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Prevalence and Antibiotic Resistance Pattern of *Escherichia coli* Isolated from Raw Dairy Milk

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ABSTRACT

E. coli is one of the most important food borne pathogen, which could be transmitted by milk and milk products. To assess the role of dairy milk as the source of drug resistant *E. coli*, we examined 50 raw dairy milk samples (25-farm milk + 25-market milk) from some selected areas of Bangladesh by cultural, morphological, biochemical and antimicrobial sensitivity tests. In the preliminary observation, the mean total aerobic mesophilic count of market and farm raw milk samples were 8.98 and 8.68 log CFU/ml, while mean coliform count were 4.20 and 3.03 log CFU/ml respectively. Thirty-three *E. coli* isolates were recovered from collected samples (66% 33 of 50) and this pathogen was more prevalent in market milk (76%, 19 of 25) than farm milk (56%, 14 of 25). In addition, most of the isolated *E. coli* exhibited resistance against ampicillin and cefotaxime. This result shows that, the raw dairy milk and its products could be a source of human drug resistant *E. coli*.

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Introduction

Diarrheagenic *Escherichia coli* continue to pose global threat for infant diarrhea, travelers' diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. *E. coli* usually harbor harmlessly to the intestinal tract of mammals; however, in the exhausted or immunosuppressed host, or when intestinal barriers are contravened, nonpathogenic strains of *E. coli* can cause serious illness (Erkmen and Bozoglu, 2016). About 70% of the childhood death in the developing countries are contributing to diarrheal diseases (Tulloch and Richards, 1993). In addition, it is considered as the second cause of child death worldwide according to the global burden disease report of the world health organization (Kosek et al., 2003). The frequencies of the pathogen vary with geographic region and depend on the socioeconomic and sanitary conditions achieved in the region or country (Black et al., 2010). About 34% of total diarrheal episodes in Bangladesh are due to diarrheagenic *E. coli* (ICDDR, 2002) and 20% of all diarrheal cases is associated with enterotoxigenic *E. coli* (Qadri et al., 2005). Bovine animals and their products are considered as one of the sources of pathogenic *E. coli* worldwide. Hassan et al. (2014) found 75% of healthy cattle in Bangladesh are the natural reservoir of *E. coli*, they also reported 43.33% shiga toxin producing *E. coli* (STEC) in cattle feces, which might be the source of STEC in Human (Hassan et al., 2017). Wang et al. (2017) reported about 75, 26 and 45% of enteropathogenic *E. coli* (EPEC), *stx1* and *stx2* genes respectively in cattle fecal samples collected from Japan. They also informed that patient's strain of atypical enteropathogenic *E. coli* (aEPEC) were closer to bovine strain (Wang et al., 2013). There are reports suggesting that ruminants could shed and spread *E. coli* to humans through fecal contamination of meat and milk (Elder et al., 2000; Asakura et al., 2001; Naidu et al., 2007).

Major modes of transmission of diarrheagenic *E. coli a*re consumption of contaminated foods and drinks, animal products and contact with farm animals (Kassenborg, 2004). Milk might be a source of *E. coli* for human and could be contaminated with food borne pathogen during milking process from the milking personnel, utensils used for milking (Rehman et al., 2014) or microorganisms may enter the udder through teat canal from the environment (Smith et al., 2007). The presence of pathogen in milk largely depends on fecal

contamination (Aycicek et al., 2005). Most bacteria either pathogenic or nonpathogenic can grow and multiply in milk due to its high nutrient contents resulting in objectionable physical changes in milk that render it of inferior quality or unfit for human consumption (Asamenew et al., 2012).

However, neither the origin nor the etiological role of raw dairy milk to human *E. coli* infection has been clarified clearly in our country. In this study, we examined whether dairy milk in the farms and local market act as a source of human *E. coli*. A total of 50 milk samples, 25 samples from local market, 25 samples from dairy farm were examined for the presence of *E. coli* and the drug resistance status of the isolated strains also divulged.

Materials and Methods

Sample Collection

We collected 25 dairy raw milk samples from five different villages and 25 samples from respective village linked five local markets located in Jessore district in the southwestern climatic zone of Bangladesh. We used sterile container for sample collection and transportation and each sample contained 150 ml of fresh raw milk. In addition, we made a survey among 50 participant in the study area by a cross sectional questionnaire including their educational qualification, personal sanitary condition, source of drinking water, consumption of raw milk and milk products to elucidate the awareness of the peoples in that zone of the country.

Determination of Total Aerobic Mesophilic Count and Coliform Count

To determine the total viable count of milk samples, 0.1 ml of each ten-fold dilution was transferred and spread on plate count agar (PCA) using a sterile glass spreader (Erkmen, 2015). The plates were then incubated at 37°C for 24 hours. Following incubation, plates exhibiting 30- 300 colonies were selected to count. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the TAMC according to ISO (1995). The results were expressed as colony forming units per ml (CFU/ml) of milk sample. The procedures for coliform count (CC) were similar to those followed in TAMC except MacConkey agar was used.

Isolation of E. coli

A quantity of 0.1 ml inoculums from dilutions was used by spread plate technique on Eosin Metheline Blue agar (EMB) (Himedia, India). Incubated at 37°C for 24 hours. Typical characteristics colony of *E. coli* on EMB agar as greenish metallic sheen was enumerated and isolated by sub culturing into EMB. Isolated organisms with supporting growth characteristics of *E. coli* were subjected to Gram's staining, sugar (dextrose, fructose, maltose, lactose and sucrose) fermentation, methyl red-voges proskauer test (MR-VP) and indole production test following the procedure mentioned by (Erkmen, 2015).

Antibiotic Sensitivity Test

Antibiotic sensitivity test of isolated *E. coli* was performed with standardized commercial antibiotic discs (Oxoid, UK) following Disc Diffusion Method (Bauer et al., 1966). Sensitivity to antibiotic was studied on Muller Hinton agar plates (Himedia, India) with ampicillin (10µg/disc), chloramphenicol (30µg/disc), ciprofloxacin (5µg/disc), gentamicin (10µg/disc), cephalexin (30µg/disc). An amount of 0.1 mL freshly grown pure culture of *E. coli* in nutrient broth (turbidity was compared with 0.5 McFarland standards) was poured on agar plates and allowed to spread gently over the entire surface with a glass rod spreader. After 5 minutes, the discs were placed at a distance of about 1 cm apart and incubated at 37°C for overnight. Based on the diameter of zones of inhibition produced around the antibiotic discs the inhibitory effect of the antibiotic to the growth of the culture was recorded and analyzed according to CLSI (2007) (Table 1).

Statistical Analysis

The data on total viable count and total coliform count of market and farm milk samples were analyzed in completely randomized design (CRD) and t test using SPSS Software (Version 16, 2007). Correlation between total viable count and total coliform count were also evaluated by Pearson Correlation Coefficient method.

Result

We conducted the survey among 50 participant in the study area by a cross sectional questionnaire which enclosed their educational qualifications, animal rearing system, sanitary condition, source of drinking water, consumption of raw milk and milk products to clarify the awareness of the peoples in that zone of the country. Among the participant, 88% were literate from primary to junior high school, only 22% of them used deep tube well for drinking water, while 66% tube well were close to the toilet and 56% respondent rear dairy cow in intensive system, 12% of them drink/eat raw milk and milk products (Table 2).

Table 1 Diameter of zone of inhibition for *E. coli**

Antibiotic	Sensitive (≥ mm)	Intermediate resistance (mm)	Resistance (≤ mm)
Ampicillin	17	14-16	13
Gentamicin	15	13-14	12
Ciprofloxacin	21	16-20	15
Chloramphenicol	18	13-17	12
Cefotaxime	18	15-17	14

*Source: Clinical and laboratory standard institute (CLSI), 2007.

Table 2 Socio economic conditions of the farmers in study area

Category	Subcategory	Percentage (%)
Educational status	Illiterate	12
	Able to signature	18
	Primary	32
	Secondary	38
Sources of drinking water	Deep tube-well	22
	Shallow tube-well	78
	Pond/river	00
	Mineral water	00
Location of tube well	Attached to toilet	56
	Near about toilet	14
	Far from toilet	30
Cow rearing systems	Intensive	56
	Semi-intensive	44
	Open	00
Raw milk or products	Eating raw products	12
	Eating processed/boiled	88

Table 3 Comparison between TAMC and CC in market and farm samples.

Samples	TAMC (Log CFU/ml)				CC (Log CFU/ml)			
	Max	Min	Average	Remarks	Max	Min	Average	Remarks
Market milk	9.62	8.35	8.98	S (P = 0.00021)	6.69	00	4.20	NS (P = 0.071)
Farm milk	9.34	8.04	8.68		6.60	00	3.03	

Note: S = Significant, NS = Not significant. The TAMC of market milk samples were significantly higher than farm milk samples and no significant difference was observed among CC of farm milk samples and market milk samples

Total Aerobic Mesophilic Count and Coliform Count

Highest total aerobic mesophilic count in market milk was 9.62 log CFU/ml, while coliform count was 6.69 log CFU/ml. In the farm milk samples, highest total aerobic mesophilic count was 9.34 log CFU/ml and coliform count was 6.60 log CFU/ml. The total aerobic mesophilic count in market milk samples were significantly higher than farm milk samples in t test ($P<0.05$). There is no significant difference between coliform count in market milk samples and farm milk samples (Table 3).

Correlation Between Total Aerobic Mesophilic Count and Coliform Count

The result presented in Fig. 1 revealed positive correlations between TAMC and CC in market milk samples. Abruptly a weak relationship was observed between TAMC and CC. The value of correlation coefficient was $R^2 = 0.0013$ and $R = 0.0365$ and regression equation was, $y = 0.1903x + 2.4992$. The result presented in Fig. 2 showed negative correlation between TAMC and CC of farm milk samples. The value of correlation coefficient was $R^2 = 0.0105$ and $R = 0.1026$ and regression equation was, $y = -0.643 x + 8.5399$.

Detection of E. coli

All the isolates upon overnight incubation at 37°C produced greenish black colored colonies with characteristic metallic sheen on EMB agar and large bright pink colored colonies with lactose fermentation on MacConkey agar (Kalin et al., 2012**)**.

In Gram's staining, the morphology of the isolated bacteria exhibited Gram-negative short rod arranged in single or paired. All the isolates fermented five basic sugars with the production of both acid and gas. The isolates were positive to MR and indole production but negative to VP test. Among the collected samples, 66% (33/50) possessed *E. coli* and the organism was more prevalent in market milk (76%, 19 of 25) than farm milk (56%, 14 of 25).

Antibiotic Resistance Pattern

We used five common antibiotics for the antibiogram of the isolated *E. coli*. Based on zone of inhibition, all of the isolates were found to be sensitive against ciprofloxacin, gentamicin and chloramphenicol while resistant to ampicillin and cefotaxime (Fig. 3)

Discussion

The demand for safe fresh milk is rising in the country with the increasing education and awareness. However, the hygienic condition of raw milk sold in local market of Bangladesh is unknown. There is no surveillance system in milk market or dairy farms in the country. If proper hygienic practice in milk collection and marketing are not followed, it might be a source of zoonotic organisms and cause serious health hazards to human (Ray and Bhunia, 2007). 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; Ogunleye et al. (2013) reported a prevalence of 80% *E. coli* in apparently healthy cattle in Nigeria, Wang et al. (2013) informed 75% prevalence of *E. coli* in bovine feces collected from Japan.

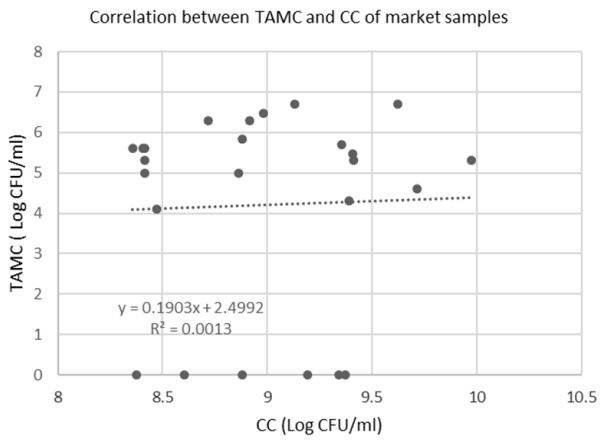

Figure 1 The correlation between TAMC and CC of market samples.

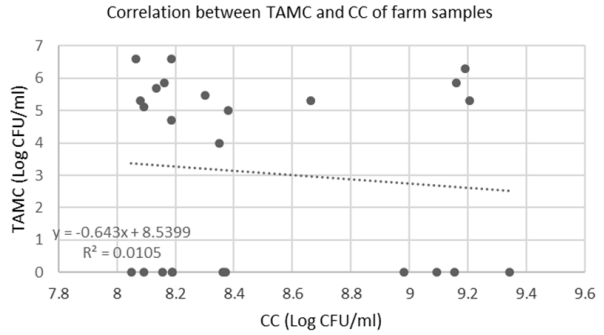

Figure 2 The correlation between TAMC and CC of farm samples

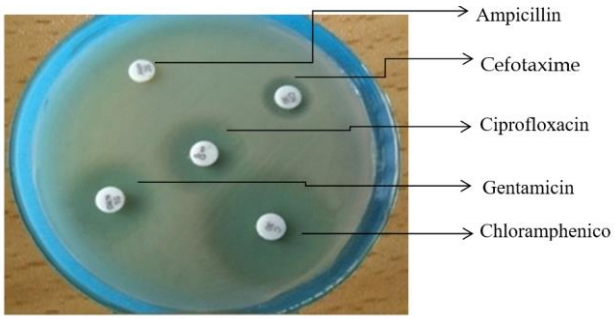

Figure 3 Antibiotic sensitivity pattern

In Bangladesh, the prevalence of *E. coli* was 23.21% in apparently healthy cattle (Masud et al., 2012). We found, 75% rectal swabs of cattle in Bangladesh harbor *E. coli* (Hassan et al, 2014). This pathogen has also been isolated from different other sources in the country by other researchers like water (Nazir et al., 2005) broiler birds (Mamun et al., 2016; Khatun et al., 2015) and layer birds (Himi et al., 2015). As the pathogen is present in feces of all mammals and birds, water and other food particles, chances of contamination of milk is high. Fecal contamination of milk could be a serious public health problem in Bangladesh but the microbiological study on dairy milk is scanty in the country. Tanzin et al. (2016) isolated 9% *E. coli* from dairy milk and buffalo milk in Bangladesh. However, these studies was limited in one dairy farm only, in addition they did not covered the local milk market of the country. However, we studied market milk besides farm milk because the milk can be contaminated in different stages of marketing also. Alam et al. (2017) isolated *E. coli* from milk samples marketed at another geographical area of Bangladesh, (Chittagong). Hadrya et al. (2012) found 4.2×10^7 CFU/ml of coliform count from raw milk at Morocco. In addition, Aaku et al. (2004) and Arenas (2004) observed that the total viable count in raw milk sample was 5.5×10^6 CFU/ml. Hossain et al. (2017) examined pasteurized, unpasteurized and UHT milk samples at greater Mymensingh area of Bangladesh and reported about log 7.4 TVC with log 3.5 CC on average in all milk samples. The TAMC and CC in market milk were 8.98 log CFU/ml and 4.20 log CFU/ml in our study, which was higher than previous studies; it might be due to insanitary condition during marketing of milk, since the TAMC and CC were not so high among farm milk of our study. Zeinhom and Abdel-Latef (2014) stated that *E. coli* was detected in 26.7 and 16% of the milk sampled from markets and farms of Egypt, respectively. Alam et al. (2017) isolated *E. coli* from 18 and 12% market milk and farm milk at Chittagong region of Bangladesh. Hossain et al. (2017) isolated *E. coli* from 32% milk samples in Mymensingh region of Bangladesh. In this study, we isolated 66 and 56% *E. coli* from market milk and farm milk respectively. This variation might be due to different geographical location, hygienic condition followed by farmers and all personnel in the milk marketing chain, different study methods also can differ the result.

Drug resistance pathogens have been increasing worldwide, leading to failures in treatment of infectious diseases in human and mammals. Uddin et al. (2011) isolated *E. coli* from raw milk in Dhaka city of Bangladesh and reported that the isolates were 100% resistant against rifampin and tetracycline while 50% resistant against nalidixic acid. Tanzin et al. (2016) isolated gatifloxacin resistant *E. coli* from milk samples in Mymensingh region of Bangladesh while Hossain et al. (2017) reported amoxicillin and erythromycin resistant *E. coli* from milk samples in this region. The isolates in our study also exhibited resistant to ampicillin and cefotaxime, which is supported by the previous studies of other researchers in the country.

Conclusion

The present study indicated that the raw milk samples in the study area are highly contaminated in both farm and local market. The problem of drug resistant bacteria is a matter of concern globally especially in developing countries, where animals live in closer to human, unaware people drink raw milk and milk products and chances of transmission of drug resistant pathogen from animal to human is high. The indiscriminate use of antibiotics in dairy animals and every steps in milk collection, processing, marketing should be closely monitored. Molecular characterization of *E. coli* isolates from milk and diarrheal patients in the country is recommended to elucidate the role of raw milk as a source of human infection in Bangladesh and a routine survey of antibiotic resistance pattern is acknowledged in the country.

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