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Original Article

Prevalence and molecular detection of the causal agents of sub-clinical mastitis in dairy cows in Sirajganj and Pabna districts, Bangladesh

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ABSTRACT

Objective: The present research work was undertaken with the objectives to investigate the prevalence and molecular detection of the causal agents of sub-clinical mastitis (SCM) in cows at milk shed areas in Sirajganj and Pabna districts, Bangladesh.

Materials and methods: A total of 300 milk samples were randomly collected from Baghabari milk shed areas of Sirajganj and Pabna districts. The milk samples were subjected for California Mastitis Test (CMT) for identifying SCM. Total 81 positive samples were then used for the isolation and identification of associated bacteria and fungi using conventional microbiological examination and biochemical tests, followed by confirmation by polymerase chain reaction (PCR) using specific primers. Besides, universal primers were used for amplification and sequencing of PCR products where specific primers were not used.

Results: The overall prevalence of SCM was 51% (n=153/300). Based on bacteriological examination and biochemical tests, several bacteria were identified in this study; the orgnaisms included *Staphylococcus sp.* (45.68%), *Streptococcus uberis* (14.81%), *Escherichia coli* (9.88%), *Proteus sp.* (19.75%), *Salmonella sp.* (1.23%), *Acinetobacter sp.* (7.41%), and fungus (1.23%). PCR technique confirmed the bacteria as *Staphylococcus aureus* (279-bp), *Streptococcus uberis* (884-bp), *E. coli* (16SrRNA 585-bp, *stx*1 606-bp, rfbO157 497-bp) and *Salmonella sp.* (*Inv-A* gene796-bp).

Conclusion: This study reveals that SCM in dairy cattle is persisting in Sirajganj and Pabna districts of Bangladesh. Hygienic practices should be improved, and providing technical intereventions may reduce the rate of SCM in the study areas.

KEYWORDS

Acinetobacter sp.; Cow; *rfb*O157; PCR; *Proteus sp.*; *Salmonella sp.*

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INTRODUCTION

Mastitis is an inflammatory condition of mammary gland characterized by physical, chemical and microbiological changes in milk (Seegers et al., 2003). However, sub-clinical mastitis (SCM) reveals no apparent change in the milk but somatic cell number is markedly increased. In dairy farms, SCM contribute up to 70%-80% of the total losses. A number of pathogens (>135) are involved for the onset of mastitis in cattle. The associated pathogens included bacteria, mycoplasma and yeast (Egwu et al., 1994). The SCM in dairy cows is crucial because it reduces milk yield (Seegers et al., 2003).

The prevalence of mastitis in cattle is higher in the farms having larger herd size as compared to those in smaller herd sizes (Radostits et al., 2000). The major etiological agents of SCM included *Staphylococci*, *Streptococci* and *E. coli* (Singh and Baxi, 1982; Rahman et al., 2014). However, from clinical case of mastitis different types of bacteria like *Staphylococci, Streptococci, Corynebacterium, E. coli* and *Bacillus* sp. have been isolated and identified by Mahbub-E-Elahi et al. (1996) at Manikganj and Bangladesh Agricultural University (BAU) Dairy Farm, Mymensingh.

Sub-clinical mastitis is a serious problem in dairy industries because there is no gross changes found in udder or glandular tissues and act as a continuous source of infection to herd mates. Depending upon the climatic condition, animal species and disease management practices, etiological agents may vary place to place and case to case. Thus, the control and prevention of mastitis is a challenge and despite of the continuous efforts, causes severe economic losses to dairy industry. Early detection of mastitis with low cost and rapid screenings in field level, hygienic farm management, biosecurity and awareness building among farmers will be helpful to control the clinical and SCM of dairy cows.

California Mastitis Test (CMT) is widely used for identifying SCM based on the presence of somatic cell number (SCN) in the milk. The present research work was undertaken to investigate the prevalence and molecular identification of causal agents of mastitis of cows in Sirajganj and Pabna districts in Bangladesh.

MATERIALS AND METHODS

Ethical statement: The samples were collected by following the international standard considering animal welfare and ethics.

Selection of study area, duration, and study animal: A total of 60 farmers from 13 villages at Sahjadpur of Sirajgonj and Shathia of Pabna district were selected randomly during July 2016 to June 2017. A structured questionnaire was developed for screening the animals. Number of cows per farm was between 3 and 50. Only apparently healthy crossbred dairy cows were considered for this study.

Sample collection: In this study, a total of 300 milk samples were collected. Once animal was considered as one sample. Before collection of milk, the teat and tips were washed with clean water, antisepsis was done with a swab soaked with 70% alcohol and then milk samples were collected aseptically from the udder during morning. All the milk samples were collected in vials which were labeled with identification number of cow.

Physical examination of milk sample: The milk samples just after collection were observed for any abnormalities in consistency, color, and presence of any other clotted blood flakes.

Detection of SCM by CMT tester: For the detection of SCM, CMT was performed as the instructions of manufacturer (CHEIL BIO Co. Ltd.). In brief, 2 mL milk and 2 mL CMT solution were mixed together in test paddle. Rotate the paddle to mix, and changes in color and gel formation was observed within 10 to 15 Sec.

Isolation of associated bacteria and fungi: The milk samples of positive results (n=81) in CMT were transferred to Department of Microbiology and Hygiene, BAU for microbiological analyses. Before incubation, the sample was allowed in normal temperature. Then 100 µL of milk sample was inoculated into 10 mL nutrient broth. Then the milk sample containing broth was incubated at 37°C for 24 h. After incubation, one loop of incubated sample was streaked on EMB agar, Mannitol Salt Agar, Salmonella-Shigella agar, and again incubated at 37°C for 24-48 h. The bacterial isolates were primarily identified through a series of bacteriological and biochemical examinations (Rahman et al., 2014; Chandrasekaran et al., 2014). The classification and specification of organism was based on the scheme presented in Bergey's Manual of Systemic Bacteriology (Halt et al., 1985). Fungus was isolated by spreading the sample on Potato dextrose agar, supplemented with broad spectrum antibiotic for prevening the growth of bacteria.

Oligonucleotide primers, PCR and sequencing: Polymerase Chain Reaction (PCR) was done for final

Table 1: Oligonucleotide primers used in this study

Name of organism	Name of primer	Oligonucleoide sequence (5′-3′)	Annealing temp (°C)	Expected band size (bp)	References
E. coli	EC16SrRNA F EC16SrRNA R	GACCTCGGTTTAGTTCACAGA 5'CACACGCTGACGCTGACCA	58	585	Schippa et al. (2010)
	EC *Stx*-1 F EC *Stx*-1 R	CACAATCAGGCGTCGCCAGCGCACTTGCT TGTTGCAGGGATCAGTCGTACGGGGATGC	56	606	Heuvelink et al. (1995)
	EC *Stx*-2 F EC *Stx*-2 R	CCACATCGGTGTCTGTTATTAACCACACC GCAGAACTGCTCTGGATGCATCTCTGGTC	56	372	Heuvelink et al. (1995)
	rfbO157F rfbO157R	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	47	497	Sanchez et al. (2010)
Salmonella spp.	Inv-A F Inv-A R	CGGTGGTTTTAAGCGTACTCT T CGAATATGCTCCACAAGGTTA	58	796	Fratamico et al. (2003)
Staph. aureus	nuc-F nuc-R	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	55	279	Dewanand et al. (2007)
Strep. uberis	S.uber-F S.uber-R	TTGTACACACCGCCCGTCA GGTACCTTAGTTTCAGTTC	52	884	Chanter et al. (1997)
Strep. agalactiae	S.agal-F S.agal-R	AAGGAAACCTGCCATTTG TTAACCTAGTTTCTTTAAAACTAGAA	62	587	Anjali and Kashyap (2017)
Strep. dysgalactiae	S.dysg-F S.dysg-R	GAACACGTTAGG GTCGTC AGTATATCTTAACTAGAAAAACTATTG	62	403	Anjali and Kashyap (2017)

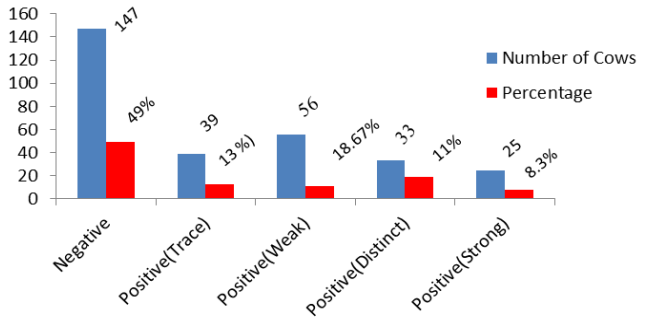

Figure 1. Prevalence of SCM in dairy cows by CMT. Except negative (49%; n=147/300) samples, 51% (n=153/300) samples were positive for SCM.

confirmation of the isolated microorganisms. The oligonucleotide primers were purchased from Bioneer, Korea (**Table 1**). The oligonucleotide primers were used for the amplification of specific gene fragments for the confirmatory identification of the isolated bacteria (**Table 1**) from the SCM cases (Tanzin et al., 2016).

The total volume of PCR mixture was 25 µL consisting of 12.5 µL PCR master mixture, 1 µL of each primers from 20 pmol stock and 5 µL of template DNA. The thermal profile of PCR of 35 cycles was consisted with an initial denaturation at 95°C for 5 min followed by 94°C for 30 Sec as denaturation, annealing at different temperature for different bacteria (**Table 1**) for 1 min, 72°C for 1 min for elongation and finally 72°C for 10 min as final extension using Thermal Cycler (Applied Biosystem, Germany). The PCR fragements were visualized by UVsolo TS Imaging System (Biometra, Jena Company) after staining with ethidium bromide.

The PCR fragements of *Proteus* sp., *Acinetobacter sp.* and *Strep. uberis* were sequenced (Bioneer, Korea) using universal primer set (8F 5'-AGTTGATCCTGGCTCAG-3'; 1492R 5'-ACCTTGTTACGACTT-3') for final confirmation as the specific primers were not used in these cases.

RESULTS AND DISCUSSION

Among the 300 cows, CMT was found to be positive in 51% (n=163/300; trace - 13%, weak 11%, distinct 18.67%, strong 8.33%) samples.

Bacteriological examinations and biochemical test followed by PCR confirmation revealed that several bacterial species and fungus were associated with the SCM. The number of samples positive for the SCM cases and the associated bacteria and fungus are illustrated in the **Figure 1** and **2**. Only one sample was found to be associated with fungus; however, the fungus was unidentified. The *E. coli* produced metallic sheen on EMB agar. The *Salmonella sp.* produced black colonies on SS agar. The *Staphylococcus sp.* grown on mannitol salt agar fermenting mannitol and produced characteristics yellowish colonies, and showed grapes like appearance under microscope after Gram stain. The *Acinetobacter sp.* produces blue colonies on EMB agar that reveals Gram positive bacteria arranged in pair under microscope; the bacterial genus was confirmed by sequencing. The *Proteus*

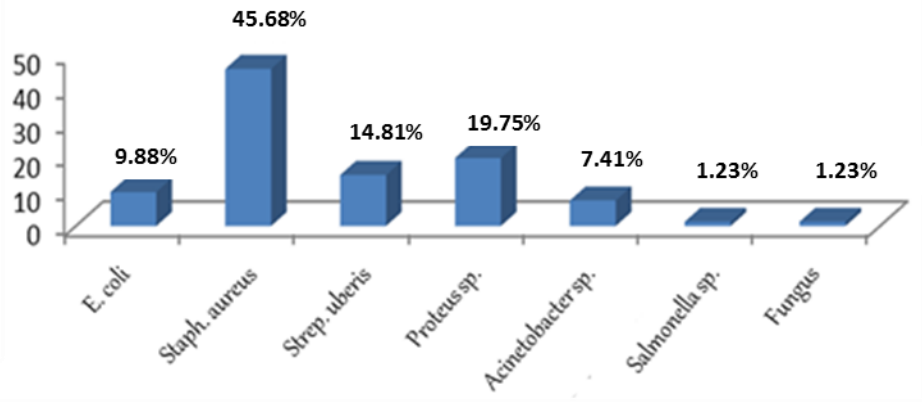

Figure 2. Percentage of different types of microorganisms prevalent in SCM. The calculation has been done based on 81 CMT positive samples.

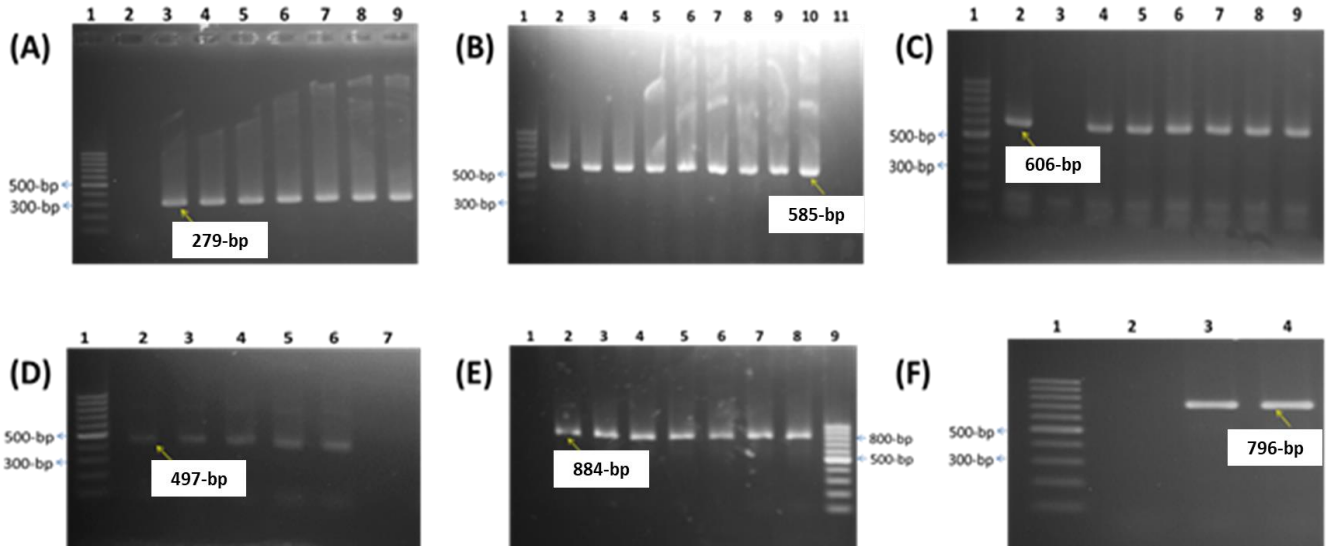

Figure 3. Molecular detection of different bacteria isolated from sub-clinical mastitis. **(A)** Amplification of *nuc* gene (279-bp) for *Staphylococcus aureus*; Lane 1: 100-bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4-9: Test sample, **(B)** Amplification of *E. coli* 16SrRNA (585-bp) specific genomic primer; Lane 1: 100-bp DNA ladder, Lane 2-9: positive for 16SrRNA; Lane 10: Positive control; Lane 11: Negative control (*E. coli*), **(C)** Amplification of *stx*-1 (606-bp) genes; Lane 1: 100-bp DNA ladder, Lane 2-6 test samples; Lane 3: Negative control; Lane 4-9 test samples, **(D)** Amplification of *rfb*O157 (497-bp) genes; Lane 1: 100-bp DNA ladder, Lane 2: positive control; Lane 7: Negative control, **(E)** PCR picture for *Streptococcus uberis*; amplification size around 884-bp, **(F)** Amplification of *Inv-A* gene (796-bp) for *Salmonella spp.*; Lane 1: 100-bp DNA ladder; Lane 2: Negative control Lane 3: Positive control; Lane 4: Test sample.

sp. produces pink color colonies on SS agar, which was also confirmed by sequencing. *Strep. uberis* was confirmed by PCR (**Figure 3**) followed by sequening of amplified amplicons. However, no other *Streptococcus spp.* were detected.

In this study, 51% (n=153/300) samples were confirmed as SCM. Kader et al. (2002) reported similar report who described the prevalence as 44.61% SCM in Bangladesh. On the other hand, higher prevalence (54%) of SCM was recorded in India by Singh and Baxi (1982). As compared with early lactation, prevalence of SCM was higher in late lactation period, as reported by Radostits et al. (2000). Prevalence of SCM was higher in high milk yielding animals as compared to low milk yielding animals (Khanal and Pandit, 2013). Previously, in Bangladesh, prevalence of SCM in dairy cows was recorded as 72.07, 66.67, 64.86 and 61.26% by CMT, SCC, WST and SFMT, respectively, as reported by Badiuzzaman et al. (2015). The highest prevalence was found for CMT (67%) and WST (62%) in the animals aging 3.5-4.5 years (Khokon et al. 2017).

In this study, 9.88% cases were associated with *E. coli*; whereas, Bradley et al. (2000) reported 14.5% of the *E. coli* mastitis, followed by *Staph. aureus* (11.8%) and *Streptococcus* species (7.9%) and *Strep. agalactiae* mastitis (13.2%), as reported by Al-kuzaay and Kshash (2013). This variation might be due to improved hygienic practices in this study area and awareness of the farmers. Gangwal and Kashyap (2017) showed prevalence of bacteria in mastitis as 28% *E. coli*, 24% *Staph. aureus*, 18% *Pseudomonas aeruginosa*, 10% *Klebsiella pneumoniae* and 2% *Strep. agalactiae*. Valbak (1990) and Olivares-Pérez et al. (2015) could isolate *Salmonella sp.* from milk samples taken from cows, as supporting our study.

PCR was applied to detect the presence of shiga-like toxin in six *E. coli* (*stx*-1 genes) isolates among eight *E. coli* strains (**Figure 3**). No *stx*2 gene could be identified in this study. However, *stx*-1 and *stx*-2 genes were reported together by Sayed (2016). Among the *E. coli* isolated, 4 harvored *rfb*O157 genes (**Figure 3**), indicating the the *E. coli* were highly pathogenic, as reported by Caine et al. (2014).

In another study, Islam et al (2014) revealed that 26.71% of milk samples were associated with *Staphylococcus sp.*; whereas, 5.48% cases were found to be positive for SCM by molecular identification. In this study, culture was done from the milk samples that demonstrated positive reaction in CMT. Mahbub-E-Elahi et al. (1996) isolated *Staphylococcus sp.*, *Streptococcus sp.*, *E. coli* and *Bacillus*, whereas Rahman et al. (1968) isolated and identified different strains of *Staphylococci* from mastitic and apparently healthy mammary glands of cows. These findings also corresponded with Shrestha and Bindari (2012) who reported highest prevalence of *Staphylococcus* followed by *E. coli*, *Streptococci* and *Corynebaceterium*. Chanda et al. (1998) reported that *Staphylococcus* was the principal organism of mastitis. *Staphylococcus* is the opportunistic bacterium which can survive the skin of the udder can infect via teat canal.

In addition to *Staphylococcus*, *E. coli* was identified in this study, which is an environmental opportunistic pathogen. Similar report was also reported by Mahbub-E-Elahi et al. (1996). *Acinetobacter sp.* was isolated from mastitis in our study, as supported by Marimuthu et al. (2014). Similary, *Proteus sp.* was identified in this study, as described by Olivares-Pérez et al. (2015). Several fungal species have been isolated from milk by Pachauri et al. (2013), as we are reporting here.

CONCLUSION

The prevalence of subclinical mastitis indicates that it is a major threat for dairy industry. The overall prevalence of sub-clinical mastitis in the study area is 51%. The associated bacterial species with the cases were *Staphylococcus sp., Streptococcus uberis, Escherichia coli*, *Proteus sp., Salmonella sp., Acinetobacter sp.* Early detection and ensuring proper preventive measures are suggested for controlling the sub-clinical mastitis in the study area.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTION

HK, MG and RI implemented the study design. HK, MSI, AY, SMR and YU participated in data collection. HK and MMM performed all the tests. HK and HR drafted, RK revised the manuscript. KHMNH critically checked the article and corrected the manuscript. All authors read and approved the final version of manuscript.

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