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Detection and Characterization of Multi Drug-resistant Extended-spectrum and pAmpC Beta-lactamases Producing *Escherichia coli* from Chicken Meat in West Bengal, India

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A B S T R A C T

Antimicrobial resistance can be seen in almost all pathogenic bacteria present in food like chicken meat, leading to treatment failure in human patients and serious public health problems. The present study aimed to detect extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamase (ACBL)-producing *Escherichia coli* from chicken meat, from different parts of West Bengal. A total of 113 raw chicken meat samples were collected during slaughter from different local markets followed by isolation and identification by standard conventional and molecular methods. About79% samples were positive for *E. coli* and among 89 isolates 17(19.1%) were positive to ESBL property and presence of the *bla*$_{CTX-M}$ gene, whereas 78 strains (87.6%) were found to possess *bla*$_{AmpC}$ gene. Antibiogram study of ESBL positive *E. coli* strains revealed resistance of these strains to ceftriaxone, ampicillin (both 100%), amoxicillin/clavulanic acid, ceftazidime, cefotaxime, tetracycline (all approx. 94%), azithromycin (70.6%) and norfloxacin (64.7%) *in-vitro* whereas imipenem (94%), amikacin (82%), gentamicin (58.8%) and ampicillin/sulbactam (71%) were quite effective against these MDR isolates. So, about 79% of chicken meat samples were found to be contaminated with *E. coli,* most of which were resistant to commonly used antibiotics which may lead to animal and human health hazards.

Introduction

India is annually producing 5.3 million metricton of meat i.e. the 5th largest in the World (DAHD, 2017) with the world's largest livestock population which plays an important role in rural economy and livelihood. It produces 21% of global chicken meat production annually. The poultry industry is a high growing vertically integrated industry in India and as well as in the state of West Bengal. West Bengal is the 2nd largest contributor with 640 thousand metric ton meat production of which chicken meat production is 328 thousand metricton (DAHD, 2017). But chicken meat can easily get spoilt with bacterial spoilage which may come from faulty handling, improper storage and poor management of the birds (Dierikx *et al.,* 2010).

Global consumption of drugs, especially antibiotics, has increased tremendously in last few decades leading to rise in their resistance among microbial populations. The incidence of extended-spectrum beta-lactamases (ESBLs)-producing *Escherichia coli* in food is quite significantly increasing nowadays all around the World. ESBL production in bacteria is governed by the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes which are easily transferred from one bacterium to another spreading the antimicrobial resistance. Among these resistance genes, the $bla_{\text{CTX-M}}$ gene is the most common gene associated with ESBL positivity in the *E. coli* (Dierikx *et al.,* 2010). This multidrug-resistant (MDR) pathogen can create a major problem during their treatment forcing the clinicians to use newer and newer antibiotics (Tenover *et al.,* 1999; Olesen *et al.,* 2004).This is leading to the increased use of last-resort antimicrobials such as carbapenems even for non-life threatening infections. These antimicrobial resistance genes of *E. coli* are easily transferrable to other pathogens conferring them resistance. In a study in Mexico, Castillo *et al.,* (2018) revealed that MDR *E. coli* associated with urinary tract infections (63%) of human beings were highly resistant (27-48%) to commonly used antibiotics. AmpC beta-lactamase (ACBL) is the first bacterial enzyme reported to destroy penicillin in Gram-negative bacteria like *Escherichia coli*. ACBL encoding gene bla_{AmpC} is found in transmissible plasmids and also in bacterial chromosomes (Reich *et al.,* 2013).ACBL producing *E. coli* strains are resistant to broad-spectrum cephalosporins but their resistance patterns are less expressed *in-vitro* than that of the ESBLs (Jacoby, 2009).

Chicken meat is a very popular and common source of animal protein worldwide. ESBL producing *E. coli* is frequently reported from chicken samples worldwide and may be pathogenic to humans causing urinary tract infections, septicemia, meningitis etc. (Grami *et al.,* 2013; Nandanwar *et al.,* 2014). Most of the countries are using a large quantity of different antimicrobials to raise poultry which are also used in human treatments. Indiscriminate use of such essential antimicrobials in animal production is likely to accelerate the resistance development of pathogens, as well as commensal organisms like *E. coli*. This would result in treatment failures, economic loss and could act as a source of the gene pool for transmission to humans (Castanon, 2007). In addition, there are human health concerns about the presence of antimicrobial residues in meat, eggs, and other animal products (Sahoo *et al.,* 2010; Darwish *et al.,* 2013).

Identification of potential MDR pathogenic bacteria is essential towards the development of better managemental procedures. With this background, the present research has aimed atthe detection and characterization of ESBL and ACBL producing MDR *E. coli* from raw chicken meat collected from different local markets of West Bengal, India and followed by *in-vitro* antibiogram to assess their resistance patterns.

Materials and Methods

Sample collection

A total of 113 chicken meat samples were collected at the time of slaughter from local markets of Nadia and Hooghly district of West Bengal during the period September to November 2018. About 10g of fresh meat samples were aseptically collected in individual vials and transported (under ice cover) to the laboratory. Primary isolation from the collected samples was attempted on the same day of collection.

Bacteriological isolation and identification

A 10% homogenized suspension of each meat sample was prepared in sterile normal saline and streaked on to MacConkey's agar (Hi-Media, India) plates following overnight enrichment in nutrient broth. The plates were incubated overnight at 37°C and the representative lactose fermenting pinkish colonies were picked up and further streaked on sterile EMB agar (Hi-Media, India) plates. Colonies producing 'metallic sheen' were selected for further morphological (by Gram's staining) and biochemical identification (Quinn *et al.,* 2011; Carter and Wise, 2004). One tentative *E. coli* isolate from each sample was taken in this study.

PCR confirmation of Escherichia coli

Identification of tentative *E. coli* isolates was confirmed from sequence of the 16S *rRNA* gene specific for this bacterium, following the protocol of Wang *et al.,* (1996) (Table 1). Briefly, genomic DNA was extracted from the over-night broth culture of *E. coli* by the conventional phenol-chloroform method. The 16S *rRNA* gene was amplified using specific bacterial primers and sequenced. Bacteria were tentatively identified by finding similarity of the sequences with SSU sequences in the NCBI GenBank and RPD (http://rdp.cme.msu.edu) databases.

Phenotypic detection of ESBL in *E. coli*

In-vitro detection of ESBL activity of the *E. coli* isolates was done by disc diffusion method (Bauer *et al.,* 1966) using both cefotaxime (30µg) and ceftazidime disks (30µg) and their clavulanate (10µg) discs as per CLSI method of Patel *et al.,* (2015). An increase of zone diameter (>5mm) in each clavulanate disk than the single drug disk is treated as phenotypical confirmation of the ESBL activity.

Molecular detection of ESBL property

Molecular confirmation of ESBL was tested in all the phenotypical ESBL positive *E.coli* strains. Bacterial genomic DNAs were extracted following the protocol of Mahanti *et al.,* (2013). Detection of the $bla_{\text{CTX-M}}$ gene was done by PCR assay as per the protocol of Weill *et al.,* (2004) (Table 1). In this method, 5µl bacterial DNA templates, 50pmol of each primer, 200mM deoxynuc1eoside triphosphate, 1U Taq DNA polymerase (Promega, USA), 2mM $MgC1_2$, and 10% dimethyl sulfoxide (DMSO) was added in a 25µl reaction mixture and subjected to amplification with following PCR conditions - 10mins of initial denaturation at 94°C followed by 30s of denaturation at 94°C, 30s of annealing at 53°C and 1min of extension at 72°C for 35 cycles and 10mins of final extension at 72°C. The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 1.5% (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India). In this study, an *Escherichia coli* serotype O2, maintained in the departmental laboratory and one *Pseudomonas aeruginosa* strain (ATCC 27853) were used as positive and negative control respectively.

Phenotypical detection of ACBL Production in Escherichia coli isolates

In-vitro ACBL activity of all *Escherichia coli* isolates was examined by cefoxitin–cloxacillin double-disc synergy (CC-DDS) test following the protocol of Tan *et al.,* (2009).

PCR detection of AmpC gene in *Escherichia coli*

All the CC-DDS positive *E.coli* strains were examined for the presence of the AmpC gene. Molecular detection of the bla_{AmpC} gene was done in all the *in-vitro* ACBL positive *Escherichia coli* isolates was performed following the protocol of Feria *et al.,* (2002) (Table 1). In this method, the total reaction volume was 25µl containing 5µl of bacterial DNA template, 100pmol of each primer, 200mM of each dNTP, 2mM $MgCl_2$, and 10% DMSO.

The PCR mixture was subjected to initial denaturation step of 5mins at 94°C; followed by 30cycles of amplification consisting of 30s of denaturation at 94°C, 30s of annealing at 57°C, 1min of elongation at 72°C and 10mins of final extension at 72°C. The PCR product was electrophoresed in 1.5% (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India) and the gel was visualized in gel documentation system (UVP, UK).

Antibiogram of ESBL gene positive *E. coli*

In-vitro antibiotic sensitivity of the ESBL gene positive *E. coli* isolates was examined using 12 commonly used antimicrobials *viz.,* ampicillin, amikacin, ampicillin/ cloxacillin, amoxicillin/clavulanic acid, ampicillin/ sulbactam, azithromycin, cefotaxime, ceftriaxone, ceftazidime, gentamicin, imipenem, and levofloxacin by disc diffusion method (Bauer *et al.,* 1966). Fresh broth cultures of the positive isolates were poured on to sterile Mueller Hinton agar (Hi-Media, India) plates followed by uniform spreading. Standard antibiotic discs (Hi-Media, India) were used as the source of antibiotics. The inhibition zone diameters were interpreted following the standard chart (Patel *et al.,* 2015).

Results and Discussion

A total of 113 chicken meat samples were examined and 89 (78.86%) samples were tested positive for *Escherichia coli*. Identification of the isolates as *E. coli* was confirmed by their pinkish colonies on Mac Conkey's agar, characteristic 'metallic sheen' on EMB agar plates, Gram's staining and positive reaction to the indole test. Identification was further confirmed by their16S *rRNA* gene sequences (Figure I).This study identified very high prevalence (78.86%) of *Escherichia coli* in poultry meat, which matches with earlier works of Reich *et al.,* (2013), Maciuca *et al.,* (2015) and Klimiene *et al.,* (2018) who reported 45%, 54% and 92% *E. coli* prevalence in chicken meat from different countries showing the significant presence of the pathogen in food chain (Dierikx *et al.*, 2010). All the *E. coli* isolates in this study showed typical cultural, morphological, biochemical as well as the 16S *rRNA* gene sequences of *E. coli* (Quinn *et al.*, 2011; Carter and Wise, 2004; Samanta, 2013).

By the *in-vitro* test, 17 (19.1%) *E. coli* isolates were detected to be ESBL producers; by PCR reaction they possessed the $bla_{\text{CTX-M}}$ gene (Figure II). A total of 78 (87.6%) *E. coli* isolates were confirmed to be ACBL (bla_{AmpC}) producer both phenotypically and genotypically (Figure III, Table 2). *E. coli* strains isolated from Hooghly district's samples showed more positivity in both ESBL and ACBL categories. Sixteen (16) isolates were found to possess both the genes (Table 2) with again Hooghly district isolates showing higher frequency. Similar or higher ESBL positivity in poultry *E. coli* isolates have been reported from different countries by Klimiene *et al.,* (2018), Casella *et al.,* (2017), Tekiner and Ozpinar (2016) and Maamar *et al.,* (2016).

The gene *bla*$_{CTX-M}$ is the most common and dominant gene among all ESBL genes (Feria *et al.*, 2002; Samanta, 2013). The present study also confirms the earlier reports and identifies it as a potential threat for even human health also (Dierikx *et al.*, 2013). The plasmid-mediated AmpC beta-lactamase enzyme is also highly prevalent (88%) among the *E. coli* strains, and the prevalence rate was higher than those observed by Casella *et al.*, (2017)(3.9%) and Van *et al.*, (2008) (23.7%). Such a high prevalence of drug resistance genes in poultry *E. coli* may increase the risk of microbial drug-resistance development in animal and human pathogens (Borjesson *et al.*, 2013).

Table.1 Target genes and their primers used in this study

Gene	Primer sequence	Size	Reference
E. coli* 16S *rRNA	ECO-1 F 5'GACCTCGGTTTAGTTCACAGA3' ECO-2 R 5'CACACGCTGACGCTGACCA3'	585 bp	Wang *et al.*,1996
***bla*$_{CTX\_M}$ consensus (ESBL)**	CTX-M F 5' CRATGTGCAGYACCAGTAA 3' CTX-M R 5' CGCRATATCRTTGGTGGTG 3'	540 bp	Weill *et al.*, 2004
***bla*$_{AmpC}$ (ACBL)**	AmpC F 5'CCCCGCTTATAGAGCAACAA3' AmpC R 5'TCAATGGTCGACTTCACACC3'	634 bp	Feria *et al.*, 2002

Table.2 Frequency of antibiotic resistance and their gene distributions among E. coli isolates from chicken meat from different sampling locations in West Bengal, India

Name of the Districts	No. of meat samples screened	No. of *E. coli* strains Isolated (%)	ESBL positivity in *E. coli* strains (%)	ACBL positivity in *E. coli* strains (%)	Gene distribution in positive *E. coli* strains		
					***bla*$_{CTX-M}$ only**	***bla*$_{AmpC}$ only**	***bla*$_{CTX-M}$ + *bla*$_{AmpC}$**
Nadia	42	32 (76.19)	6 (18.76)	27 (84.38)	0	21	6
Hooghly	71	57 (80.28)	11 (19.29)	51 (89.47)	1	41	10
Total	113	89 (78.76)	17 (19.10)	78 (87.64)	1	62	16

Table.3 Resistance pattern of 17 ESBL positive *E. coli* isolates obtained from chicken meat in West Bengal

Sl. No.	Antimicrobials (Conc. in μg)	Isolates sensitive		Isolates intermediately sensitive		Isolates resistant	
		No.	%	No.	%	No.	%
1.	Amikacin (AK - 30)	14	82.35	3	17.65	0	0
2.	Amoxicillin / Clavulanic acid (AMC - 20/10)	0	0	1	5.88	16	94.12
3.	Ceftriaxone (CTR 30)	0	0	0	0	17	100
4.	Ampicillin/Sulbactam (A/S - 10/10 mcg)	12	70.59	5	29.41	0	0
5.	Ampicillin (AM - 10)	0	0	0	0	17	100
6.	Ceftazidime (CAZ - 30)	0	0	1	5.88	16	94.12
7.	Imipenem (IPM - 10)	16	94.12	1	5.88	0	0
8.	Gentamicin (GEN - 10)	10	58.83	7	41.17	0	0
9.	Norfloxacin (NX - 10)	0	0	6	35.29	11	64.71
10.	Cefotaxime (CTX - 30)	0	0	1	5.88	16	94.12
11.	Azithromycin (AZM - 15)	3	17.65	2	11.76	12	70.59
12.	Tetracycline (TE - 30)	0	0	1	5.88	16	94.12

Figure.1

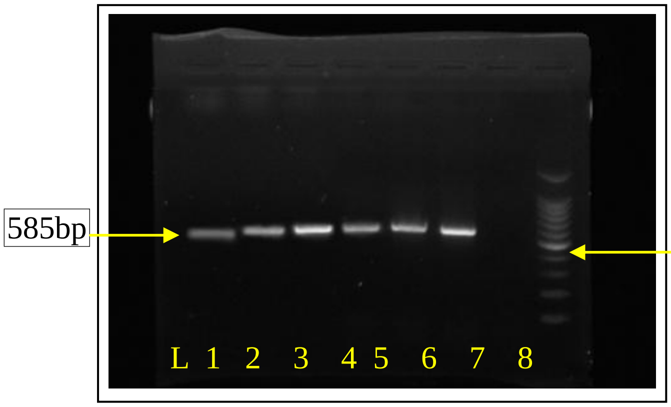


Figure.2

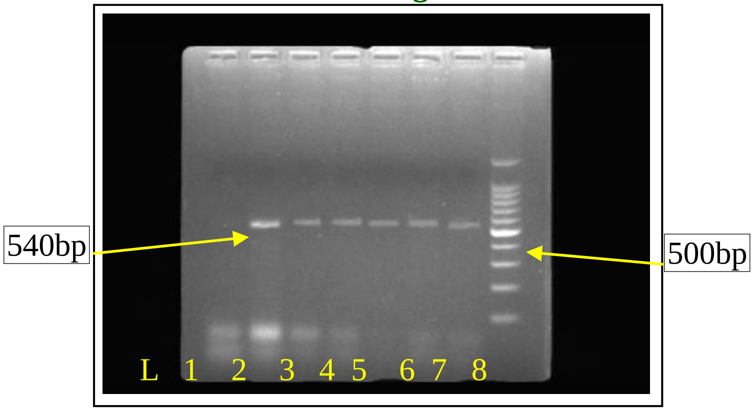
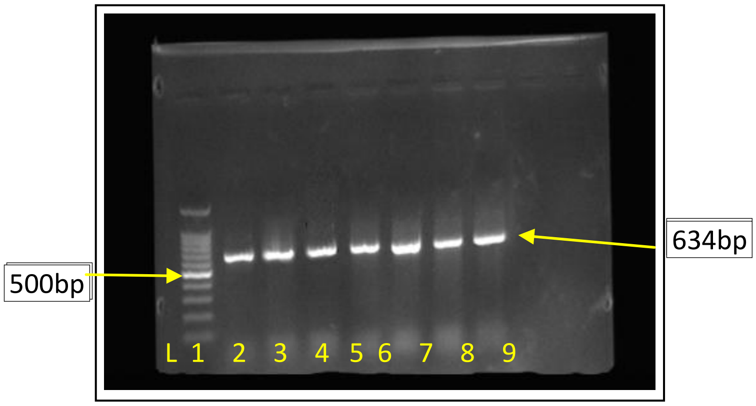


Figure.3



In-vitro antibiogram of the ESBL-positive *E.coli* isolates revealed multi-drug resistance with high-level (64-100%) resistance to ampicillin, ceftriaxone, cefotaxime ceftazidime, amoxicillin-clavulanic acid (all beta-lactams), norfloxacin (fluoroquinolones) and azithromycin (macrolides), tetracycline (tetracycline) (Table 3). Isolates were however sensitive to drugs like amikacin, gentamicin, imipenem, and ampicillin-sulbactam. The ESBL-positive *E. coli* isolates were resistant to most of the antimicrobials and thereby confirmed to possess MDR properties *i.e.* those were resistant to at least 3 different classes of antibiotics (Reich *et al.*, 2013; Maamar *et al.*, 2016; Van *et al.*, 2008; Beninati *et al.*, 2015). Again, Tekiner and Ozpınar (2016) reported that *E. coli* from raw chicken meat were resistant to cefotaxime (62.1%), ceftazidime (55.2%), cefoperazone (51.7%) and cloxacillin (20.6%). Van *et al.*, (2008) also reported multidrug-resistance among poultry meat *E. coli*, which were resistant to tetracycline (77.8%), ampicillin and amoxicillin (both 50.5%),gentamicin (24.2%) and norfloxacin (17.2%) although few other studies indicated that ESBL positive *E. coli* strains are sensitive to few beta-lactams and aminoglycosides like amikacin, imipenem, ampicillin-sulbactam, and gentamicin (Castillo *et al.*, 2018; Tekiner and Ozpınar, 2016). The high positivity of multidrug-resistant *E. coli* in human food items can increase the potential transmission risk of microbial drug resistance to human pathogens leading to treatment failure in near future. This rapid increase in the development and spread of antibiotic resistance is a matter of serious concern (Van *et al.*, 2008; Ryu *et al.*, 2012). In recent years, enough evidences are showing excessive use of antimicrobial agents and antimicrobial resistance from animals and poultry (Mathew *et al.*, 2009). Antibiotic usage has increased markedly over the last few years with the intensification of poultry farming practices, in many countries including India (Castanon, 2007; Sahoo *et al.*, 2010). Major reasons for use of antibiotics in food-producing animals include prevention and treatment of infections, growth promotion and improvement in production in the poultry farm (Mehdizadeh *et al.*, 2010; Haldorsen *et al.*, 2008).

Therefore it can be concluded that approx. 79% of the chicken meat samples examined in this study, were contaminated with *E. coli*; 19% and 87% of these bacteria were positive for ESBL and ACBL property. They were resistant to commonly used antimicrobials other than amikacin, imipenem, gentamicin, and ampicillin/sulbactam. This drug resistance even to third-generation cephalosporins in pathogens like *Escherichia* *coli* is increasing in India as well as in other countries raising serious concern for animal and human health.

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Conflict of Interest

No competing interest exists among the authors.

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