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RESEARCH ARTICLE

Frequency of Extended Spectrum Beta Lactamase Producing *Escherichia coli* in Fresh and Frozen Meat

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ABSTRACT

A cross sectional study was performed to determine the frequency of *Escherichia coli* in fresh and frozen meat samples followed by antimicrobial resistance profiling and to detect different extended spectrum beta lactamases (ESBLs) genes. A total of 100 samples of fresh and frozen meat (n=50 each) were collected from different butcher shops and supermarkets. Equal numbers of specimens were collected from chicken and mutton. Samples were processed for isolation and identification of *E. coli* by standard microbiological, biochemical and molecular characterization. The resistance pattern was detected by Kirby-Bauer disk diffusion method while presence of ESBLs was checked by double disk synergy test and PCR. The results of present study showed that among 100 meat samples, potentially pathogenic *E. coli* was isolated from 36 samples with greater contamination 20/50 (40%) in chicken samples in comparison to mutton 16/50 (32%). Similarly, the frequency of *E. coli* was more pronounced in fresh meat 30/50 (60%) rather than frozen 4/50 (8%). The highest resistance pattern (100%) was observed against ampicillin, ciprofloxacin, vancomycin and tetracycline followed by cefotaxime (91.6%) and (n=27) isolates were found multi drug resistant (MDR). The double disk synergy test found 17 (47.22%) ESBL producing isolates while *bla* CTX-M gene was identified in 5 (29.41%) isolates followed by *bla* OXA-48 in 4 (23.52%) samples and *bla* TEM gene in 1 (5.88%). This study revealed that vigilant control procedures should be implemented all over the food chain and effective surveillance should also be performed at national level to minimize the spread of MDR and ESBL producing *Escherichia coli* from raw meat.

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INTRODUCTION

Escherichia coli is considered top ranked foodborne pathogen within *Enterobacteriaceae* family. Most of *E. coli* strains are non-pathogenic but few are responsible for gastrointestinal issues including diarrhea in infants, children and adults, urinary tract infections and meningitis (Gallardo *et al*., 2017). Poultry is recognized as main source of *E. coli* dissemination among environment, human community and animals. The pathogenic strains of *E. coli* in poultry often results in difficult to treat infections posing a direct threat to poultry industry and human health. Extraintestinal pathogenic *E. coli* (ExPEC) causes severe losses in poultry in form of mortality and morbidity due to colibacillosis and also recognized as avian pathogenic *Escherichia coli* (APEC) (Bélanger *et al*., 2011). These ExPEC can also cause variety of human extraintestinal diseases like neonatal meningitis, urinary tract infections and sepsis (Ranjan *et al*., 2017). Despite of these ExPEC, poultry gut also accommodates various pathogenic intestinal *E. coli* (Lutful Kabir, 2010). Enterohemorrhagic *E. coli* (EHEC) is also a common intestinal reservoir of cattle, sheep, goat and chicken. EHEC is transmitted to human community primarily via consumption of contaminated raw milk, meat and undercooked meat products (Sethulekshm *et al*., 2016).

Meat and meat products are among the most important edible commodities of animal origin like cattle, sheep and poultry. Contaminated meat is a vital source of food borne illness due to the fact that it has favorable conditions and nutrients for the efficient growth of pathogenic microorganisms such as proteins, fermentable carbohydrates, minerals and growth factors (Datta *et al.*, 2012). Pathogenic strains of *Escherichia coli* are cause of foodborne illness in humans when ingested. Contamination of meat during slaughtering process can occur as a result of direct or indirect contact with feces, contaminated tools, skin, equipments, personnel and their clothings (Adzitey, 2015). The irrational and excessive usage of antibiotics for the treatment of food animals is the reason behind development of multidrug resistance in microorganisms creating a serious public health threat. This resistance is shifted to humans by consumption of meat and its products (Mouiche *et al*., 2019).

There are various strategies which bacteria can adopt to attain drug resistance including enzymatic degradation of drugs, active efflux and alteration in the target site of drugs. The degradation by beta lactamase enzymes is the key mechanism for acquired resistance against beta lactam antibiotics. The increasing trend and spread of extended spectrum beta lactamase (ESBL) producing bacteria are posing a serious threat because they can degenerate clinically important 3rd as well as 4th generation cephalosporins but unable to inactivate carbapenems (Rahman *et al.,* 2018).

The ESBLs are mostly produced among *Enterobacteriaceae*, predominantly in *Escherichia coli*. Moreover, *E. coli* encoding ESBLs are resistant to more than two classes of antimicrobials and referred as multidrug resistant (MDR), presenting a serious challenge in healthcare settings as very few treatment options are left for treatment (Meletis, 2016). Many studies on ESBL producing *E. coli* from clinical settings have been conducted in Pakistan during past few years but there are very few reports regarding their prevalence in meat samples from Pakistan. Due to these facts, the current research was planned to find out incidence of ESBL producing *E. coli* in fresh and frozen meat samples from Pakistan.

MATERIALS AND METHODS

Samples collection: Total (n=100) fresh and frozen meat samples (poultry and mutton) were collected from various butcher shops and super markets. Fifty samples were collected from each category and equal numbers of samples were collected from chicken and mutton. The samples were carefully packaged into polyethylene bags, labeled properly then transferred in the icebox to the laboratory for further processing.

Isolation and identification of *Escherichia coli:* A 25 g of each meat specimen was mixed aseptically in the 225 ml of sterilize peptone water and subjected to overnight enrichment followed by inoculation on MacConkey's agar or Eosin Methylene Blue (EMB) agar (Oxoid, UK) and incubated for 24-48 hours at 37°C aerobically Pinkish colonies on MacConkey agar or distinct metallic green colonies on EMB agar indicated positive growth for *E. coli* which was further confirmed by Gram's staining and biochemical tests (Nawaz *et al*., 2019). Preservation of isolates was done in brain heart infusion with 30% glycerol and stored at -80°C (Rahman *et al*., 2018).

Molecular confirmation of *Escherichia coli:* Molecular confirmation of isolates was done by polymerase chain reaction (PCR) targeting the *uid*A (b-glucuronidase) gene. The DNA was separated using commercial Kit (Thermo Scientific, USA), PCR was performed in thermal cycler (Bio Rad, USA) and products were visualized using 1% gel by electrophoresis as described by (Maynou *et al*., 2017).

Antimicrobial susceptibility testing: After the confirmation of *E. coli*, antibiotic susceptibility profile of all isolates was investigated against commonly used antimicrobials including ampicillin, amoxicillin/ clavulanic acid, Amikacin, Cefotaxime, Ciprofloxacin, Ceftriaxone, Ceftazidime, Gantamicin, Tetracycline, Vancomycine, Imipenem, Meropenem and Dorepenem. The result analysis was done according to the guidelines of Clinical Laboratory Standards Institute as described by (Nawaz *et al*., 2019).

Phenotypic detection of ESBL production: Modified double disc synergy test (MDDST) was used for detection of ESBL producing *E. coli* using cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg) and amoxicillin-clavulanate (20/10 µg) discs. The strains ATCC 35218 and ATCC 25922 were used as negative and positive controls of *E. coli*, respectively. Amoxicillin-clavulanate (20/10 µg) disc was placed to center of the agar plate and other antibiotics discs were placed at 15 to 20mm distance. The ESBL production considered as positive as any increase in zone in the direction of disc of amoxicillin-clavulanate (Saleem *et al*., 2017).

Identification of ESBL encoding genes: The isolates of *Escherichia coli* showing positive phenotypic ESBL detection were processed for the identification of extended spectrum beta lacatamase encoding genes using specific primers by PCR (Table 1). The targeted ESBL genes were *bla CTX-M, bla TEM* and *bla OXA*$_{-48}$ (Hosseinzadeh *et al*., 2018; Nawaz *et al*., 2019).

Statistical analysis: The variables were presented in form of percentages (%). The value ($P<0.05$) was considered as significant. All the statistical analyses were performed by Stata 11 software (Stata Corp, USA).

RESULTS

The results of present study showed that out of 100 meat samples, (n=36) were identified as *Escherichia coli* on the basis of standard microbiological and molecular methods (Fig. 1). The prevalence of *E. coli* was recorded higher 20/50 (40%) in chicken meat as compared to 16/50 (32%) in mutton. Moreover, the detection of *E. coli* was found significantly more pronounced in fresh meat samples 30/50 (60%) rather than frozen meat 4/50 (8%). Greater trend of *E. coli* was found in fresh chicken meat 44.40% than frozen chicken 11.10% ($P=0.021$). Similarly, significant variation was found between the fresh mutton and frozen mutton ($P=0.007$) as described in Table 2.

Table 1: Name and sequences of primers used for PCR amplification

Primers Name	Sequence	Product size	References
uidA-F	ATCACCGTGGTGACGCATGTCGC	486bp	(Maynou *et al.*, 2017)
uidA-R	CACCACGATGCCATGTTCATCTGC		
bla OXA-48 F	GCGTGGTTAAGGATGAACAC	438bp	(Hosseinzadeh *et al.*, 2018)
bla OXA-48 R	CATCAAGTTCAACCCAACCG		
bla TEM F	ATGAGTATTCAACATTTCCG	862bp	(Eguale *et al.*, 2017)
bla TEM R	GACAGTTACCAATGCTTAATCA		
bla CTX-M F	CGTCACGCTGTTGTTAGGAA	780bp	(Bello-López *et al.*, 2017)
bla CTX-M R	ACGGCTTTCTGCCTTAGGTT		

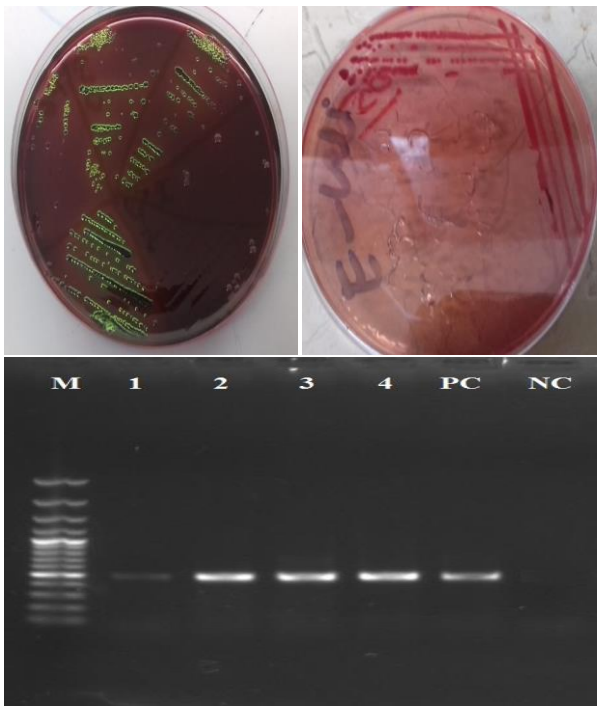

Fig. 1: Confirmation of *Escherichia coli* isolates on the basis of cultural and molecular methods. A: Brilliant green colonies with metallic sheen on Eosin Methylene Blue (EMB) agar. B: Dark pink colonies on MacConkey agar. C: Gel showing 486bp band for *uid*A gene. Lane M: 100bp Ladder, Lane 1-4: Positive samples, Lane PC: Positive control, Lane NC: Negative control.

Table 2: Frequency of *Escherichia coli* from fresh and frozen meat samples

Samples Type	No. of samples collected	Positive *E. coli* samples	Positive *E. coli* samples percentage	P Value
Fresh chicken meat	25	16	44.40%	
Frozen chicken meat	25	04	11.10%	0.021*
Fresh mutton	25	14	38.90%	
Frozen mutton	25	02	5.50%	0.007*
Total	100	36	36.00%	

*Statistically significant at (P<0.05).

Table 3: Percentage resistance pattern of *Escherichia coli* against different antibiotics

Antibiotics Used	Resistance (%age)	Intermediate resistant	Sensitive (%age)
Amoxicillin (AMP)	24 (66.67%)	10 (27.78%)	2 (5.55%)
Vancomycin (VA)	36 (100%)	0 (0%)	0 (0%)
Gentamicin (CN)	24 (66.67%)	3 (8.33%)	9 (25%)
Ampicillin/Clavulanate (AMC)	36 (100%)	0 (0%)	0 (0%)
Cefotaxime (CTX)	33 (91.67%)	2 (5.5%)	1 (2.78%)
Tetracycline (TET)	36 (100%)	0 (0%)	0 (0%)
Ceftriaxone (CRO)	22 (61.12%)	12 (33.33%)	2 (5.55%)
Ciprofloxacin (CIP)	36 (100%)	0 (0%)	0 (0%)
Ceftazidime (CAZ)	24 (66.67%)	8 (22.22%)	4 (11.11)
Amikacin (AK)	23 (63.89%)	1 (2.78%)	12 (33.33%)
Imipenem (IMP)	5 (13.89%)	7 (19.44%)	24 (66.67%)
Doripenem (DOR)	14 (38.89%)	9 (25%)	13 (36.11)
Meropenem (MEM)	9 (25%)	12 (33.33%)	15 (41.66%)

Antimicrobial susceptibility profile of *Escherichia coli*: Different isolates of *E. coli* showed variable pattern of antibiotic susceptibility against various antimicrobial agents. The resistance to not less than 3 different class of antibiotics was observed in (n=27) 75% of the *E. coli* isolates and these isolates were therefore referred as MDR. All the *E. coli* isolates (100%) were found resistant to ampicillin, ciprofloxacin, vancomycin and tetracycline while 91.6% showed resistance against cefotaxime. Furthermore, 66.66% isolates were detected resistant to three drugs, ceftazidime, gentamicin and amoxicillin /clavulanic acid. The highest sensitivity was observed to imipenem (66.66%) as shown in Table 3.

Frequency of ESBL producing *Escherichia coli*: The double disk synergy test showed that phenotypically 17 out of 36 (47.22%) isolates of *E. coli* were ESBL producers. Among these ESBL positive (n=17) isolates, *bla* CTX-M gene was identified from 5 (29.41%) isolates followed by *bla* OXA-48 from 4 (23.52%) and *bla* TEM gene was detected from 1 (5.88%) isolate.

DISCUSSION

Over the last decade, ESBL producing *E. coli* has been frequently contaminating the meat of food animals and posing a global threat to public health and food security. Mostly these isolates are MDR in nature, which increase the challenge for their treatment and eradication. The multi drug resistance is getting more serious in Pakistan where antibiotic usage is not strictly regulated (Rahman *et al*., 2018).

The present study was designed to isolate and identify *E. coli* from fresh and frozen meat samples followed by estimation of their antimicrobial susceptibility and presence of ESBL genes. In this study, the prevalence of *E. coli* was found 36% (36/100) and similar findings with 35.40 % prevalence were reported in Nepal (Saud *et al*., 2019) and 20.40% in chicken meat in Iran (Safarpordehkordi *et al*., 2014). Conversely, a much higher prevalence (75%) of *E. coli* was reported in 2015 (El-Tawab *et al*., 2015) and 49% in raw chicken meat in Bangladesh (Rahman *et al*., 2017). The variation in the results recorded by different scientist was due to contamination of collection sites and type of meat samples. It was found that *E. coli* was more prevalent in fresh chicken and mutton meat samples then in frozen meat. The poor hygienic handling and processing is mainly responsible for the *E. coli* contamination in meat. Same type of findings was also recorded in Saudi Arab (Hemeg, 2018) as the growth of *Escherichia coli* cells is impaired at temperatures below 21°C and stops at 7.5°C.

The antimicrobial susceptibility profiling was done for (n=36) *E. coli* isolates against 14 different antibiotics. The results showed highest resistance pattern (100%) against ampicillin, ciprofloxacin, vancomycin and tetracycline followed by cefotaxime (91.6%). The results finding of Adzitey (2015) and Dehkordi *et al*. (2014) showed higher resistance to ciprofloxacin and ampicillin, while the results of Nahar *et al*. (2018) showed 100% resistance to ampicillin and cefotaxime. *E. coli* isolates 75% (n=27) were categorized as multi drug resistant *Escherichia coli* (MDR-EC). The increased trend of multi drug resistant *E. coli* was also reported by Ahmed *et al*. (2009) in Japan, Adzitey (2015) in Ghana, Altalhi *et al*. (2010) in Saudi Arabia, Dehkordi *et al*. (2014) in Iran and Abdel-Rahman *et al*. (2015) in Egypt. This difference in results of antibiotic susceptibility profile was due to excessive use of antibiotics in commercial poultry and veterinary practice for both prevention and treatment. Among the carbapenems, highest resistance was found against doripenem (38.89%) and meropenem (25%) which is comparable to the findings of Batool *et al*. (2016) in Pakistan and Ahmed *et al*. (2009) in Japan while these results are in contrast with the findings of Zhao *et al*. (2018).

The results of present study showed 47.22% (n=17) isolates were ESBL producers phenotypically using double disk synergy test which is very close to the findings of Hussain *et al*. (2017) with 46% from India and Rahman *et al*. (2018) with 47.6% in Pakistan. A much-elevated level (70%) of ESBL producing *E. coli* was detected by Saleem *et al*. (2017) in Pakistan and Nahar *et al*. (2018) from Japan. This elevated level of ESBL producing *E. coli* in the meat of food animals is posing a potential threat to humans. It was also observed that among the phenotypically ESBL positive (n=17) *E. coli* isolates; *bla* CTX-M gene was detected in 5 (29.41%) isolates which is also supported by Chishimba *et al*. (2016) in Zambia with 13% presence of *bla* CTX-M, 58% in South Africa by Montso *et al*. (2019) and 75% in Ghana by Eibach *et al*. (2018). Furthermore, *bla* OXA-$_{48}$ was detected in 4 (23.52%) and *bla* TEM gene was found in only 1 (5.88%) isolate. The results of Falgenhauer *et al*. (2019) also supported the fact that *bla* CTX-M is the most prominent type of ESBL present in meat samples followed by *bla* TEM.

Conclusions: The results of current study concluded that poultry and mutton meat samples were contaminated with MDR-*Escherichia coli* and this contamination was more pronounced in fresh meat samples. Chicken meat is highly contaminated with *E. coli* than mutton and increased occurrence of ESBL-EC in meat robustly suggest for involvement of efforts to drop this emerging challenge of drug resistance.

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Authors contribution: ZN and ABS conceived and designed the study; MM and MUQ performed the experiments; BA and RA compiled the data and results; MAZ, SA and AR wrote and critically reviewed the manuscript.

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