



Molecular characterization and antimicrobial resistance of *Escherichia coli* in dairy products of Dhaka, Bangladesh

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Abstract

This study aimed to identify and evaluate the occurrence and antibiotic susceptibility of *E. coli* in various dairy products. Physical, biochemical, and molecular tests were used to identify and characterize the *E. coli* isolates. The study found that *E. coli* was present in 16% of raw milk, 8% of cheese, 6% of butter, and 10% of ice cream samples. No *E. coli* O157:H7 or its toxin *stx1* was identified in any samples. The antibiotic susceptibility test revealed that the highest susceptibility was to Azithromycin, Gentamycin, and Ciprofloxacin, while the lowest susceptibility was to Amoxicillin/clavulanic acid, Tetracycline, and Trimethoprim/sulfamethoxazole. Interestingly, 36.84% of *E. coli* isolates showed multidrug resistance, which is a serious health concern as they may transmit and develop antibiotic resistance in the human body. The study highlights the need for continued surveillance and monitoring of dairy products for food safety and public health purposes.

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Introduction

E. coli is a gram negative, rod-shaped, anaerobic bacterium which normally inhabits in human and animal intestinal tracts (Abd El Tawab *et al.* 2015). Many diseases can be occurred through pathogenic *E. coli* (Ribeiro *et al.* 2019). *E. coli* can be categorized into enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) according to their virulence genes (Deb Roy *et al.* 2018).

The pathogenic strain of *E. coli* can cause several illnesses both in humans and animals. *E. coli* has the capability to produce serious diseases like diarrhea and other systemic diseases, urinary tract infection etc. *E. coli* STEC strains mediated infection in human has been reported in several countries (Kaddu-Mulindwa *et al.* 2001; Leelaporn *et al.* 2003). About 20% of diarrheal cases in Bangladesh are caused due to ETEC *E. coli* (Qadri *et al.* 2005).

Milk is very high nutritious food. Milk and dairy products contains nutritional components including proteins, lipids, minerals, and vitamins which are important for maintenance

and growth of human body (Ababu *et al.* 2020). Milk and dairy products are very common food of billions of people worldwide. Sometimes raw or ununpasteurized milk can cause illness as those milk can contain pathogens which are contaminated either by animals or environment due to improper milk processing in non-hygiene environment (Rehman *et al.* 2014). Ice cream is one of the most delicious foods. Peoples of all ages specially children loves ice cream. Milk is the main ingredient of ice cream. Pathogens can use ice cream as their growth media as milk is the main component of ice cream which has almost neutral pH, high nutritional value and longer storage time. The main sources of ice cream contamination are the ingredients (especially water and raw milk) used during production as well as flavoring agents, utensils and handling during production system.

Butter is another milk derived product that is produced by churning the cream from cows' milk. Butter is the form of highly concentrated fluid milk. About one third of world's total milk is used for butter preparation.

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Butter contains high amount of milk fat but the protein concentration is very low. Besides, butter is enriched with vitamin A, calcium, phosphorus and vitamin D. Cheese is milk-derived fermented food. The health benefits of cheese including the body formation enhance strong immune system, strong hair and proper fluid balance in body. In Bangladesh, cheese is consumed widely in several food items. The manufacturing process of cheese in unhealthy conditions is public health concern. As the main component of cheese production is raw milk, there have possibility to contamination of cheese with pathogenic bacteria like *E. coli* during the pre-production to post production process.

As *E. coli* inhabits in intestines of warm-blooded animals (Lara *et al.* 2016) so, there have possibility to transmit to milk from animal body. The main reason is the fecal contamination during the improper handling of milk (Garbaj *et al.* 2016; Sharafati *et al.* 2014). The products that are derived from raw milk including ice cream, butter and cheese can introduce pathogenic bacteria in human body. The out breaks of food borne diseases have been reported due to the consumption of raw milk and processed dairy items (CDC, 2022).

Occurrence of pathogens in dairy products are serious health issue especially who consumed dairy products that are produced from raw or unpasteurized milk. Pathogenic bacteria in foods are responsible for foodborne diseases and antimicrobial resistance.

In dairy industry, Bovine mastitis is known as one of the serious diseases (Seegers *et al.* 2003). Bovine mastitis is also one of the major problems of Bangladesh in term of milk production reduction and loss of milk quality (Biswas *et al.* 2020). Several microorganisms such as virus, bacteria, mycoplasma and yeast are responsible for mastitis (Rahman *et al.* 2013). Among the bacteria, *E. coli* is mostly responsible for bovine mastitis worldwide (Barkema *et al.* 1998; Lan *et al.* 2020; Tenhagen *et al.* 2009; Verbeke *et al.* 2014).

The microorganisms responsible for milk contamination have been investigated throughout the world (Hassan *et al.* 2014). Limited number of works has been reported on the identification of *E. coli* in food items like dairy products in Bangladesh (Alam *et al.* 2006; Islam *et al.* 2008; Hessain *et al.* 2015). Still now only a few researches have been conducted on characterization of *E. coli* at molecular level in dairy products.

Therefore, the aim of this current study was to characterize *E. coli* in dairy products at molecular level from different

locations of Dhaka district Bangladesh. The isolated bacteria were subjected to antimicrobial susceptibility testing after detection using *16s rRNA* gene.

Materials and methods

Sample collection

In this research, about 50 fresh raw milk samples, 50 pasteurized milk samples, 50 cheese samples, 50 butter samples and 50 local made ice cream samples were randomly collected from different areas of Dhaka district including Savar, Ashulia, Dohar, Keraniganj, Nawabganj and Dhamrai. Raw milk samples were collected directly from farmers while pasteurized milk samples, cheese and butter samples were collected from local stores. About 5 ml of milk samples were collected in sterile falcon tubes from June, 2022 to August 2022. After collection, the samples were properly stored on ice box and transported to the lab for further analysis and stored at -20°C until culture experiments and DNA extraction.

E. coli identification

E. coli was enriched and isolated following protocol with minor modifications. About 500 µl of raw milk samples were inoculated into 4.5 ml of LB (Luria Bertani) media (Alpha Bioscience, UK) and incubated at 37°C for overnight. About 100 µl of bacterial culture was inoculated onto Eosin Methylene Blue Levine (EMB) agar (Hi-media, India) and incubated at 37°C for 24 hours. The suspected colonies were further streaked on to EMB agar plates for isolation of pure colonies. In case of other milk product samples, 10 g of samples were homogenized with 90 ml of LB broth. About 100 µl of homogenates were spread on MacConkey agar (Tulip Diagnostics Ltd., India) and EMB agar plates respectively and incubated 37°C for overnight. The grown colonies were confirmed as *E. coli* through colony morphology on MacConkey agar as well as several biochemical tests (Momtaz *et al.* 2013). Final confirmation was accomplished by *16S rRNA* gene amplification.

Genomic DNA extraction

Bacterial DNA was extracted using AxyPrep™ Multi source Genomic DNA Mini prep Kit (Corning, USA) following the prescribed protocol. DNA quality was verified through Nanodrop quantification.

Detection of *16s rRNA* gene

Universal primers (16S-for (5'-GCTGGAT-CACCTCCTTTC-3') and 23S-rev (5'-AGTGCCAAGG-CATCCACC3') were used for *16S rRNA* gene amplification.

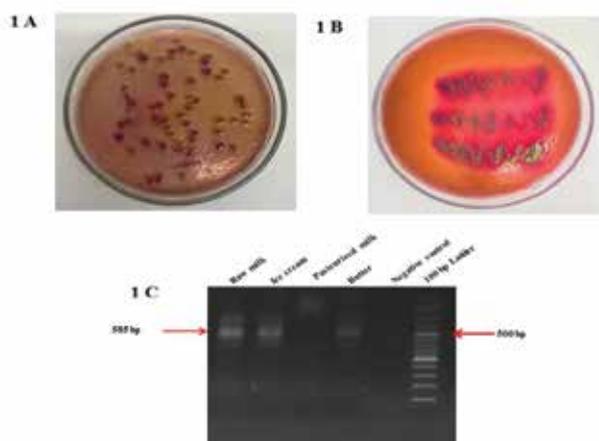


Fig. 1. Identification of *E. Coli* from milk and dairy products. 1 A: *E. coli* colonies on MacConkey agar; 1 B: metallic green glossy colonies on EMB Agar; 1 C: Agarose gel image after PCR

PCR reaction (25 μ l) consisted of primers (0.2 μ M each), DNA template (1 ng), 2X PCR master mix (12.5 μ l) and nuclease free H₂O. The PCR was performed in Aeris™ Thermal Cycler (Esco Life sciences, Singapore) and the thermal cycler conditions were as follows: (i) 95°C for 1 min; (ii) 40 cycles of 45 sec at 95°C, 45 sec at 52°C and 1 min at 72°C; (iii) 7 min at 72°C. The PCR products were justified by gel electrophoresis.

Detection of *E. coli* O157:H7 and its toxin *Stx1*

Specific primers (listed in Table 2) were used to identify *E. coli* O157: H7 (Forward: CGGACATCCATGTGATATGG; Reverse: TTGCCTATGTACAGCTAATCC) and its toxin *stx1* (Forward: ATAAATCGCCATTCGTTGACTAC;

Reverse: AGAACGCCCACTGAGATCATC) as previously described method (Paton AW and Paton JC 1998). The expected amplicon size of *E. coli* O157:H7 and *stx1* was 259 bp and 180 bp respectively. The 25 μ l reaction mix consisted of primers (0.2 μ M each), DNA template (1 ng), 2X PCR master mix (12.5 μ l) and nuclease free H₂O. Amplification was subjected to 35 cycles where denaturation temperature was 95°C for 1 min; 65°C for 2 min for 10 cycles, then 60°C for 15 cycles; elongation at 72°C for 1.5 min from cycles 25 to 35. The PCR products were then analyzed by 2% agarose gel electrophoresis.

Antimicrobial susceptibility testing

In the next step, *E. coli* isolates were subjected to antimicrobial susceptibility tests following standard guidelines (Humphries *et al.* 2021) by using Kirby-Bauer disc diffusion method. Antibiotics including Ampicillin (10 μ g), Azithromycin (Azm) 15 μ g, Amoxicillin/clavulanic acid (30 μ g), Ceftazidime (30 μ g), Ceftriaxone (30 μ g), Chloramphenicol (30 μ g), Ciprofloxacin (10 μ g), Gentamycin (10 μ g), Imipenem (Imi) 10 μ g, Kanamycin (30 μ g), Norfloxacin (30 μ g), Tetracycline (30 μ g) and Trimethoprim/sulfamethoxazole (10 μ g) (HiMedia Laboratories Pvt. Ltd, Mumbai, India) were tested. In brief, bacterial suspensions prepared on sterile phosphate buffered saline (PBS). Then, a sterile swab stick was used to collect bacterial suspensions and later spread on agar plate surface. After placing the antibiotic disks for each plates, incubated at 37°C for 24 h. Later, the inhibition zones on agar plates were recorded according to CLSI criteria. Finally, antibiotic susceptibility was measured for each antimicrobial agent. In Antimicrobial Susceptibility Testing, *E. coli* ATCC 25922 was used as positive control. Each test was repeated for at least three times for better clarification of the results. The calculation of Multiple Antibiotic Resistance (MAR) index was performed following the prescribed

Table I. Isolation of *E. coli*, *E. coli* O157:H7 and virulence gene *stx1* from raw, pasteurized milk and other dairy products

Samples	Total number of samples	<i>E. coli</i> Positive	Occurrence (%)	<i>E. coli</i> O157:H7	STEC (<i>stx1</i>)
Raw milk	50	8	16	ND	ND
Pasteurize milk	50	0	0	ND	ND
Cheese	50	4	8	ND	ND
Butter	50	3	6	ND	ND
Ice cream	50	5	10	ND	ND

*ND: Not detected

formula (Krumperman, 1983). The isolates that showed resistance to three or more classes of antimicrobials are titled as multidrug resistant (MDR) (Magiorakos *et al.* 2012).

Statistical analysis

Data derived from *E. coli* isolates were analyzed through SPSS/16.0 software. Statistical difference tests were performed with chi-square at 5% significance level.

from this study. The metallic sheen colonies were isolated from 8 (16%) out of 50 raw milk samples, 4 (8%) out of 50 cheese samples, 3 (6%) out of 50 butter samples and 5 (10%) out of 50 ice cream samples. No *E. coli* isolates were identified in pasteurized milk samples. At least three colonies were collected from each sample type and subjected to molecular characterization. PCR amplification was carried out by using 16s and 23 universal primers and amplified products were confirmed the presence of 16sRNA gene by gel electrophoresis (Fig. 1C).

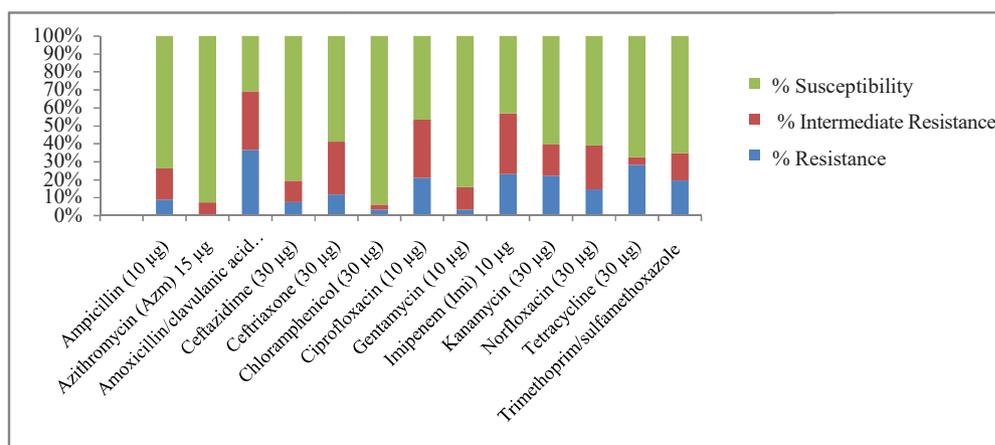


Fig. 2. Percentage of Antimicrobial Susceptibility of *E. coli*

Table II. Primers used in this study

Primer ID	Sequence
16S Fw	5'-GCTGGATCACCTCCTTTC-3'
23S Rv	5'-AGTGCCAAGGCATCCACC3'
stx1-Fw	ATAAATCGCCATTCGTTGACTAC
stx1-Rv	AGAACGCCCACTGAGATCATC
rfbO157:H7-Fw	CGGACATCCATGTGATATGG
rfbO157:H7-Fw	TTGCCTATGTACAGCTAATCC

Results and discussion

Isolation of *E. coli* from collected milk and dairy products

About 50 raw milk samples, 50 pasteurized milk samples, 50 cheese samples, 50 butter samples and 50 local made ice cream samples were analyzed in the laboratory using different cultural, biochemical and staining methods. The red/pink colonies grown on MacConkey agar (Fig. 1A) were further streaked on EMB agar and *E. coli* metallic green glossy colonies with a dark or purple center were confirmed as *E. coli* strains (Fig. 1B). Table 1 outlined the overall findings

Identification of *E. coli* O157:H7 and virulence gene *stx1*

PCR genotyping using *16S rRNA* housekeeping gene primers confirmed the presence of *E. coli* DNA in 20 isolates. About twenty *E. coli* isolates were detected from raw milk, cheese, butter and ice cream samples. The *E. coli* samples were further screened for the presence of *E. coli* O157:H7 and virulence gene (*stx1*). None of the samples found positive for *E. coli* O157:H7 and *stx1* gene.

Study of antimicrobial resistance and calculation of multiple antibiotics Index of identified E. coli strains

Table 3 represents the antibiotic resistance profile and multiple antibiotics index of identified *E. coli* strains. In this analysis, maximum number of the isolates (13) was resistant to Tetracycline which was followed by Amoxicillin (10), Ciprofloxacin (7), Trimethoprim/sulfamethoxazole (4), Imipenem (3), Norfloxacin (2), Kanamycin (1) and Ceftriaxone (1) respectively. From this analysis it was observed that, three isolates from raw milk samples were resistant to three antibiotics, three isolates were resistant to two antibiotics, two isolates were resistant to single antibiotic. Among the *E. coli* isolates from cheese samples, one isolate was resistant to four antibiotics, two isolates were resistant to two antibiotics and one isolate was resistant to single antibiotic. Among the *E. coli* isolates from butter samples, one sample was resistant to three antibiotics; another isolate was resistant to two antibiotics. Among the *E. coli* isolates from ice cream samples, two

isolates were resistant to three antibiotics, one isolate was resistant to two antibiotics and two isolates were resistant to single antibiotic. Multidrug resistance analysis showed that, 36.84% *E. coli* isolates were resistant to three or more antibiotics which might be due to the failed antibiotic therapies and may pose potential threat to human health in developing antibiotic resistance. Fig. 2 represents the susceptibility pattern of tested antibiotics. The susceptibility to Ampicillin, Azithromycin (Azm), Amoxicillin/clavulanic acid, Ceftazidime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamycin, Imipenem (Imi), Kanamycin, Norfloxacin, Tetracycline and Trimethoprim/sulfamethoxazole were 73.7%, 92.7%, 30.7%, 80.6%, 58.9%, 93.9%, 46.6%, 84%, 43%, 60.4%, 60.9%, 67.4% and 65.1% respectively.

The milk samples were collected maintaining proper hygienic conditions. However, 16% of collected raw milk samples contained *E. coli*. There have some differences in *E. coli* prevalence of this present study with other studies throughout

Table III. Antibiotic Resistance Profile and Multiple Antibiotic Resistant Index of Individual *E. coli*

Samples	No. of Antibiotics	Antibiotic resistance	MAR Index
Raw milk			
Sample -1	2	Am	0.2
Sample-2	3	Am-Tet-Cef	0.3
Sample-3	3	Cip-Tet-St	0.3
Sample-4	2	Kan-Imi	0.2
Sample-5	3	Nor-Cip-tet	0.3
Sample-6	2	Tet-Am	0.2
Sample-7	2	Tet	0.2
Sample-8	2	Am-Cip	0.2
Cheese			
Sample-1	2	Am-Imi	0.2
Sample-2	4	Am-Cip-tet-st	0.4
Sample-3	2	Tet	0.2
Sample-4	2	Cip-Tet	0.2
Butter			
Sample-1	3	Tet-Cip-St	0.3
Sample-2	2	Tet-Am	0.2
Ice cream			
Sample-1	2	Am-Tet	0.2
Sample-2	4	Am-Cip-tet-St	0.4
Sample-3	2	Tet	0.2
Sample-4	3	Nor-Imi-Am	0.3
Sample-5	2	Tet	0.2

Am: Amoxicillin, Tet: Tetra cycline, Kan: Kanamycin, Nor: Norfloxacin, Cip: Ciprofloxacin, Cef: Ceftriaxone, St: Trimethoprim/sulfamethoxazole, Imi:Imipenem.

the world (Kumar and Prasad 2010; Silva *et al.* 2001; Disassa *et al.* 2017) but, *E. coli* contamination in milk and dairy products is a growing challenge throughout the world. Lot of peoples especially peoples from developing countries consumed unpasteurized milk and milk products throughout the world. Reports indicated the presence of pathogenic bacterial species including *E. coli* in milk and milk products (Claeys *et al.* 2013). Presence of *E. coli* in raw milk has been reported in a study from Bangladesh (Uddin *et al.* 2011). Another study identified about 18% of *E. coli* contaminated raw milk samples in Chittagong (Alam *et al.* 2017). In a study in Dinajpur district, Bangladesh has detected *E. coli* in commercial ice cream brands which indicated lack of maintaining hygienic practices throughout the manufacturing process (Hasan *et al.* 2015). Pathogenic bacterial (including *E. coli*) contamination of ice cream was identified in other studies in Bangladesh (Hussain *et al.* 2012; Sohel *et al.* 2022). In Pakistan, about 57% of the raw milk samples and 51.66% of milk products were detected contaminated with *E. coli* (Soomro *et al.* 2002).

Molecular characterization of microorganisms derived from milk and dairy products are done worldwide (Gebeyehu *et al.* 2022; Hassani *et al.* 2022; Manishimwe *et al.* 2021; Tóth *et al.* 2020). Occurrence of *E. coli* in milk samples indicates contamination by fecal. Milk can be contaminated in the processing stages after milking. It might get contaminated from the outer surface of udder, hands of peoples dealing with processing, storage equipment and surrounding environments. Not every *E. coli* that present in milk and milk products are harmful for human health except the pathogenic *E. coli* that contains bacterial toxins that can transmit diseases to human body.

The microbial quality of the milk can be affected by feeding style and housing strategies (Pendleton *et al.* 2013). The use of water for washing the milking equipment might be another source of contamination (Canizalez-Roman *et al.* 2013). Moreover, milk that has been stored contains high amount of gram negative bacteria (Kaper *et al.* 2004; Martín *et al.* 2010; López-Saucedo *et al.* 2003; Qadri *et al.* 2005).

The pathogenic bacteria that are found in milk are responsible for major dairy-related disorders. One of the major health concerns in Bangladesh is the presence of pathogenic bacteria in ice cream. Some microbiological assessment studies on ice cream have been conducted in Bangladesh (Hossain *et al.* 2012; Sohel *et al.* 2022). In this analysis, about 10% of the analysed ice cream samples that were collected from local sellers contained *E. coli*. Some of the ingredients like milk,

water might be contaminated by *E. coli* during the preparation process. There have evidence of presence of *E. coli* in milk (Islam *et al.* 2016; Bag *et al.* 2012) and drinking water in Bangladesh (Hasan *et al.* 2022). A study from Iran reported about 15% of the analyzed ice cream samples contained *E. coli* (Hosseini-Naveh *et al.* 2019) while in Belgium *E. coli* strains such as *E. coli* O145 and O26 were detected in ice cream samples (De Schrijver *et al.* 2007). Another study reported about 51.4% of homemade ice cream samples harbored *E. coli* in Costa Rica (Windrantz and Arias, 2000).

Shiga toxin-producing *E. coli* (STEC) are considered as dangerous pathogen causing food borne diseases. In this study, neither *E. coli* O157:H7 nor *stx1* were detected from the isolated *E. coli* isolates. Now-a-days, *E. coli* is identified as one of the major etiologies of bovine mastitis (Green *et al.* 2005). The major reasons for recurrence of infection might be because of infection from environment or those organisms were persistent in the mammary gland. In comparison with other reports (Zhang *et al.* 2018), the finding of this present study indicated that *E. coli* contamination in studied milk, cheese, butter and ice cream samples were relatively lower which might be because of maintaining proper hygienic regulations during collecting of raw milk, preparation of cheese, butter and ice cream samples.

Conclusions

The presence of *E. coli* in raw milk, pasteurized milk, cheese, butter and ice cream was assessed through physiological, biochemical and molecular tests. Our study has demonstrated that, some of these dairy products contained *E. coli* which might be because of contamination during milking and further processing steps. As most of the milk products are prepared from non-pasteurized milk therefore, proper safety precautions are needed to prevent *E. coli* contamination. The peoples who involved in milk processing should be trained on regular basis by the regulatory authorities. Antibiotic resistant pattern of *E. coli* isolates indicated an alarming condition which needs more attention. Pathogenic bacteria may transmit antibiotic resistant genes through their plasmids to the human and thus, develop antibiotic resistance in human body. Therefore, regular food monitoring is strongly recommended to control foodborne diseases especially in developing countries like Bangladesh.

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Declaration of competing interest

The authors have declared no conflict of interest.

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