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HIGH RATE OF ESBL PRODUCING *ESCHERICHIA COLI* FROM RETAIL CHICKEN CARRYING *BLA*$_{CTX-M}$ GENE ON PLASMIDS MAINLY CARRYING FREPB REPLICON

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ABSTRACT

Antimicrobial resistance is increasingly reported worldwide, however, there is scarcity of data from Pakistan. Particularly, data from food-producing animals and their products is totally lacking. Here, we report on the ESBL-genotypes, integron types, insertion sequence, plasmid replicon typing and susceptibility to commonly used-antimicrobials of *E. coli* recovered from poultry-meat in live bird market of District Mardan, Khyber Pakhtunkhwa, Pakistan. Random *E. coli* isolates were picked up for screening of ESBL-production and -genotype, antimicrobial resistance profile through disc diffusion method and integron and plasmid replicon typing. Of the 33 *E. coli* multidrug resistant isolates (resistant to tetracycline, ampicillin, cefotaxime and novobiocine), 28 (phylogroup B2=11, A=9 and D=8) were ESBL producers harboring *bla*$_{CTX-M}$ (*bla*$_{CTXM-9=}$25 and *bla*$_{CTXM-1}$=3, *bla*$_{CTXM-15}$=3), while, none of them were carrying *bla*$_{SHV}$ or *bla*$_{TEM}$. Majority of these ESBL producers were carrying class-1 integron (n=21), although 11 isolates were carrying IS*CR1*, which found to be linked with *bla*$_{CTX-M}$ among 5 isolates (45.5%). PCR based plasmid replicon typing revealed that FrepB replicon was found most dominantly and carried by 27 isolates followed by B/O replicon. This suggests that poultry raw meat may contribute to spread of ESBL-producing *E. coli* or ESBL- genes.

Keywords: ESBL; food-producing animals; integron; replicon typing; IS*CR;* multidrug resistant; *E.*

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INTRODUCTION

Antimicrobial resistance (AMR) is increasingly reported from all over the world. AMR seems even more challenging for developing countries like Pakistan mainly due to unrestricted use of antimicrobials and lack of surveillance programs to monitor development of drug resistance (Khan *et al*., 2010; Mitema, 2010; Khan *et al*., 2019; Tasbihullah *et al*., 2020; Shafiq *et al*., 2019). Particularly, resistance to drugs which are proven safe and effective like β-lactams and carbapenems is highly quite frightening as these drugs are losing its efficacy and reputation due to emerging AMR. Bacteria achieve resistance to β-lactams by acquiring the ability to express extended spectrum β-lactamases (ESBL) enzymes that inactivate many antimicrobials including cephalosporins (third and fourth-generation) and monobactams, but could not deactivate cephamycins and carbapenems (ur Rahman *et al*., 2018a; ur Rahman *et al*., 2018b). Generally, *Escherichia coli* encoding for ESBLs have also been found resistant to at least more than two different classes of antimicrobials and are termed as multidrug resistant (MDR). These MDR pathogens are presenting a serious challenge in healthcare settings for both animals and humans.

Genes that encode ESBLs are being classified into three main categories: *Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$. The most predominant *Bla*$_{CTXM}$ type is further comprised of five further sub-groups (*Bla*$_{CTXM-1}$, *Bla*$_{CTXM-2,}$ *BlaC*$_{TX-M-8,}$ *Bla*$_{CTX-M-9}$ and *Bla*$_{CTX-M-25)}$ and more than 160 variants have been reported thus far (http://www.lahey.org/studies). Literature study indicate that *Bla*$_{CTXM-9-}$, *Bla*$_{CTXM-15}$ and *Bla*$_{CTXM-1}$ are the most successful variants for being adapted widely all over the world (Andrea *et al*., 2013; Shafiq *et al*., 2019; ur Rahman, *et al*., 2018a), while *Bla*$_{CTXM-15}$ and *Bla*$_{CTX-M-9}$ have been reported as dominant variants in Asia that have been recovered from different origins including food animals (Timofte *et al*., 2014). Furthermore, increased isolation rate of MDR bacteria, particularly *E. coli*, from food-producing animals have rendered them reservoir of different drug resistance-conferring genes mainly those encoding for ESBL enzymes (Geser *et al*., 2012). Most often, these ESBL encoding genes are carried on conjugative plasmids and have been found associated with other mobile elements such as insertion sequence common region 1 (IS*CR1*) and integrons (Ali *et al*., 2017; Ali *et al*., 2016; Aqib *et al*., 2019; Khattak *et al*., 2018; ur Rahman *et al*., 2018a). Of the three major integron classes, integron 1 and IS*CR-1* have been shown associated with transmission of antibiotic resistance genes (Ali *et al*., 2016). Due to crucial role of conjugative plasmids in dissemination of resistance elements, various classification systems such as Inc typing (which relies on incompatibility groups) are commonly used (Carattoli *et al*., 2005) helping to trace the spread and evolution of plasmids (Datta & Hedges, 1971).

Recently poultry industry in Pakistan has been modernized for intense production to meet the increasing demand of meat; however, this has been achieved, partly, at the cost of excessive use of antibiotics. Antimicrobials are excessively used in the existing production system not only for treatment purposes, but major part of it goes for prevention of diseases and as growth promoters. However, epidemiological data regarding development of resistance in microbes isolated from poultry is rare in Pakistan. This is for the first time that we report on the isolation rate of poultry meat-associated *E. coli* capable of producing ESBL enzymes in District Mardan, Pakistan.

MATERIALS AND METHODS

Ethics: The current study was approved from the ethical committee of Abdul Wali Khan University Mardan. All work described here is carried out according to the local institutional and national guidelines and legislations.

Collection of samples and location: Samples from poultry meat (*n=100*) were collected from butcher shop right at the time of slaughtering at live bird market in District Mardan, KP during January 2018 to March 2018. Samples were obtained from 11 different shops located within the city with one sample per bird and not more than 3 samples per day per shop were collected. A piece of leg, liver, heart or spleen was collected from a freshly slaughtered chicken, and each piece was processed separately. Samples were transported in a sterile bag in icebox maintaining low temperature and were immediately processed for culturing.

Isolation and screening of cefotaxime- resistant-*E. coli:* Samples shortly after arrival were minced manually and homogenized within the sample bag after addition of sterile saline (1ml) and were directly streaked onto MacConkey agar (Difco^{TM} Becton Dickinson, Sparks, MD USA) containing 1mg/L concentration of cefotaxime and incubated for 24 hours at 37 ºC. Pink candidate colonies were further purified and streaked onto eosin methylene blue agar, gram stained and confirmed by API-20E kit (bioMérieux, Marcy I'Etoile, France) and specie specific PCR assay as described earlier (Tantawiwat *et al*., 2005).

Phenotypic screening of ESBLs producers: Confirmed cefotaxime-resistant *E. coli* isolates were further investigated for ESBL production by double disk synergy test following guidelines of Clinical and Laboratory Standard Institute (CLSI) using antibiotic disc of cefotaxime (30 µg), cefotaxime plus clavulanic acid (30/10 µg), ceftazidime (30 µg), ceftazidime plus clavulanic acid (30/10 µg) (Oxoid, Hampshire, United Kingdom) for ESBL production (CLSI., 2014). Results were interpreted as per guidelines of CLSI.

ESBL-genotyping of *E. coli* isolates producing ESBLs: Bacterial DNA from phenotypically confirmed ESBL producers was extracted through boiling method as described earlier (Ali *et al*., 2016). PCR assay was used for identification of $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}, as described previously using specific primers (Chen *et al*., 2010). Primers and PCR assay conditions are described in **Supplementary Table 1.**

Antibiotic susceptibility testing: Mueller-Hinton agar (Difco^{TM}) has been used for the antibiotic susceptibility of ESBLs isolates against 21 different antibiotics following the standard Kirby-Bauer disk diffusion method and results were interpreted as per guidelines of CLSI (CLSI., 2014). List of antibiotics was comprised of β-lactam and non- β-lactam antibiotics. ESBLs confirmed *E. coli* were confirmed as multi-drug resistant (MDR) when found resistant to at least two antibiotics of different classes.

Phylogenetic grouping: A triplex PCR was performed targeting *yjA*, *chuA* and the *TspE4* for classification of isolates into specific phylogroups as reported previously (Clermont *et al*., 2000).

Detection of integrons and IS*CR1:* All three classes of integrons: class 1, 2 and 3 were investigated by PCR assay as described earlier (Dillon *et al*., 2005). Furthermore, presence of insertion sequence IS*CR1*was also confirmed by PCR as described previously (Ali *et al*., 2016), while, association of IS*CR1* with ESBL genes was identified as described elsewhere (Ali *et al*., 2016).

PCR-based Inc/rep typing: Five multiplex PCR were performed targeting different replicon types as described previously (Carattoli *et al*., 2005).

RESULTS

Frequency of ESBL–producing *E. coli* isolated from retail meat: Randomly, a total of 33 *E. coli* isolates were screened initially, of which, 28 isolates were confirmed as ESBL-producers by double disk synergy test. The occurrence of *E. coli* with the ability to produce ESBL enzymes among all isolates are described in **Table 1.** These ESBL producers were recovered from all three organs investigated, however, all isolates recovered from heart were found to be ESBL producers.

ESBL genotyping indicates predominance of *bla_{CTX-M}*: All 28 under study population of *E. coli* isolates were carrying *bla_{CTX-M}* while *bla_{SHV}* and *bla_{TEM}* could not be amplified from a single isolate **(Table 2).** Further sub typing of *bla_{CTX-M}* indicated that *bla_{CTXM-9}* was carried by 89.3% (n=25) isolates, while 3 (10.7%) were harboring *bla_{CTXM-1}*. Furthermore, 3 isolates were also carrying additional *$bla_{CTXM-15}$* along with *bla_{CTXM-9}*, however, none of the isolates were carrying *bla_{CTXM-8}* and *bla_{CTXM-2}*.

Antibiotic susceptibility testing showed MDR phenotypes: Susceptibility against commonly used 21 different antimicrobials including β-lactams (ampicillin, amoxicillin/clavulanic acid *etc.*), cephalosporins (cefotaxime, ceftazidime, cefepime *etc.*), carbapenems (meropenems, imipenems, etrapenam), tetracycline (tetracycline, doxycycline), quinolones (ciprofloxacin), aminoglycosides (gentamycin, kanamycin,amikacin), floroquinolones (ciprofloxacin, norfloxacin, levofloxacin), monobactams (aztreonam), lincosamides (clindamycin), fosfomycin, sulfonamides (trimethoprim-sulfamethoxazole), phenicol (chloramphenicol), tigecycline, novobiocine and polymyxin B (colistin) (Table 3). All isolates were found resistant to ampicillin, cefotaxime, tetracycline, trimethoprim-sulfamethoxazole and novobiocine, while increased numbers of isolates were found susceptible to imipenem (89.2%), etrapenem (89.2%), meropenemes and fosfomycin (85.7%).

Phylogenetic classification indicates B2 as predominant group: Phylo- group B2 was the most prevalent (11/28, 39.2%) followed by group A (9/28, 32.1%), and group D (8/28, 28.5%), respectively (**Table 4**).

Class 1 integron was found as predominant: Twenty-one (75%) were carrying integron 1, while, integrons of class-2 and class-3 were detected in 25% and 7.1, respectively (**Table 4).**

CTXM encoding genes were found linked to IS*CR1*: In the recent past, we screened a number of ESBL-producing *E. coli* isolates for the presence of ESBL encoding genes in the integron-integrase variable regions; however, none of the ESBL gene was located in the variable region suggesting no involvement of integron in dissemination of ESBL genes. Interestingly, we found that majority of ESBL genes were found linked to rather IS*CR1*indicating its role in fast dissemination of these genes (Ali *et al*., 2016). Thus based on our past experience, we speculated the role of IS*CR1*elements in dissemination of ESBL genes. We thus investigated presence of IS*CR1*elements and its association with ESBL genes. Results showed that 11 isolates (39.28%) were harboring IS*CR1*. Of these, IS*CR1*element was found linked with *bla_{CTX-M}* among 5 isolates (45.5%) suggesting its role in mobilization of *bla_{CTX-M}* **(Table 4)**. Interestingly, of these 5 IS*CR1*-carrying ESBL-producing isolates, 4 were harboring *bla_{CTXM-9}* variant, while only one isolate was carrying *bla_{CTXM-1}*.

PCR based plasmid replicon typing indicated majority of isolates were carrying FrepB Inc type: A total of 5 triplex and 3 simplex PCR reactions were performed for plasmid replicon typing. Our results indicated that 27 isolates were carrying FrepB type of plasmid. Furthermore, replicon type B/O was carried by 10 (35.3%) isolates. Strikingly, a total of 16 isolates were carrying a single Inc/Rep type (FrepB) of plasmid, while 7 isolates were carrying two Inc types (FrepB and B/O) of plasmids. Finally, five isolates were carrying multiple (more than two) plasmids of different Inc/Rep groups with one isolate (Sss8sc4) carrying a total of 11 types (**Table 5**). Overall, our results suggest that plasmid FrepB is the most dominant Inc type of plasmid found in *E. coli* isolates under study.

Table 1. Isolation of MDR ESBL-producing *E. coli* isolates.

Source of samples	Total samples	No of *E. coli* isolates n (%)	ESBL phenotypes n (%)	MDR phenotypes n (%)
Spleen	42	21 (50.0)	18(85.7)	18(85.7)
Liver	20	6 (30.0)	4(66.7)	4(66.7)
Heart	38	6 (15.8)	6(100)	6(100)
Total	100	33(33.0)	28(84.8)	28(84.8)

Table 2. Distribution of ESBL encoding genes among MDR ESBL producers.

#	ESBL genes	Sample nature/origin			Frequency	Percentage
		Liver	Spleen	Heart		
1	bla_{CTX-M}	4	18	6	28/28	100.0
2	bla_{CTXM-9}	3	16	6	25/28	89.3
3	bla_{CTXM-1}	1	2	0	3/28	10.7
4	bla_{CTXM-2}	0	0	0	0/28	00.0
5	bla_{CTXM-8}	0	0	0	0/28	00.0
6	bla_{SHV}	0	0	0	0/28	00.0
7	bla_{TEM}	0	0	0	0/28	00.0

Table 3. Antimicrobial susceptibility profile of ESBL producing *E. coli* isolates (n=28).

S.no	Antimicrobial agent	Abb.	conc.(µg)	Susceptible (%)	Intermediate (%)	Resistance (%)
1	Ciprofloxacin	CIP	5 µg	4/28(14.2)	0/28(0)	24/28(85.7)
2	Tetracycline	TE	30 µg	0/28(0)	0/28(0)	28/28(100)
3	Meropenem	MEM	10 µg	15/28(53.5)	8/28(28.5)	5/28(17.8)
4	Doxycycline	DO	30 µg	2/28(7.1)	1/28(3.5)	25/28(89.2)
5	Gentamycin	CN	10 µg	18/28(64.2)	5/28(17.8)	5/28(17.8)
6	Imipenem	IPM	10 µg	10/28(35.7)	10/28(35.7)	8/28(28.5)
7	Aztreonam	ATM	30 µg	5/28(17.8)	12/28(42.8)	11/28(39.2)
8	Ceftazidime	CAZ	30 µg	7/28(25)	13/28(46.2)	8/28(28.5)
9	Cefepime	FEP	30 µg	19/28(67.5)	4/28(14.2)	5/28(17.8)
10	Ampicillin	AMP	10 µg	0/28(0)	0/28(0)	28/28(100)
11	Norfloxacin	NOR	10 µg	4/28(14.2)	5/28(17.8)	19/28(67.5)
12	Trimethoprim-sulfamethoxazole	SXT	1.25/23.75 µg	0/28(0)	0/28(0)	28/28(100)
13	Colistin sulphate	CT	µg	0/28(0)	1/28(3.5)	27/28(96.4)
14	Chloramphenicol	C	30 µg	7/28(25)	0/28(0)	21/28(75)
15	Enrofloxacin	ENR	5 µg	2/28(7.1)	0/28(0)	26/28(92.8)
16	Cefotaxime	CTX	30 µg	0/28(0)	0/28(0)	28/28(100)
17	Fosfomycine	FOS	50 µg	24/28(85.7)	4/28(14.2)	0/28(0)
19	Novobiocine	NV	30 µg	0/28(0)	0/28(0)	28/28(100)
20	Tigecycline	TGC	15 µg	0/28(0)	28/28(100)	0/28(0)
21	Etrapenam	ETP	10 µg	25/28(89.2)	0/28(0)	3/28(10.7)
22	Co-amoxicalv	AMC	20/10µg	11/28 (39.2)	13/28(46.2)	4/28(14.2)

Table 4. Genotypic characterization of ESBL-producing *E. coli* isolates.

No	ID	location	PG	ESBL genotype			Integron typing			ISCR 1	IS*CR1*+ ESBL	Resistance/Intermediate phenotype
				CT XM	SH V	TE M	Int. 1	Int. 2	Int. 3			
1	ss3c1	MC	B2	CTXM-9	-	-	+	-	-	+	+	CAZ,MEM,CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA,CTX, NV, TGC, AMC
2	ss26sc1	GK	B2	CTX-M-9+ CTX-M-15	-	-	+	-	-	+	-	CIP.AMP,SXT,TE,CN,ATM,DO,C,DA,CTX, NV, TGC,IMP, MEM
3	ls5sc1	CC	D	CTXM-9	-	-	+	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C, DA,CTX, NV, TGC
4	ss16sc1	CC	D	CTXM-9	-	-	+	-	-	+	+	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C, DA,CTX, NV, TGC,IMP, MEM
5	ss12sc2	SKM	B2	CTXM-9	-	-	+	-	-	+	-	CIP,AMP,NOR,SXT,TE,ATM,DO,CAZ,DA,CTX, NV, TGC, AMC
6	ss3c2	MC	D	CTXM-9	-	-	+	+	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA,CTX,NV, TGC, AMC,IMP
7	ss10c1	MC	A	CTXM-9	-	-	+	-	-	+	-	CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA,CTX ,NV, TGC,IMP
8	hs19asc1	SKM	A	CTXM-9- CTX-M-15	-	-	-	-	-	+	-	CAZ,CIP,AMP,SXT,TE,ATM,DO,DA,CTX, NV, TGC,IMP
9	ss12sc1	SKM	D	CTXM-9	-	-	+	-	-	+	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,CTX, NV, TGC, AMC
10	hs9sc2	MC	A	CTXM-9	-	-	+	-	-	+	+	CAZ,CIP,AMP,NOR,SXT,TE,C, DA,CTX, NV, TGC
11	ss15sc1	CC	A	CTXM-9	-	-	+	+	-	-	-	CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA, CTX, TGC, FOS
12	hs11sc1	CC	B2	CTXM-9	-	-	+	-	-	-		CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO, DA,CTX, NV, TGC, AMC
13	ss30sc1	GK	B2	CTXM-9	-	-	-	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA,CTX, NV, TGC,AMC
14	ls5sc4	CC	B2	CTXM-9	-	-	+	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,DA,CTX, NV, TGC
15	s22sc1	GK	B2	CTXM-9	-	-	+	+	-	+	-	CAZ,MEM,CIP,AMP,NOR,SXT,TE,CN, DO,C,DA,CTX, NV, TGC, FOS, AMC,DA
16	ss14sc1	CC	B2	CTXM-1	-	-	-	-	-	+	+	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C,DA,CTX, NV, TGC,DA
17	hs12sc1	SKM	B2	CTXM-9	-	-	-	-	-	+	-	CAZ,CIP,AMP,NOR,SXT,TE,CN,DO,C,DA,CTX, NV, TGC,DA
18	ss13sc1	SKM	A	CTXM-1	-	-	+	+	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,DO,DA,CTX,NV, TGC, AMC,NV
19	ss26sc2	GK	B2	CTXM-9	-	-	-	-	-	+	-	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C,NV,DA,TGC
20	ss18sc1	CC	D	CTXM-9	-	-	-	-	-	+	-	CAZ,AMP,SXT,TE,ATM,DO, AMC,NV,DA,TGC
21	hs13sc1	SKM	D	CTXM-9	-	-	+	+	-	-	-	CAZ,AMP,NOR,SXT,TE,ATM,DO,C, FOS, AMC,NV,DA,TGC
22	ss8sc4	MC	D	CTXM-9	-	-	+	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,MEM,CN,ATM,DO,IPM,C,AMC,NV,D A,TGC
23	ss21sc1	GK	A	CTXM-9	-	-	+	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C, AMC,NV,DA,TGC
24	ss25sc1	SKM	A	CTXM-9+ CTX-M-15	-	-	+	+	-	-	-	CAZ,AMP,SXT,TE,ATM,DO,C, NV,DA,TGC
25	Ls25sc1	SKM	A	CTXM-1	-	-	+	-	-	+	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,NV,DA,TGC
26	Hs38sc1	SKM	A	CTXM-9	-	-	+	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,NV,DA,TGC
27	Ss36sc1	GK	B2	CTXM-9	-	-	-	-	-	-	-	CIP,AMP,SXT,TE,CN,DO,C,NV, DA,TGC
28	Ls26sc1	GK	D	CTXM-9	-	-	+	+	+	+	+	CAZ,MEM,AMP,NOR,SXT,TE,CN,ATM,IPM,NV,DA,TGC

PG phylogroup, MC Malakand chaok, CC college cowk, SKM Sheikh Maltoon, GK Gajju khan market, Int.1 integron 1, Int.2 Integron 2, Int.3 Integron 3, CiprofloxacinCIP, Tetracycline TE, MeropenemeMEM, Doxycycline DO,Gentamycin CN, Imipenem IPM, Aztreonam ATM, Ceftazidime CAZ, Cefepime FEP, Ampicillin AMP, Norfloxacin NOR, Trimethoprim-sulfamethoxazole SXT, Colistin sulphate CT, Chloramphenicol C, Enrofloxacin ENR, Cefotaxime CTX, Fosfomycine FOS, Clindamycin DA, Novobiocine NV, Tigecycline TGC, Etrapenam ETP,

Table 5. plasmid replicon typing of ESBL producers.

S. No	ID	Inc Group	Multiplex Inc/Rep PCR results															Simplex Inc/Rep PCR		
			1			2			3			4			5			1	2	3
			Hi1	Hi2	i1	X	L/M	N	FiA	FiB	W	Y	P	F	A/C	T	FIIS	FrepB	K/B	B/O
1	Hs9sc2	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
2	Ss30	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
3	Hs11sc1	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4	Ss26sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
5	Ss18	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
6	Ss12sc2	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
7	Ss3sc1	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
8	Hs19	Hi1/Hi2/i1/FrepB/K/B	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
9	Sss8sc4	Hi1/Hi2/i1/FiA/FiB/W/Y/P/F/ FrepB/K/B	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	-
10	Ss25sc1	Hi1/Hi2/i1 FrepB/K/B	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
11	Ls5sc4	FiA/FiB/W/Y/P/F/FrepB/B/O	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-	+
12	Ss14sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
13	Ss16sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
14	Ss12sc1	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
15	Ls5sc1	FrepB/B/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
16	Ss15	Y/P/F/FrePB	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
17	Ss21sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
18	Hs12sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
19	Hs13sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
20	Ss26sc2	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
21	Ss13sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
22	Ss10sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
23	Ss3sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
24	Ss22sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
25	ls25sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
26	Hs38sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
27	ss36sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
28	ls26sc1	FrepB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
total		FrepB	3	3	3	0	0	0	2	2	2	2	2	2	0	0	0	27	2	10

DISCUSSION

AMR is known to evolve in response to consistent and excessive use of antimicrobials and its emergence mimic the phenomenon of toxin production in response to signals present in the surroundings of microbes (ur Rahman and van Ulsen 2014; ur Rahman *et al*. 2014; Piet *et al*. 2016). Applications of antibiotics in poultry industry in Pakistan is not strictly regulated and monitored thereby raising concern of emergence of antimicrobial resistant microorganisms supported by the recent increased MDR reports (Ali *et al*., 2016; Ali *et al*., 2017; Naeem *et al*., 2006; ur Rahman., *et al*., 2018a; ur Rahman *et al*., 2018b; ur Rahman *et al*., 2019; Younas, *et al*., 2019). For the very first time in Pakistan and particularly in the North West Frontier province-the Khyber Pakhtunkhwa- we report on the genotypic characteristics of *E. coli*isolated from poultry ready to sale in Mardan KP Pakistan. Current data showed that overall 84.8% *E. coli* were ESBL producers (Table 2). An agreement with this, 81% of poultry feces samples in the Netherlands contained ESBL producing *E. coli* (Blaak *et al*., 2015). However, our findings show higher incidence rate of *E. coli* producing enzymes isolated from poultry as compared to previously published report from Bangladesh (30%) (Hasan *et al*., 2012) and France (10.7%) (Girlich *et al*., 2007). Possibility exists that this observed heightened incidence rate of ESBL-producing *E. coli* may be linked to over use or constant addition of antibiotics to poultry during production. It goes along with high rate of incidence of ESBL-producing *E. coli* human patients that were hospitalized in Pakistan (Abrar *et al*., 2017; Rahman *et al*., 2016 Riaz *et al*., 2012)) as well as from community and environment (Ullah *et al*., 2009) suggesting widespread presence of ESBL carrying isolates. However, current observation may not be considered generalized, as we could analyzed a limited number of samples from only one city of district Mardan of Khyber Pakhtunkhwa province, Pakistan.

Mobile elements such as integrons and insertion sequences are considered crucial in the dissemination of resistance conferring elements and emergence of MDR bacteria. In line with previous reports, our results reveal a predominant occurrence of class 1 integron among ESBL-positive *E. coli* (Ali *et al*., 2016; Dillon *et al*., 2005; Gu *et al*., 2008; Yao *et al*., 2007). Integrons of class 1 generally carries a variable region comprising a gene cassette arrays encoding other different resistance elements. Strikingly, this study identified that the $bla_{\text{CTX-M}}$ variants were mainly found associated with the IS*CR1*. These results corroborate with previous findings of our lab (Ali *et al*., 2016) and other studies from different countries of the world (Kar *et al*., 2015). Notably, our results of the most abundant ESBL genotype ($bla_{CTX\text{-}M}$) and its strong association with the IS*CR1*elements in plasmid of FrepB replicon type indicate that the IS*CR1* is more likely involved in mobilizing these resistance-conferring elements and is probably directly responsible for its dissemination.

Conclusion: In conclusion, this study indicate that the broiler meat obtained from live bird market of District Mardan, Pakistan, carry *E. coli* harboring drug resistance elements. Majority of these isolates were ESBL producers encoding predominantly $bla_{CTX\text{-}M}$ variant linked to IS*CR1* elements and on a plasmid that mainly carrying FrepB type of replicon. These findings suggest an urgent and effective intervention to discourage further spread of antimicrobial dissemination and initiation of an overall structural surveillance program.

Novelty Statement: Extended spectrum β lactamase (ESBL)-producing multi drug resistant *Escherichia coli* is increasingly reported from all around the world. *E. coli*, quite often, carries ESBL-encoding genes on different plasmids which help them fast dissemination. Information and analysis of plasmids carrying ESBL genes is totally lacking from Pakistan. In this paper, we describe ESBL-producing *E. coli* encoding various types of ESBL encoding genes carrying on different plasmids Inc types

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Conflict of interest: Nothing to declare

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Table 1 Primers used for identification of target genomic regions described in the study.

Primer name	Target gene	Sequence (5´-3´)	Size – bp	References
β-lactamases				
CTX-M –F	*bla*$_{CTXM}$	CGCTTTGCGATGTGCAG	~550	(Villegas *et al*., 2004)
CTX-M –R		ACCGCGATATCGTTGGT		
CTXM1-F[1]	*bla*$_{CTXM-1}$	GCT GTT GTT AGG AAG TGT GC	~490	(Shibata *et al*., 2006)
CTXM1-R		CCA TTG CCC GAG GTG AAG		
CTXM2-F[2]	*bla*$_{CTXM-2}$	ACG CTA CCC CTG CTA TTT	~450	(Shibata *et al*., 2006)
CTXM2-R		CCT TTC CGC CTT CTG CTC		
CTXM8-F	*bla*$_{CTXM-8}$	CGC TTT GCC ATG TGC AGC ACCGTC GCT	~307	(Pitout *et al*., 2004)
CTXM8-R		CAG TAC GAT CGA GCC		
CTXM9-F[3]	*bla*$_{CTXM-9}$	GCA GAT AAT ACG CAG GTG	~490	(Shibata *et al*., 2006)
CTXM9-R		CGG CGT GGT GGT GTC TCT		
CTX-M-U1	*bla*$_{CTXM-15}$	ATGTGCAGYACCAGTAARGTKATGGC	~900	(Mulvey *et al*., 2003)
CTX-M-U2		TGGGTRAARTARGTSACCAGAAYCAGCGG		
SHV –F	*bla*$_{SHV}$	GGG TTA TTC TTA TTT GTC GC	~567	(Chang *et al*., 2001;
SHV –R		TTAGCGTTGCCAAGTGCTC		Yao *et al*., 2007)
TEM-F	*Bla*$_{TEM-1, -52, -71,}$	ATA AAA TTC TTG AAG ACG AAA	~1086	(Yao *et al*., 2007)
TEM-R	$_{-104-105, -138,}$	GAC AGT TAC CAA TGC TTA ATC		
Integrons and integron variable region				
intI1-F	*intI1*	CCT CCC GCA CGA TGA TC	280-bp	(Dillon *et al*., 2005)
intI1-R		TCC ACG CAT CGT CAG GC		
intI2-F	*intI2*	AAA TCT TTA ACC CGC AAA CGC	439-bp	(Dillon *et al*., 2005)
intI2-R		ATG TCT AAC AGT CCA TTT TTA AAT TCT A		
intI3-F	*intI3*	AGT GGG TGG CGA ATG AGT G	599-bp	(Dillon *et al*., 2005)
intI3-R		TGT TCT TGT ATC GGC AGG TG		
Specific to *E. coli*				
UAL	*UidA*	TGG TAA TTA CCG ACG AAA ACG GC	147-bp	(Tantawiwat *et al*.,
UAR		ACG CGT GGT TAC AGT CTT GCG		2005)
***E. coli* phylogrouping**				
ChuA-F	*ChuA*	GAC GAA CCA ACG GTC AGG AT	279-bp	(Clermont *et al*.,
ChuA-R		TGC CGC CAG TAC CAA AGA CA		2000)
YjaA-F	*YjaA*	TGA AGT GTC AGG AGA CGC TG	211-bp	(Clermont *et al*.,
YjaA-R		ATG GAG AAT GCG TTC CTC AAC		2000)
TspE4C2-F	*TspE4C2*	GAG TAA TGT CGG GGC ATT CA	152-bp	(Clermont *et al*.,
TspE4C2-R		CGC GCC AAC AAA GTA TTA CG		2000)
ISCR1	IS*CR1*	CGC CCA CTC AAA CAA ACG	469-bp	(Kiiru *et al*., 2013)
		GAG GCT TTG GTG TAA CCG		

[1]The PCR primers used can detect genes for CTX-M-1 and several variants, such as CTX-M-3 and CTX-M-15.
[2]The PCR primers used can detect genes for CTX-M-2 and several variants, such as CTX-M-20 and CTX-M-31.
[3]The PCR primers used can detect genes for CTX-M-9 and several variants, such as CTX-M-14 and CTX-M-16.