Bangl. J. Vet. Med. (2017). 15 (2): 141-146 ISSN: 1729-7893 (Print), 2308-0922 (Online)

ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM MILK, BEEF AND CHICKEN MEAT IN BANGLADESH

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ABSTRACT

Food-borne pathogens causing infections and intoxications can affect everyone. *Escherichia* (*E*) *coli* is one of the major food borne bacterial pathogens. This study was conducted to investigate the prevalence of *E. coli* in milk, chicken meat and beef and to determine the multi-drug resistance profile of *E. coli* in Mymensingh district, Bangladesh. A total of 169 samples including milk (n=108), chicken meat (n=51) and beef (n=10) were collected from Bangladesh Agricultural University (BAU) dairy farm, American dairy farm, Gazipur and retail markets of municipal area during July 2016 to June 2017. *E. coli* were isolated and identified by colony characteristics on selective agar like Eosine-methylene blue (EMB) agar, Salmonella-Shigella (SS) agar, Gram staining, biochemical test and Polymerase Chain Reaction (PCR). The overall prevalence of *E. coli* in all food samples was 37.86%. A total of 32 (29.63%) milk, 25 (49.02%) chicken meat and 07 (70%) beef samples were *E. coli* positive through conventional method. Among 64 samples only 23 samples (35.94%) were confirmed by PCR. Multi-drug resistant *E. coli* were detected by disc diffusion test using 10 commonly used antibiotics. Antibiogram study showed that *E. coli* isolated from chicken meat were resistant to oxytetracycline (92%), sulphonamide-trimethoprim (84%), amoxycillin (76%) and erythromycin (60%). *E. coli* isolated from beef sample were resistant to erythromycin (85.71%) and oxytetracycline (71.43%) and sensitive to ciprofloxacin (100%), gentamicin (100%) and neomycin (100%). However, all isolates of *E. coli* were found sensitive to amikacin (100%). *E. coli* isolated from milk sample were 100% sensitive to gentamicin followed by neomycin, ciprofloxacin, azithromycin, oxytetracycline and erythromycin. Overall 50% of *E. coli* isolates of food were found multi-drug resistant. About 28.13%, 57.14% and 76% of the *E. coli* isolates originated from milk, beef and chicken meat respectively were multi-drug resistant. The higher prevalence of *E. coli* in chicken meat, beef and milk indicates unhygienic production and processing of these foods. Presence of multi-drug resistant *E. coli* in these foods might pose serious public health threats. The antibiogram profile of the isolates will help therapeutic decision making in the treatment of colibacillosis in cattle and poultry in Bangladesh.

Keywords: *E. coli*, milk, beef and chicken meat, antibiogram, multidrug resistance, PCR.

INTRODUCTION

Foods of animal origin like chicken meat, beef and milk are rich in proteins which are very essential to body growth and development. However, foods of animal origin also act as a vehicle and medium to transmit various microorganism causing health hazards, disease and death. Food borne diseases are a growing public health problem all over the world which cause an estimated 48 million illnesses and 3,000 deaths each year in the United States (Scallan *et al*., 2011). In developed countries, up to 30% of the population suffer from food borne diseases each year, whereas in developing countries up to 2 million deaths are estimated per year (WHO, 2007a, b).

E. coli is one of the major food borne bacterial pathogen. Majority of the *E. coli* are non-pathogenic but few of them are highly pathogenic causing watery and bloody diarrohea e.g., *E. coli* 0157:H7 which is associated with life threatening disease such as hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Raw milk is considered a high-risk food as it is highly nutritious and serves as an ideal medium for bacterial growth. Several factors are responsible for milk contamination such as poor hygienic milking conditions, contaminated equipment, milking utensils and milk handlers with poor personal hygiene (Mohamed *et al.*, 2014).

E. coli is most common species of facultative anaerobe found in the Gastro-Intestinal tract of both man and animals and the most commonly encountered pathogen in the *Enterobacteriaceae* family, therefore the presence of such organism in foods is the indication of fecal contamination (Mohamed *et al.,* 2014). Due to the increase consumption chicken, milk and beef, the risk of exposure to various animal origin pathogens such as pathogenic *E. coli* has also increased (Shivani *et a1*., 2014*;* Lisa, 2013).

Food is also an important factor for the transfer of antimicrobial resistance. Such transfer can occur by means of residues of antibiotics in food like poultry meat (Jhonson *et al*., 2007), through the transfer of resistant food-borne pathogens or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms (Pesavento *et al*., 2007). In the process of food production many kinds of antimicrobials are used for preventing and controlling diseases, enhancing growth and increasing feed efficiency in food producing animals (CDC, 2005). Due to the indiscriminate uses of antibiotics and agricultural use of antimicrobials (David *et al*., 2002) the incidence of multiple drugs resistance in *E. coli* has been increased (Khan *et al*., 2005; Sharada *et al.*, 2010).

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Bangladesh is a densely populated agricultural country with >160 million and most of them have close interaction with animals. Salauddin (2015) isolated *E. coli* in all the samples of broiler and found some isolates of *E. coli* were multi drug resistant. Islam *et al*. (2010) characterize shiga-toxin producing *Escherichia coli* in raw meat, raw milk in Bangladesh .Food borne diseases including colibacillosis are a significant and widespread global public health threat reported in Bangladesh (Samad, 2011; Zakaria *et al*., 2011). Hence the objectives of this study were to isolate and identify antimicrobial resistant *E. coli* from chicken meat, beef and milk samples in Bangladesh.

MATERIALS AND METHODS

Collection of samples

The samples were collected randomly from farms and local markets situated in Mymensingh and Gazipur district of Bangladesh. A total of 169 (51 poultry meat, 10 beef and 80 milk) samples have been tested from July 2016 to June 2017. Aseptically collected meat samples have been placed in sterile plastic bags and then brought to the Medicine laboratory of veterinary science at BAU using icebox. Milk samples have been collected from BAU dairy farm, American dairy farm, Gazipur, surrounding other local farm and market. Aseptically 8-10 ml of milk was collected in test tube directly from teat of lactating cow and local market and send to the Medicine laboratory using icebox. Five to ten grams of chicken breast meat or beef were collected aseptically in zipper bag and send to the laboratory using ice box.

Sample preparation

Five to ten grams of meat were mixed with 10 ml of peptone (0.1%) water then homogenized suspension was prepared using sterilized pestle and mortar.

Isolation and identification of *E. coli*

The homogenized samples were then transferred into nutrient broths (5 ml/test tube), nutrient agar and others specific media (EMB agar and SS agar, Hi-media, India). In every step, samples were incubated at 37^0C for 24 hours. The positive samples were then subculture several times to be pure culture. Gram staining and Biochemical test (five basic sugars) were done to be confirmed (Cheesbrough, 1985). Antibiotic disc diffusion (CLSI, 2012) test were done for *E. coli* in Muller- Hinton agar (Hi-Media, India).

Polymerase chain reaction (PCR)

DNA extractions were performed through boiling method (Hassan *et al*., 2012). PCR assay were applied in all the 64 isolates to confirm the *E. coli*. For the detection of *E. coli* a highly conserved region such as 16S rRNA was targeted. The nucleotide sequence (5'-3') of the primer ECO-1Foward GACCTCGGTTTAGTTCACAGA and ECO1Reverse-CACACGCTGACGCTGACCA with amplicon size 585 bp (Fratamico *et al*., 2000) were used. PCR reaction mixture for single sample were 20 µl consisting of RNAse free water 5 µl, PCR master mixture (Thermo Scientific, EU) 10 µl, genomic DNA 3 µl and primer 2 µl. The PCR amplification was done by Initial denaturation at 94^0C for 3 minutes followed by 35 cycle of denaturation at 94^0 C for 45 second, annealing at 55^0C for 45 sec and extension at 72^0C for 60 secon. The final extension was at72^0C for7 min. PCR amplify products were subjected to gel (1% agarose, Takara, Japan) electrophoresis with ethidium bromide fluorescence (100 v for 30 minutes) and visualized in gel documentation system via UV transilluminator (302 nm).

Detection of multi-drug resistant *E. coli*

Antimicrobial sensitivity test

Antimicrobial susceptibility of *E. coli* was performed by the disc diffusion test applied on Muller-Hinton agar (Hi-media, India) *in vitro* using 10 commercially available antibiotics (Oxoid, UK) e. g., oxytetracycline (30µg), ciprofloxacin (5µg), gentamicin (10µg), erythromycin, (15µg), azithromycin (15µg), sulphonamide-trimethoprim (25µg), neomycin (10µg), amoxicillin (10µg), doxycycline (10µg) and amikacin (30µg) according to the guidelines of the CLS1 (2012).

RESULTS

Prevalence

Based on cultural, staining and biochemical characteristics, the overall prevalence of *E. coli* in food was 37.86% and in milk 29.63%, chicken meat 49.02 % and in beef 70 % (Table 1).

Table 1. Prevalence of *E. coli* in food

Food Samples	Milk	Chicken meat	Beef	Total
No. of samples tested	108	51	10	169
No. of culture positive samples	32	25	07	64
Prevalence (%)	29.63	49.02	70	37.86

Cultural, staining and biochemical characteristics

Cultural colony characteristics showed that *E. coli* produces turbid growth on nutrient broth and smooth white to grayish white colony on nutrient agar with peculiar fetid odor, dark with metallic sheen on EMB agar and slight pinkish smooth colonies on SS agar. On Gram staining *E. coli* were found Gram negative short rods and arranged as single, paired or in short chain. The five basic sugars e. g., dextrose, maltose, lactose, sucrose and mannitol were fermented by most of the isolates producing acid and gas but few isolates fermented all basic sugar except sucrose.

Molecular detection

The result of PCR is presented in Figure 1. The amplified size of PCR product was 585 bp indicated or reconfirmed as *E. coli* isolate in the food samples.

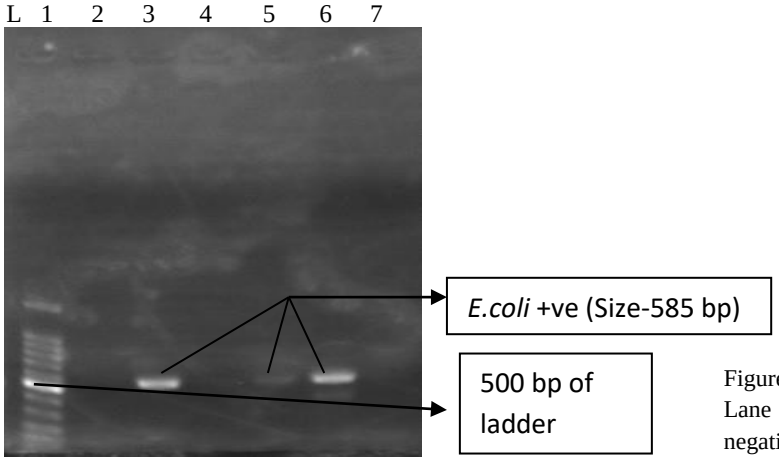

Figure 1. Amplification of PCR product; Lane:1:100 bp ladder, Lane 3,5,6: Specific for *E.coli* (Size-585 bp). Lane 2, 4 and 7 negative

Antibiogram study

The results of antibiotic sensitivity test have been shown in Table 2. *E. coli* isolates of chicken meat were highly resistant to oxytetracycline (92%) followed by sulphonamide-trimethoprim (84%), amoxycillin (76%), erythromycin (60%). *E. coli* isolates of beef samples were highly resistant to ciprofloxacin and gentamicin (100%) followed by erythromycin (86%) and oxytetracycline (71%). All isolates of *E. coli* were highly sensitive to amikacin. *E. coli* isolates of milk samples were highly sensitive to gentamicin (100%) followed by ciprofloxacin, neomycin, azithromycin, oxytetracycline and erythromycin. But they were resistant to amoxicillin (50%), followed by sulphonamide-trimethoprime (47%), doxycycline (44%).

Table 2. Antibiogram study of *E. coli* isolates from different food samples

Antibiotics	Chicken meat (n=25)		Milk (n=32)		Beef (n=07)	
	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)
Oxytetracycline	23(92)	2(8)	05(33.33)	10(66.67)	05(71.43)	2(28.57)
Ciprofloxacin	11(44)	14(56)	05(15.63)	27(84.38)	0	7(100)
Gentamicin	04(16)	21(84)	0	32(100)	0	7(100)
Erythromycin	15(60)	10(40)	13(40.63)	19(59.38)	06(85.71)	1(14.29)
Azithromycin	08(32)	17(68)	09(28.13)	23(71.88)	02(28.57)	5(71.43)
Sulphonamide and Trimethoprim	21(84)	4(16)	15(46.88)	17(53.13)	02(28.57)	5(71.43)
Neomycin	02(08)	23(92)	02(6.25)	30(93.75)	0	7(100)
Amoxycillin	19(76)	6(24)	16(50)	16(50)	03(42.86)	4(57.14)
Doxycycline	12(48)	13(52)	14(43.75)	18(56.25)	02(28.57)	5(71.43)
Amikacin	0	11(100), n=11	0	6(100), n=6	0	7(100)

The prevalence of multi-drug resistant *E. coli* isolated from different food samples was presented in Table 3. About 50% of the total isolates of *E. coli* were multi-drug resistant. However, 76% of the chicken meat *E. coli* isolates was multi-drug resistant.

Table3. The prevalence of multi-drug resistant *E. coli* isolates

Antibiogram applied	Milk	Chicken meat	Beef	Total
No. of *E. coli* isolates	32	25	07	64
Multi-drug resistant	09	19	04	32
Overall prevalence (%)	28.13	76.0	57.14	50.0

DISCUSSION

The overall prevalence of *E. coli* in foods of animal origin was 37.86%. Jakaria *et al.,* (2012) reported a higher prevalence of 78.86% in cloacal sample of chicken. Rahman *et al.* (2004) reported a prevalence of 21.09% in dead or moribund chicken. Barua *et al.*, (2007) reported that the highest prevalence of *E. coli* was observed in ready-to-eat (RTE) milk products (76%), followed by RTE meat products (35.21%). This variation in prevalence with current study might be due to the variation in the selection of sample. Arslan and Ayi (2011) reported 42.9 % prevalence from retail meat sample including chicken meat. Our results indicate that there is a high prevalence of *E. coli* in chicken meat, milk and beef which suggest that the production and processing of these foods are not hygienic. The farmers and people involved in every stage of food production and processing should be educated about food hygiene.
Relatively higher numbers of *E. coli* isolates were obtained in conventional method (37.87%) than molecular technique (35.94%). Similar finding was also reported by Koskinen *et al.* (2010) who reported culture identified a species not targeted by the PCR test in 44 samples from clinical mastitis and in 9 samples from subclinical mastitis and dissimilar with Salauddin (2015) who reported all isolates of *E. coli* were 16S rRNA positive.
Multi- drug resistant *E. coli* isolates were found for 10 commonly used and market available antibiotics. Although we did not check the pathogenicity of the isolates, the gene responsible for multi-drug resistance may transfer to consumer via food and results in serious public health hazard as because Boarlin *et al*., (2005) reported antimicrobial resistance is more frequent in pathogenic than in other porcine *E. coli* strains, and also shows that the resistance genes found in ETEC isolates are different from those of other porcine *E. coli* isolates and that clear associations exist between specific resistance and virulence genes. Jhonson *et al.* (2007) also reported that the drug resistant human isolates were similar to poultry isolates and thus, concluded that many drug-resistant human fecal *E. coli* isolates may be originated from poultry. This resistance occurs due to possessing of resistant gene found in single and multiple size plasmids in *E. coli* isolates.
The highest prevalence of multi-drug resistant *E. coli* isolates was obtained from chicken meat 76%. Adesiyun *et al.* (2007) reported *E. coli* which was resistant to at least three or more antimicrobial agents. Álvarez-Fernández *et al*. (2013) reported that 91.7 % *E. coli* isolates of poultry were multi-drug resistant. Indiscriminate use, improper selection, improper dose, incorrect duration of antibiotics at flock level may be responsible for such a higher occurrence of MDR. Hassan *et al.* (2013) reported 22.7% MDR *E. coli* isolates from bird samples.

In our study, about 92% of *E. coli* isolates of chicken meat were resistant to oxytetracycline followed by sulphonamide and trimethoprim (84%), amoxycillin (76%), erythromycin (60%). Smith *et al.* (2007) also reported similar findings. Jhonson *et al.* (2007) stated that *E. coli* isolates in poultry were resistant to trimethoprim-sulphamethoxazole, quinolones and cephalosporins. Oxytetracycline and potentiated sulphonamides are commonly used antibiotics in Bangladesh. Hence in therapeutic decision these drugs should be used with caution and only after antibiotic sensitivity testing.

Almost 100% of the *E. coli* isolated from beef samples were resistant to ciprofloxacin and gentamicin followed by erythromycin (86%) and oxytetracycline (71%) which is similar to the findings of Jhonson *et al*. (2007). Similarly, use of ciprofloxacin and gentamicin (widely used antibiotics in both cattle and poultry practice) should be done only after sensitivity testing.

All isolates of *E. coli* were highly sensitive to amikacin which differ with the findings of Arya *et al*. (2008) who reported that STEC strains are resistant to amikacin (80%), tetracycline (63%), ciprofloxacin (20%). Amikacin is a recently introduced drug in poultry farm. Rationale use of this drug may prevent development of resistant isolates of *E. coli* in future. *E. coli* isolates of milk sample were highly sensitive to gentamicin followed by ciprofloxacin, neomycin, azithromycin, oxytetracycline and erythromycin. Miles *et al.* (2006) reported that *E. coli* isolates were resistant to tetracycline (82.4%) which is similar to our result.

E. coli isolated from beef sample were highly sensitive to ciprofloxacin, gentamicin and neomycin which are supported by the findings of Hossain *et al*. (2008).

CONCLUSIONS

The higher prevalence of *E. coli* in milk, chicken meat and beef indicates unhygienic production and processing of these foods. Presence of multi-drug resistant *E. coli* in these foods may pose serious public health threats. The antibiogram profile of the isolates may help therapeutic decision making in cattle and poultry practice in Bangladesh. Further studies on pathogenicity and detection of antibacterial resistant genes as well as genetic evolution can be performed.

ACKNOWLEDGEMENT

The authors are grateful to Ministry of Education, The People's Republic of Bangladesh for the financial support of this study (Project No.267/2015 MoE).

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