MOLECULAR DETECTION AND CHARACTERIZATION OF *ESCHERICHIA COLI* ISOLATED FROM RAW MILK SOLD IN DIFFERENT MARKETS OF BANGLADESH

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ABSTRACT

The study was intended for molecular detection of *E. coli* isolated from raw cow's milk. A total of 20 milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh. Milk samples were cultured onto various culture media for the isolation of bacteria. The isolated bacteria were identified by studying staining characteristics, cultural properties on different selective media, biochemical tests, catalase and coagulase test, and finally by PCR. Out of 20 samples, 15 (75%) milk samples were found positive for *E. coli.* 15 *Escherichia coli* isolates were amplified by *16S rRNA* gene based PCR. Antimicrobial sensitivity test was carried out to ascertain the susceptibility of the organism to various antibiotics. Its results showed that the *E. coli* isolates were resistant to amoxycillin (86.67%) and erythromycin (73.33%) but sensitive to azithromycin (53.33%), ciprofloxacin (86.67%), gentamicin (86.67%), norfloxacin (80%) and streptomycin (66.67%).

Key words: *Escherichia coli, 16s rRNA* gene, raw milk

INTRODUCTION

Milk is considered as one of the high nutritional quality foods. Whole milk is the milk as it comes from the cow and contains about 3.5% milk fat. Research continues to expand the positive role of milk and milk products on individual's health. Evidence goes well beyond bone health to include its effects on immunity, mild hypertension, reducing selected cancers (Giovannucci *et al*., 1998), supporting weight management strategies and increasing satiety in dieters, among other positive effects. However, it is an excellent medium for growth and transmission of different bacterial pathogens to humans (Donkor *et al.*, 2007). *Escherichia coli* is a Gram-negative, rod-shaped bacterium, a member of the family *Enterobacteriaceae* and commonly found in the lower intestine of warm-blooded organisms (Singleton, 1999*).*It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk and contaminated raw vegetables and sprouts. Its significance as a public health problem was recognized in 1982, following an outbreak in the United States of America. EHEC produces toxins, known as vero-toxins or Shiga-like toxins because of their similarity to the toxins produced by *Shigella dysenteriae.* The pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans and animals. Most strains of *E. coli* live as commensals, many perhaps all are opportunistic pathogens of human and animals (Levine, 1984). Though it is a part of normal enteric microflora, but it is capable of producing serious diarrheal diseases, as well as other systemic diseases, especially infection of the urinary tract. E. coli and other facultative anaerobes constituted about 0.1% of gut flora (Eckburg *et al*., 2005) and oro-fecal transmission is the major route through which pathogenic strains of the bacterium cause disease. Over the last half-century it has become increasingly obvious that there are a number of different pathogenic groups of *E. coli.* Pathogenic *E. coli* strains are categorized into pathotypes on the basis of their virulence genes. At least six known pathotypes associated with gastrointestinal infections have been recognized, apart from those opportunistic "nonpathogenic strains" causing urinary tract infections, diarrhoea, septicemia, and meningitis in humans and a number of similar diseases in animals. Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with Shiga toxigenic *E. coli* (STEC) strains have also been described in Latin America, India and other developing countries (Kaddu-Mulindw *et al.*, 2001; Leelaporn *et al.*, 2003). In Bangladesh, the predominant group of *E. coli* associated with childhood diarrhoea is enterotoxigenic *E.coli*; accounting for approximately 20% of all diarrheal cases (Qadri *et al*., 2005).

Several works have been done throughout the world regarding the milk and microorganisms of contaminated milk (Hassan *et al*., 2014). In Bangladesh, a few works have been done on isolation and molecular characterization of *E. coli* from raw milk of cattle and buffalo (Alaine *et al.*, 2006; 1slam *et al.*, 2008; Hossain *et al.,* 2011, Hossain, 2015). Moreover very few works have been reported in Bangladesh on molecular detection of *E. coli* with other pathogenic organisms in cow's raw milk in a specific time period. So, keeping the above facts in mind, the present study was designed with an objective to molecular detection and characterization of bacteria isolated from raw milk samples. We studied the antimicrobial susceptibility patterns of isolated bacteria with the detection of *E. coli* using *16s rRNA gene.*

MATERIALS AND METHODS

Sample collection

A total of 20 cow's raw milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh using falcon tube. The samples were collected from January 2015 to June 2015 and investigation was carried out following collection. The collected milk samples were immediately transported on ice to the Microbiology Laboratory at the Department of Microbiology and Hygiene of BAU and District Veterinary Hospital, Sirajgonj for bacteriological analysis.

Isolation and identification of *E. coli*

The collected milk samples were performed 10 fold dilution using 0.1% peptone water and diluted samples were inoculated onto MacConkey (MC) agar by pour plate method and incubation at 37^0C for 24 hours. Then sub cultured onto eosin methylene blue (EMB) agar, MC agar by streaking to obtain pure culture. These isolates were preserved for further bacterial identification. The isolates were identified as *E. coli* on the basis of Gram staining, colony morphology on MC agar, EMB agar, biochemical characterization of the isolates (using sugar fermentation test, indole and MR-VP test), catalase and coagulase tests. Further the isolates were confirmed by amplification of *E. coli* specific *16S rRNA* gene.

Bacterial Genomic DNA extraction:

A pure bacterial colony of *E. coli* was mixed with 100 μl of distilled water which were boiled for 10 minutes then immediately kept on ice for ice shock. Finally centrifugation was done at 10000 rpm for 10 minutes. The supernatant were collected and used as DNA template for PCR (Engler and Kelley, 2000).

Identification of *E. coli* by PCR

Escherichia genus specific PCR was performed to amplify *16S rRNA* gene of *E. coli*. Two different primers pairs were used for this purpose, *16S rRNA* gene (F 5'-GACCTCGGTTTAGTTCACAGA-3' and R 5'-CACACGCTGACGCTGACCA-3') according to the methods described by Schippa *et al*. (2010). Each 20 μl reaction mixture consists of 3 μl genomic DNA, 10 μl PCR master mixtures (Promega, USA), 1 μl of each of the two primers with the final volume adjusted to 20 μl with 5μl of nuclease free water. Amplification was done by initial denaturation at 95°C for 5 minutes, followed by denaturation at 94^0C for 45 sec, annealing temperature of primers was 55°C for 45 sec and extension at 72°C for 1 minutes. The final extension was conducted at 72°C for 5 minutes. The total reaction was performed at 30 cycles. The amplified PCR products were resolved by electrophoresis in 2% agarose gel at 100v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator.

Antibiotic Sensitivity Test

All isolates of *E. coli* were tested for antimicrobial drug susceptibility against antimicrobial agents by disc diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007). Sensitivity pattern of the isolates were determined against ciprofloxacin, azithromycin, gentamicin, amoxicillin, streptomycin, erythromycin and norfloxacin. Antimicrobial testing results were recorded as sensitive, intermediate sensitive and resistant according to zone diameter interpretative standards provided by CLSI (2007).

RESULTS AND DISCUSSION

20 raw milk samples were analyzed in the laboratory by different cultural, biochemical, staining and molecular methods to detect *Escherichia coli* in raw milk. Out of 20 samples, 75% (n=15) were contaminated with *E. coli.* In MC agar plates, the samples showed bright pink or red colonies in MC agar were identified as *E. coli* (Figure 1). In EMB agar colonies produced by *E. coli* are smooth, circular, black color colonies with metallic sheen (Figure 2). The microscopic examination of Gram's stained smears from MC agar and EMB showed that the isolated bacteria were Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain (Figure 3). *E. coli* showed negative result to catalase and coagulase test. DNA extracted from *E. coli* isolates was used in the PCR assay. PCR primers targeting *16S rRNA* gene of *E. coli* amplified 585 bp fragments of DNA confirmed the identity of *E. coli.* Result of PCR for *E. coli* is shown in Figure 4. Isolated *E. coli* were analyzed for the antibiotic susceptibility. The results of antimicrobial susceptibility test showed that 8 (53.33%) isolates were susceptible to azithromycin, 10 (66.67%) isolates were susceptible to streptomycin, 13 (86.67) isolates were susceptible to gentamicin, 12 (80%) isolates were susceptible to norfloxacin, 11 (73.33%) isolates were susceptible to tetracycline and 10 (66.67%) isolates were susceptible to streptomycin. Furthermore, 12 (86.67%) isolates were resistant to amoxycillin and 11 (73.33) isolates were resistant to erythromycin (Figure 5).

Figure 1. Pink color Colonies of *E. coli on* MC agar

Figure 2. Metallic sheen colonies on EMB by *E. coli*

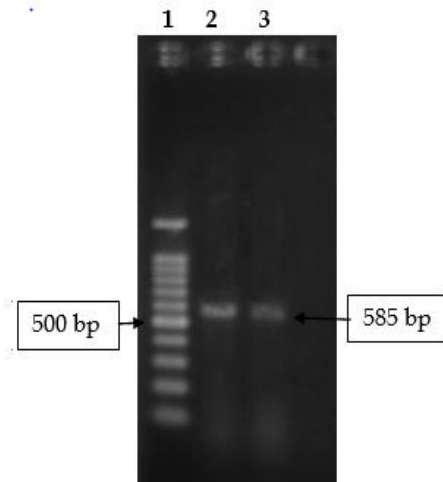Figure 3. Gram negative, pink colored, small rod shape *E. coli* under light microscope. (100x)

Figure 4. *16S rRNA* gene based PCR for *E. coli* .Lane 1: 100 bp DNA ladder, Lane 2, 3: Tested sample was positive for *16S rRNA* gene.
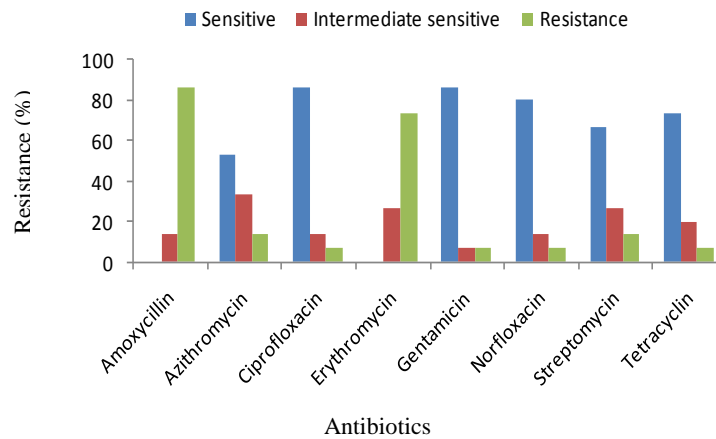
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Figure 5. Antibiotic resistance patterns of *E. coli* against commonly used antibiotics in Bangladesh

The present study was structured for identification and characterization of *Escherichia coli* from cow's raw milk sample. Total 20 samples were collected and analyzed. Out of 20 samples, 15 samples were revealed the positive result for *E. coli*. *E. coli* was identified and confirmed by cultural examination, morphological studies, staining characters and biochemical tests and finally PCR were performed for the amplification of specific gene (*16s rRNA* gene) of isolated bacteria. Milk is the best media for the growth of many bacteria in which some of them are pathogenic. As we know fresh milk is enriched with pathogenic and non pathogenic bacteria which can be transmitted to human by milking and consumption of milk. *Coliform* bacteria present in the fresh raw milk might be hazardous if proper boiling of milk is not done during consumption. It causes disease if proper hygienic procedure is not maintained during milking. In most cases, milk containing *E. coli* was obtained from animals with subclinical mastitis. Zafolon *et al.* (2008) studied at Nova Odesa, São Paulo and showed that the prevalence of *E. coli* was 57.3%. The results obtained in our study are likewise higher when compared to those formerly documented (Soomro *et al.,* 2002; Kumer and Prasad, 2010; Hossain, 2015). Based on observations made throughout the collection of samples, we concluded that the improper hygiene practice and poor management before and during milking may have contributed to the contamination of milk with *E. coli,* and the communal farms are more vulnerable in this case. The incidence at a considerable high percentage indicates the alarming situation both for dairy farming and for public health. The presence of *E. coli* in the milk sample is an appealing as well as an important finding of this study. Results of antimicrobial susceptibility test showed that most of the isolates of *E. soli* were sensitive to azithromycin, streptomycin, gentamicin, norfloxacin and ciprofloxacin but resistant to amoxicillin and erythromycin, which was closely related with Memon *et al.* (2013) and Bedada *et al.* (2011).

CONCLUSION

The results of this study concluded that raw milk available for consumers have a high *E. coli* contamination. Thus the results of the present study warn the need for more precaution. The present research was undertaken with a view to isolating and characterizing *Escherichia coli* present in cow's raw milk sample collected from different upazila markets of Bangladesh. Out of 20 samples, 15 (75%) milk samples 15 (75%) milk samples were found positive for *E. coli*. 15 *Escherichia coli* isolates were amplified by *16S rRNA* gene based PCR. Resistant pattern against broad spectrum antibiotic (e.g., amoxicillin) delimitate an alarming situation which needs special consideration.

ACKNOWLEDGEMENTS

REFERENCES

1. Alain M, Nur-A-Hasan, Ahsan S, Pazhani GP, Tamura K, Ramamurthy T, Gomes DJ, Rahman SR, Islam A, Akhtar F, Shinoda S, Watanabe H, Faruque SM and Nair GB (2006). Phenotypic and molecular characteristics of *Escherichia coli* isolated from aquatic environment of Bangladesh. *Microbiology and Immunology* 50: 359-370.
2. Bedada BA and Hico A (2011). Mastitis and antimicrobial susceptibility test at Asella, oromia Regional state, Etiopia. *Journal Microbiology and Antimicrobials* 3: 228-232.
3. CLSI (2007). Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. Clinical and Laboratory Standards Institute M100-S17: 1. Wayne, Pennsyslvania. pp. 32-50.
4. Donkor ES, Aning KG and Quaye J (2007). Bacterial contaminations of informally marketed raw milk in Ghana. *Ghana Medical Journal* 41: 58-61.
5. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE and Relman DA (2005). Diversity of the human intestinal microbial flora. *Science* 308: 1635-1638.
6. Engler MD and Kelley LC (2000). A rapid DNA isolation procedure for the identification of *Campylobacter jejuni* by the polyinerase chain reaction. *Letters in Applied Microbiology* 31: 421-426.
7. Giovannucci E, Rimm EB, Wolk A, Ascherio A, Stampfer MJ, Colditz GA and Willett WC (1998). Calcium and fructose intake in relation to risk of prostate cancer. *Cancer Research* 58: 442-447.
8. Hassan J, Parvej MS, Rahman MB, Khan MSR, Rahman MT, Kamal T and Nazir MHMNH (2014). Prevalence and characterization of *Escherichia coli* from rectal swab of apparently healthy cattle in Mymensingh, Bangladesh. *Microbes and Health* 3: 12-14.
9. Hossain AM, Al-Arfaj AA, Zakri AM, El-Jakee JK, Al-Zogibi OG, Hemeg HA and Ibrahim IM (2015). Molecular characterization of *Escherichia coli* O157:H7 recovered from meat and meat products relevant to human health in Riyadh, Saudi Arabia. *Saudi Journal of Biological Sciences* 22: 725-729.
10. Hossain MF (2015). Study on microbial assessment of milk collected from different markets of Mymensingh, Gazipur and Sherpur districts with particular emphasis on the molecular detection and antimicrobial resistance of the isolated bacteria. MS thesis, submitted to the department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202.
11. Islam MA, Mondol AS, de Boer E, Beumer RR, Zwietering MH, Talukder KA and Heuvelink AE (2008). Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. *Applied and Environmental Microbiology* 74: 5414-5421.
12. Kaddu-Mulindw DH, Aisu T, Gleier K, Zimmermann S and Beutin L (2001). Occurrence of shiga toxin-producing *Escherichia coli* in fecal samples from children with diarrhoea and from healthy zebu cattle in Uganda. *International Journal of Food Microbiology* 66: 95-101.
13. Kumar R and Prasad A (2010). Detection of *E. coli* and *Staphylococcus* in milk and milk products in and around Pantnagar. *Veterinary World* 3: 495-496.
14. Levine MM (1984). *Escherichia coli* infections.Germander (ed), Bacterial vaccines, Academic Press, Inc, New York. pp: 187-235.
15. Leelaporn A, Phengmak M, Eampoklap B, Manatsathit S, Tritilanunt S, Siritantikorn S, Nagayama K, Iida T, Niyasom C and Komolpit P (2003). Shiga toxin- and enterotoxin-producing *Escherichia coli* isolated from subjects with bloody and nonbloody diarrhoea in Bangkok, Thailand. *Diagnostic Microbiology and Infectious Disease* 46: 173-180.
16. Qadri F, Svennerholm AM, Faruque AS and Sack RB (2005). Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment and prevention. *Clinical Microbiology Reviews* 18: 465-483.
17. Schippa S, Lebba V, Barbaro M, Nardo GD, Totino V, Checchi MP, Longhi C, Maiella G, Cucchiara S and Conte MP (2010). A distinctive microbial signature' in celiac pediatric patients. *BMC Microbiology* 10: 175.
18. Singleton P (1999). Bacteria in Biology, Biotechnology and Medicine, 5[th]Edn. Wiley. pp. 444-454.
19. Soomro AH, Arain MA, Khaskheli M and Bhutto B (2002). Isolation of *Escherichia Coli* from Raw Milk and Milk Products in Relation to Public Health Sold under Market Conditions at Tandojam, *Pakistan Journal of Nutrition* 1: 151-152.
20. Zafolon LF, Langoni H, Benvenutto F, Castelani L and Broccolo CR (2008). Aspectos epidemiológicos da mastitecausadapor *Staphylococcus aureus*. *Veterinária e Zootecnia* 15: 56-65.