

Original Article

Antibiotic resistance profile of bacteria isolated from raw milk samples of cattle and buffaloes

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ABSTRACT

Objectives: The objective of this study was to isolate and identify *Staphylococcus aureus* and *Escherichia coli* from raw milk samples of cattle and buffalo, and to evaluate the antibiotic sensitivity pattern.

Materials and methods: A total of 34 milk samples were collected twice from 17 different healthy cattle (n=14) and buffaloes (n=3) at one-month interval, and analyzed in laboratory by staining, cultural and biochemical characteristics followed by polymerase chain reaction targeting *nuc* gene of *S. aureus* and 16S rRNA of *E. coli*. Antibiotic sensitivity pattern of the isolated bacteria was assessed using the disc diffusion method.

Results: Confirmation of the isolates as *S. aureus* and *E. coli* were carried out by PCR using *nuc* gene, 16S rRNA gene specific primers specific for *S. aureus* and *E. coli* respectively. A total of 12 samples (35.29%; 11 from cattle, 1 from buffalo) were found to be positive for *S. aureus*; 5 and 7 during first and second month, respectively. The *E. coli* were found in three samples (2 from cattle, 1 from buffalo); one in first month and two in the second month. The antibiotic sensitivity test using 4 commonly used antibiotics indicated that the most of the isolates were resistant to Gatifloxacin and one isolate showed intermediate resistance to Ofloxacin while sensitive to Ciprofloxacin and Levofloxacin.

Conclusion: Two different species of bacteria i.e., *S. aureus* and *E. coli* are contaminating with milk samples. The pathogenic bacteria can be controlled effectively by using Ciprofloxacin and Levofloxacin in the case of mastitis in cattle and buffaloes in Bangladesh.

KEYWORDS

Antibiotic resistance, *Escherichia coli*, Milk, PCR, *Staphylococcus aureus*

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INTRODUCTION

Milk is considered as an excellent medium for growing of many microorganisms. Milk can be contaminated with several bacteria during milking process from the milking personnel, utensils used for milking (Rehman et al., 2014). Besides, microorganisms may enter the udder through teat canal, and the bacteria may come out through milk (Smith et al., 2007). *Staphylococcus aureus* and *Escherichia coli* are the two major contaminants of milk. The presence of the pathogen in milk largely depends on fecal contamination, and the presence of pathogen in feces mainly originates from feed contamination (Aycicek et al., 2005).

Food borne diseases are of great concern around the world. However, this is an important issue in developing countries where poor sanitation is maintained during collection and processing of milk from cattle and buffaloes (Le et al., 2003). *S. aureus* is an important pathogen for dairy ruminants causing inflammatory reactions, and the organism is believed to cause 30-40% mastitis (Akineden et al., 2001; Asperger and Zangeri, 2003; Cabral et al., 2004; Katsuda et al., 2005). The organism can be excreted directly from udder through milk (Rehman et al., 2014). Presence of *S. aureus* in milk indicates the hygienic standard followed during milking process. Information on antibiotic resistance against *S. aureus* can be useful in treating the disease caused by the organism (Jahan et al., 2015).

E. coli is one of the important bacteria of gut flora (Eckburg et al., 2005). Among the pathogenic *E. coli*, Shiga toxigenic *E. coli* (STEC) strains have been reported mostly in Latin America, India, Bangladesh and many other developing countries (Kaddu-Mulindw et al., 2001; Rehman et al., 2014). Pathogenic *E. coli* have been isolated by several researchers in Bangladesh (Nazir et al., 2005; Khatun et al., 2015; Himi et al., 2015) from fecal samples of healthy cattle (Hassan et al., 2014), raw milk of cattle and buffaloes (Alam, 2006; Islam et al., 2008; Hossain et al., 2011; Jahan et al., 2015). In Bangladesh, about 20% of all diarrheal cases is associated with enterotoxigenic *E. coli* (Qadri et al., 2005). Moreover, very few works have been reported in Bangladesh on molecular detection of pathogenic organisms from raw cow milk and buffalo milk. Besides, selection of

appropriate antibiotic against the *S. aureus* and *E. coli* is crucial for proper treatment of mastitis in cattle and buffaloes. Therefore, the objective of this study was to isolate and identify *S. aureus* and *E. coli* from raw milk samples of cattle and buffalo, and to evaluate the antibiotic sensitivity pattern.

MATERIALS AND METHODS

Sample collection and ethical approval: The study was design to investigate the bacteriological analysis of raw milk of healthy cows and buffaloes collected from Bangladesh Agricultural University (BAU) Dairy Farm. A total of 34 raw milk samples, regardless of quarter of udder were collected. Fourteen samples were collected twice from 14 different cattle at one-month interval. Similarly, 6 samples were collected from 3 different buffaloes at one-month interval. In all cases, about 10 mL of fresh milk was collected from each animal by using sterile test tubes after dispatching few drops of milk first. All samples were collected as per standard sample collection procedure without harming or giving stress to any animal. The collected milk samples were immediately transported on ice to the Bacteriology Laboratory at the Department of Microbiology and Hygiene of BAU for bacteriological analysis. Upon arrival in the Laboratory, milk samples were kept incubated at 37°C for 2 h then cultured in 5% sheep blood agar, Salmonella Shigella (SS) agar, MacConkey agar, Eosin Methylene (EMB) agar and Mannitol salt (MS) agar by spreading method.

Isolation of *Staphylococcus aureus*: Primary culture was done in nutrient broth for enrichment of bacteria. Pure culture of *S. aureus* was obtained based β -hemolysis on blood agar, Gram staining, morphological study, biochemical characteristics, catalase test, coagulase test, as per the procedures mentioned by Cheesbrough (1985) and Jahan et al. (2015).

Isolation of *E. coli*: MC agar media were streaked aseptically with 200 μ L of milk sample and incubated at 37°C for overnight. Appearance of bright red or pink color colony was used to culture in EMB agar. Appearance of the colony of pink/red and greenish black with metallic sheen was considered positive for *E. coli* in EMB agar. The positive colonies were sub cultured into EMB agar to obtain pure colony. The pure colonies were

Table 1: Oligonucleotide primer used in the study

Primer	Primer Sequence (5'-3')	Target gene	Amplicon size	References
ECO-1	GAC CTC GGT TTA GTT CAC AGA	16S rRNA	585-bp	Hassan et al. (2014)
ECO-2	CAC ACG CTG ACG CTG ACC A			
Nuc F	GCG ATT GAT GGT GAT ACG GTD	nuc	279-bp	Jahan et al. (2015)
Nuc R	AGC CAA GCC TTG ACG AAC TAA AGC			

subjected for sugar fermentation, biochemical tests, as per the procedure described by Cheesbrough (1985) and Nazir et al. (2005).

Molecular characterization by PCR: Bacterial DNA was extracted by Wizard genomic DNA purification kit (Promega, USA). The details protocol - bacterial colony was taken from pure culture with pipette tip and dissolve into 1 mL PBS in eppendorf tube, the tube is placed in vortex for proper mixing and centrifuged at 13,000 rpm for 2 min. Then, supernatant is discarded, 600 μ L nuclei lysis solution is added and mixed by gentle pipetting, kept in 80°C for 5 min, 3 μ L RNase solution is added, mixed by pipetting and incubated in 37°C for 45 min and cool to room temperature, 200 μ L of protein precipitation solution was added and vortex for mixing, incubated in ice for 5 min, centrifuged at 13,000 rpm for 5 min, protein precipitated, so pellet is discarded and supernatant containing the DNA is collected, supernatant is transferred into another eppendorf tube containing 600 μ L isopropanol and mixed by pipetting, centrifuged at 13,000 rpm for 2 min, supernatant is discarded, 600 μ L 70% ethanol is added and mixed with the pellet, centrifuged at 13,000 rpm for 2 min, aspirate the ethanol and air dry the pellet for 10-15 min, rehydrate the DNA pellet in 100 μ L of DNA rehydration solution for 1 h at 65°C or overnight at 4°C.

PCR mixture was prepared by the following method. To make 25 μ L of PCR master mixture, 12.5 μ L of 2 X Master mixtures (Promega, USA), 1 μ L of each primer (10 pmol/ μ L), 2 μ L of DNA template and remaining 8.5 μ L deionized water were added in PCR tube.

Amplification of 16S rRNA gene: Amplification was performed on a thermocycler (Eppendorf Personal, Germany) where PCR tubes were set on the wells of the thermocycler plate. The machine was run according to the program initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 60 sec, with a final extension at 72°C for 3 min.

Amplification of nuc gene: Amplification was done by initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing temperature of primers was 55°C for 45 sec and extension at 72°C for 1.5 min. The final extension was conducted at 72°C for 10 min. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed in UV light using a Gel documentation system (Biometra, Germany).

Antibiotic sensitivity test: *In vitro* antibiotic sensitivity test was done as per recommendation of the Clinical and

Laboratory Standard Institute (CLSI, 2012). The antibiotic discs impregnated gatifloxacin (5 mcg), Ofloxacin (5 mcg), Ciprofloxacin (5 mcg) and Levofloxacin (5 mcg) (Oxoid, UK) were used in this study.

RESULTS AND DISCUSSION

Among 34 samples, 12 (32.29%) showed β -hemolysis on 5% cattle blood agar with circular, small, smooth raised whitish colony. Islam et al. (2007b) reported that 89.3% *S. aureus* from bovine origin were hemolytic. This variation was due to the difference in sample origin indicating that raw milk contained less association with *S. aureus* as compared with feces of cattle from where the bacteria were isolated by Islam et al. (2007b). After overnight incubation on MS agar media, some plates showed yellow colony and some plates showed whitish colony. All the suspected *S. aureus* which produced β -hemolysis on 5% blood agar were able to ferment mannitol salt agar characterized by the formation of yellow colony and white/transparent colony indicated other *Staphylococcus* spp., as indicated by Cheesbrough (1985), Begum et al. (2007) and Islam et al. (2007a, b). In Gram staining, the organism revealed as Gram positive, violet colored, cocci shaped and arranged in grapes like cluster under light microscope. Catalase test was performed to differentiate *Staphylococci* (catalase producer) from *Streptococci* (non-catalase producer). Hydrogen peroxide (H_2O_2) was broken down into water and oxygen. Production of oxygen was indicated by bubble formation. All *S. aureus* isolates were catalase positive. A total of 12 samples were found as catalase positive, as described by Cheesbrough (1985). All the isolates of *Staphylococci* gave positive reaction in coagulase test indicated that the isolates were pathogenic *S. aureus*. The positive result was confirmed by the formation of curd like clotting compare to negative control where there is no formation of curd like clotting. Isolation of by cultural, morphological and biochemical tests were performed according to methods described by Cheesbrough (1985), Hummerjohann et al. (2014) and Jahan et al. (2015). In Bangladesh, *S. aureus* has been isolated from several sources like bovine (Begum et al., 2007; Islam et al., 2007a), pus, exudate, surgical wound swab, burn ulcer exudate, aural swab and diabetic ulcer exudate of human (Islam et al., 2007b).

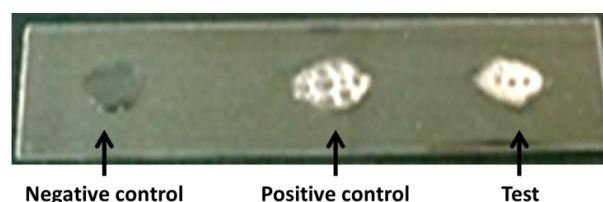


Figure 1: Slide catalase test of *Staphylococcus aureus*

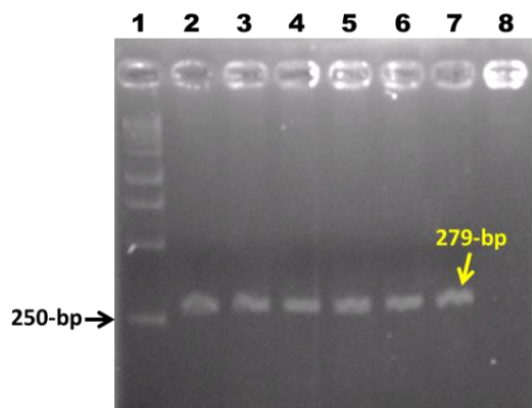


Figure 2: PCR to detect *nuc* gene in *Staphylococcus aureus*. Lane 1: 1 kb DNA ladder (Promega, USA), Lane 2-6 : Tested isolates, Lane 7 : Positive control, lane 8 : Negative control.

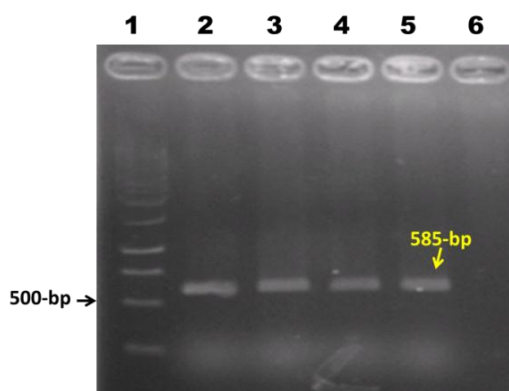


Figure 3: PCR to detect 16S rRNA in *Escherichia coli*. Lane 1: 1 kb DNA ladder (Promega, USA), Lane 2-4 : Tested isolates, Lane 5 : Positive control, lane 6 : Negative control.

After overnight incubation, 3 samples showed bright pink or red colonies on MC agar, were identified as *E. coli*. For presumptive identification of *E. coli*, the selective colony on McConkey agar for each sample was sub-cultured successively onto Eosin Methylene Blue (EMB) agar giving greenish-black colonies with metallic sheen. In Gram staining, the organism revealed as Gram-negative, small rod shaped, pink colored arranging single or paired. All the isolates fermented the five basic sugars (dextrose, sucrose, lactose, maltose and mannitol) and sorbitol producing acid and gas, as reported by Cheesbrough (1985), Roy et al. (2012) and Chandrasekaran et al. (2014). All the isolates were catalase positive, VP test negative, indole test positive, and methyl-red positive, as reported by Nazir et al. (2005) and Roy et al. (2012).

In our study, we could amplify a 279-bp amplicon of *nuc* gene (Figure 2). This finding was inclined with the reports of Jahan et al. (2014) and Hummerjohann et al. (2014). Jahan et al. (2014) isolated *S. aureus* from raw cow milk samples in Bangladesh while Hummerjohann et al. (2014) isolated Methicillin-resistant *S. aureus* from Swiss

raw milk cheese. Here, we could isolate *S. aureus* from both cow milk and buffalo milk differing from the findings of Jahan et al. (2014) and Hummerjohann et al. (2014).

The bacterial isolates identified as *E. coli* through cultural, morphological and biochemical characteristics were confirmed by PCR (Figure 3), as reported by Nazir et al. (2005), Schippa et al. (2010), Hassan et al. (2014), Himi et al. (2015) and Khatun et al. (2015). The *E. coli* contamination found in raw milk might be due to cross contamination of milk with feces or lack of hygienic measures during collection and processing of milk. There are some reports of isolation of *E. coli* from rectal swab of bovine animals throughout the world; for example, Ogunleye et al. (2013) reported a prevalence of 80% in apparently healthy cattle in Nigeria. In Bangladesh, Masud et al. (2012) conducted experiment on isolation of *E. coli* from rectal swab of apparently healthy cattle and found 23.21% prevalence of *E. coli*. In another study, Hassan et al. (2014) reported 75% rectal swabs of cattle were associated with *E. coli*. *E. coli* has also been isolated from different sources like water (Nazir et al., 2005), broiler and layer (Nazir et al., 2005; Nasrin et al., 2007; Roy et al., 2012), diarrheic stool of infant (Nazir et al., 2007) and diarrheic calves (Nazir et al., 2007) in Bangladesh. Many of these studies have applied molecular technique *i.e.*, PCR for the confirm detection of bacterial species. However, to the best of our knowledge, this is the first report on molecular based detection of *E. coli* and *S. aureus* from raw milk samples of apparently healthy cattle and buffaloes in Bangladesh.

Four antibiotics were used against the isolated bacteria. Among these, all *S. aureus* and *E. coli* were found to be sensitive to Ciprofloxacin, Levofloxacin, and Ofloxacin and showed intermediate resistance to Gatifloxacin. Himi et al. (2015) isolated *E. coli* from Bangladesh and the isolates were sensitive to Ciprofloxacin and Levofloxacin. We found some intermediate resistant isolates to Gatifloxacin. The possession of such factors by the *E. coli* isolates signifies the fact that the intermediate resistance organisms may gain resistance property due to the indiscriminate use of antibiotics. The *E. coli* should be considered as hazardous to health and advocate the preventing risk factors. However, in the present study Ciprofloxacin and Levofloxacin were proved to be the best antibiotics to treat *E. coli* infection/mastitis in cattle since they were highly effective.

CONCLUSION

Mastitis is the most prevalent disease in developing country which causes a huge loss in dairy sector and also

has public health significance for consumers of milk and dairy products. Prevalence study of mastitic organisms in healthy animals is essential to reveal out the epidemiology of mastitis outbreaks in Bangladesh. The bacterial infection in the dairy farm of the study area was increasing day by day, which is a matter of concern in the livestock sector of Bangladesh. From the present study, it can be concluded that *S. aureus* and *E. coli* is prevalent in milk produced by healthy bovine animals in Mymensingh region of Bangladesh. But the sample size in this study was very small and study period was also short. This study should perform with a large sample size and for a long period of time will reveal the actual data of milk organisms in Bangladesh.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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None to declare.

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