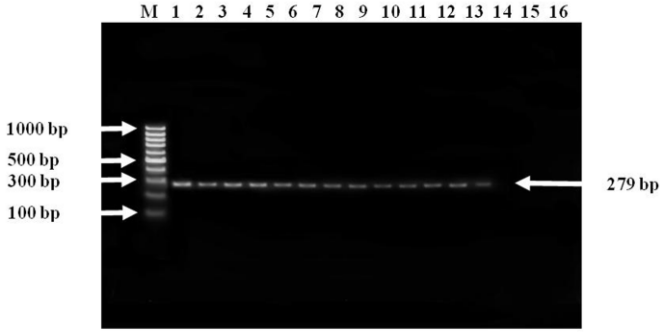

Fig. 1. 1.5% agarose gel Electrophoresis stained with ethidium bromide shows band of nuc gene specific DNA for the *Staphylococcua aureus* in PCR method. M=100bp DNA marker; Lane 1=Positive control; Lanes 2-13= PCR product of isolated coagulase positive *Staphylococcua aureus* (279bp); Lanes 14-16=Isolated Coagulase positive *Staphylococcua aureus* showing negative results.

Sulfamethoxazole-trimethoprim (70%) were also sensitive. 90% tested coagulase positive *Staphylococcus aureus* were resistant to Ampicillin. Already 5 antibiotic resistant *staphylococcus aureus* were found. In case of Coagulase negative staphylococcus spp. these were found sensitive to 7 antibiotics and acquired resistant properties against 3 antibiotics. The antibiotic sensitivity test revealed that coagulase negative *Staphylococcus* spp. was sensitive to Ciprofloxacin (100%), Gentamicin (100%), Sulfamethoxazole-trimethoprim (100%), Erythromycin (80%), Kanamycin (80%) and Tetracycline (60%). On the

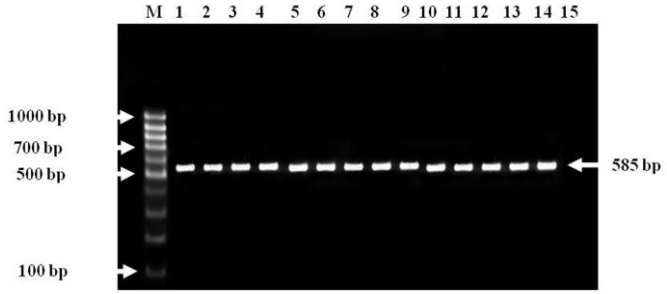

Fig. 2. Results of 16srRNA gene specific PCR product of *E.coli* isolated from CMT positive milk samples, analyzed by using 1.5% agarose gel electrophoresis. M=100bp DNA marker; Lane 1= Positive control of *E.coli*; Lanes 2-14= PCR products of isolated *E. coli* (585bp); Lane 15=Negative control.

other hand, Ampicillin, Amoxicillin and Oxytetracycline were less sensitive. On the other hand, most *E.coli* isolates were found resistant against 9 antibiotics and found sensitive to only 3 antibiotics. The antibiotic sensitivity test showed that highest number of E.coli isolates were sensitive to Ciprofloxacin (90%) and completely resistant to Ampicillin (100%) and Amoxicillin (100%). *Bacillus* spp. isolated from milk samples was found highly sensitive against 5 antibiotics compared to other antibiotics of the panel. The antibiotic sensitivity test indicated that highest number of other *Bacillus* spp. were susceptible to Ciprofloxacin (100%) and Kanamycin (100%) and least to Ampicillin and Amoxicillin.

IV. Discussion

In this study, four different types of bacteria were isolated from 52 CMT positive milk samples. The isolated bacteria were coagulase positive *Staphylococcus aureus*, coagulase negative *Staphylococcus* spp. *E. coli.* and *Bacillus* spp. In this study, 27 (49.09%) isolates were coagulase positive *Staphylococcus aureus*, 10 (18.18%) isolates were coagulase negative *Staphylococcus* spp., 15 (27.27%) isolates were *E.coli* and 3(5.45%) were *Bacillus* spp. These findings nearly supported the findings of Mahbub-E-Elahi *et al.* [12] in which isolated and identified bacteria were *Staphylococcus aureus* (31.33%), coagulase negative *Staphylococcus* spp. (18.0%), *Streptococcus* sp. (14.0%), *Corynebacterium pyogenes* (8.0%), *E. coli* (6.0%), *Bacillus* sp. (4.7%), and 18.0% unidentified bacteria from the cases of bovine mastitis. References [13], [14] and [15] shows that Staphylococci as the chief pathogens of mastitis. Compared to an earlier study of Mahbub-E-Elahi *et al.* [12] the incidence of *E. coli* was rather higher increased (27.27%) which might be attributed to the indiscriminate use of penicillin which might have led to the elimination of gram positive organisms in certain cases.

Screening of pathogenic from non-pathogenic *Staphylococcus aureus* and *E.coli* isolates of milk samples in this study were performed by gene specific (nuc gene, 16srRNA, STX1 and STX2 genes) PCR. In this present study 13(48.15%) coagulase positive *Staphylococcus aureus* among 27 isolates were gave positive result against nuc gene. All isolated coagulase negative *Staphylococcus* spp. gave negative result against nuc gene. This result was closely in agreement with Mansoor Khakpoor *et al.* [16] findings in Iran. In their study 52.63 %(10 of 19 *S. aureus*

by culture) *S. aureus* were detected by nuc gene specific PCR. Present study slightly differs with Amin *et al.* [17] findings. They screened 33.8% coagulase positive *Staphylococcus aureus* by PCR. All the isolated *E.coli* (100%) was confirmed by PCR with *E.coli* specific 16srRNA in current study but negative in case of Stx1 and Stx2 gene. This finding in case of 16srRNA gene completely agreed with Amin *et al.* [17] findings. In their study all media cultured isolates of E.coli were confirmed by PCR. Present study completely differed with Van Kessel *et al*. [18] and Farzan *et al.* [10] findings. Van Kessel *et al*. [18] detected Shiga toxin genes (Stx1 and Stx2) enrichments in 15.2% of the bulk tank milk samples and Farzan *et al.* [10] indicate that 78 (26%) of 300 Iranian milk and dairy samples were contaminated with *E. coli* among them 93.5% were found to be positive on two target genes, Stx1 and Stx2. None of the isolated *E.coli* of present study was shiga toxin producing thus they were negative to Stx1 and Stx2 gene.

Further in the present investigation antibiogram studies were also conducted for the isolates by using nine antibiotics which were used frequently in Satkhira for the treatment of mastitis. Ciprofloxacin, Gentamicin and Sulfamethoxazole-trimethoprim were found to be more effective antibiotic among all the tested antibiotics against all the bacteria isolated in the present study which is completely agreed with Bedada *et al.* [19] finding. The antibiotic sensitivity test revealed that coagulase positive *Staphylococcus aureus* isolates were found sensitive to six antibiotics and resistant to five antibiotics. All coagulase positive *Staphylococcus aureus* were sensitive to Ciprofloxacin (100%) and the least number of *S. aureus* were sensitive to Tetracycline (40%). Gentamicin (80%) and Sulfamethoxazole-trimethoprim (70%) were also sensitive. 90% tested coagulase positive *Staphylococcus aureus* were resistant to Ampicillin. Already five antibiotic resistant *staphylococcus aureus* were found. These findings of antibiotic sensitivity assays were somewhat in agreement with the findings of Abera *et al.* [20] and Mansoor Khakpoor *et al.* [16]. They also found Ciprofloxacin, Gentamicin and Sulfamethoxazole-trimethoprim sensitive against coagulase positive *Staphylococcus aureus.* In case of Coagulase negative staphylococcus spp. these were found sensitive to seven antibiotics and acquired resistant properties against three antibiotics. The antibiotic sensitivity test revealed that coagulase negative *Staphylococcus* spp. was sensitive to Ciprofloxacin (100%), Gentamicin (100%), Sulfamethoxazole-trimethoprim (100%), Erythromycin (80%), Kanamycin (80%) and Tetracycline (60%). On the other hand, Ampicillin, Amoxicillin and Oxytetracycline were less sensitive. This finding of antibiotic sensitivity assays was somewhat in agreement with the findings of Farzana *et al.* [21] and Linda and Ynte [22]. On the other hand, most *E.coli* isolates were found resistant against nine antibiotics and found sensitive to only three antibiotics. The antibiotic sensitivity test showed that highest number of E.coli isolates were sensitive to Ciprofloxacin (90%) and completely resistant to Ampicillin (100%) and Amoxicillin (100%). These findings were more or less similar with the findings of Bedada *et al.* [19]. The antibiotic sensitivity test indicated that highest number of other *Bacillus* spp. were susceptible to Ciprofloxacin (100%) and Kanamycin (100%) and least to Ampicillin and Amoxicillin. Almost similar antibiogram profiles were also recorded by Kurjogi *et al.* [23]. Indiscriminate use of antibiotic for treatment purposes in cows results in variation of antibiogram profile which might be due to enzymatic degradation, mutation at binding sites, down regulation of outer membrane proteins, efflux pumps and transduction of genes in bacterial isolates.

V. Conclusion

In this study Isolation and identification of bacteria from CMT positive milk samples were carried out on the basis of colony morphology in different media (nutrient broth, nutrient agar, blood agar, Mannitol salt agar, MacConkey agar, Sorbitol MacConkey agar and Eosin Methylene Blue agar), staining characteristics and biochemical properties studies (sugar fermentation reaction, coagulase, catalase, MR, VP and Indol). A total of 37 *S* spp. bacteria were isolated from 52 CMT positive milk samples in different cultural media. Of the 37 *Staphylococcus* spp. 27 isolates revealed characteristics colony morphology such as small gray-white smooth colony on NA, beta hemolytic colony on BA, yellowish color colony and media on MSA. This isolates were Gram-positive cocci and arranged in grape like cluster and fermented the 5 basic sugars with the production of acid. This isolates gave positive reaction in MR test, catalase test, coagulase test and negative in Indole and VP test. Rest 10 isolates exhibited non hemolytic colony on BA and pinkish colony on MSA with no color change and were also gram positive cocci. This isolates showed negative result on coagulase test. Rest biochemical test results were same. 15 *E.coli* isolates revealed characteristics colony morphology such as smooth, circular, white to grayish white colony in nutrient agar. Smooth, circular, black color colonies with metallic sheen were produced in EMB. Rose pink lactose fermenter colonies were produced on MCA. Pink color colony produced on SS and SMA agar. Morphologically *E. coli* isolates were hanging drop test positive and gram negative short rod arranged in single or paired. The *E. coli* isolates revealed a complete fermentation of 5 basic sugars by producing both acid and gas. The isolates also revealed positive reaction in MR test and Indole test but negative reaction in VP test. The cultural characteristics of 3 isolated *Bacillus* spp. revealed that large, creamy colonies with ß hemolysis were produced on BA; thick, grayish-white colonies were produced on NA. In Gram's staining isolates revealed large rod shaped arranged in chain. All the isolates fermented dextrose, sucrose, lactose, maltose and mannitol with the production of acid. The isolates also exhibited negative reaction in VP test, negative reaction in MR and indole positive. The bacteria isolated from 52 CMT positive milk samples were coagulase positive *Staphylococcus aureus* 27 (49.09%) followed by *Escherichia coli* 15 (27.27%), coagulase negative *Staphylococcus* (CNS) 10 (18.18%) and Bacillus spp. 3 (5.45%) in CMT positive milk samples. In this present study, 13(48.15%) coagulase positive *Staphylococcus aureus* among 27 isolates gave positive result against nuc gene. All isolated *E.coli* (100%) were confirmed by PCR with *E.coli* specific 16srRNA in current study but negative in case of Stx1 and Stx2 gene. Further, antibiogram studies were also conducted for the isolates by using 9 antibiotics which were used frequently in Satkhira district for the treatment of mastitis. Antibiogram profile of this study

revealed that, all the isolated bacteria were sensitive to Ciprofloxacin only. Overall observation of this study clearly indicated that the indiscriminate use of different kinds of antibiotics for the treatment of mastitic cow's of Satkhira district certainly initiated the emergence of multidrug resistant *Staphylococcus aureus* and *E. coli* organisms which are definitely potential threat for dairy as well as human health of this region. In relation to the present study, further investigation might be performed on the following aspects: Genome analysis to correlate drug resistance with pathogenicity of the isolated organisms need to be studied, Identification of other important bacteria causing mastitis such as, *Pseudomonas* spp.,*Streptococcus dysagalactiae*, *Streptococcus agalactiae* might be done to determine their pathogenicity in relation to CM and SCM. Further study to detect the role of close association of biofilm producing *Bacillus* spp. and Methicillin resistant coagulase positive *Staphylococcus aureus* for the development of multidrug resistant property of the organisms present in the udder of SCM and CM crossbred cows might be done.

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