# *Genotypic characterization of multidrug resistant* Escherichia coli *isolates reveals co-existence of ESBL- and carbapenemase- encoding genes linked to* ISCR1

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**Summary**
Antimicrobial resistance in food-producing animals has not yet judiciously been reported from Pakistan. Here, we report on the isolation rate of poultry-associated multidrug resistant extended spectrum β-lactamase (ESBL) -producing *Escherichia coli* in Peshawar, Pakistan. A total of 200 samples, 50 from retail-poultry meat, 50 from sick birds, 50 from the boiler farm-environment, and 50 from human beings working on or exposed to poultry were analyzed for isolation of ESBL-producing *E. coli*, ESBL-encoding genes and antimicrobial susceptibility. A total of 81 *E. coli* isolates [(50.0% Phylogroup-A, 33.3% D and 16.7% phylogroup B2)], were recovered, 36 (44.4%) of them were found to be ESBL-producers. PCR revealed that $bla_{CTXM}$ was the most prevalent (14/36 = 38.9%) ESBL-encoding gene followed by $bla_{SHV2}$ (9/36 = 25%). Strikingly, co-occurrence of multiple ESBL- and/or carbapenemase-encoding genes in a single isolate was observed, and combination of $bla_{CTXM}$ + $bla_{SHV2}$ was the most predominant (19.4%) followed by $bla_{CTXM}$ + $bla_{NDM1}$ + $bla_{OXA-48}$ (11.1%) and $bla_{CTXM}$ + $bla_{OXA-48}$ (8.8%). All these ESBL producers were found to be multidrug resistant (MDR) and were carrying either integron 1 (48.5%) or 2 (51.5%). Finally, 14 of the 36 isolates were also found positive for variable region and insertion sequence common region 1, which was found linked to ESBL/carbapenemase encoding genes in 5/14 isolates suggesting its role in dissemination.

## *Introduction*

Antimicrobial resistance (AMR) is a global emerging threat. Particularly, resistance to *β*-lactams and carbapenems is quite alarming as these drugs are proven safe and efficient, but are losing its effect due to emerging AMR. Bacteria achieve resistance to *β*-lactams through production of extended spectrum *β*-lactamases (ESBL) that inactivate antimicrobials including third-, fourth-generation cephalosporins and monobactams (El Salabi *et al.* 2013, ur Rahman *et al.* 2018b). Carbapenemases on the other hands are enzymes that can inactivate carbapenem drugs-the most effective and last resort of antibiotics. ESBLs and carbapenemases are predominantly produced in *Enterobacteriaceae*, mainly in *Escherichia coli* that is utilized as crucial resistance mechanism against cephalosporins and carbapenems (Nordmann *et al.* 2011, ur Rahman *et al.* 2018b). Furthermore, generally, *E. coli* encoding for ESBL and carabepenemases are resistant to more

than one classes of antimicrobials and hence are multidrug resistant (MDR), and thus presenting a serious challenge in healthcare settings. In addition to implications of antimicrobials for prophylaxis and as growth promoters, recurrent bacterial infections encourages excessive and constant usage of these antimicrobials thereby resulting emergence and development of resistance against these compounds (ur Rahman *et al.* 2018b). Emergence of resistance against carbapenem and cephalosporins is particularly more worrisome as limited options of treatment are left for patients infected with these resistant microbes.

Three major categories, $bla_{CTXM}$, $bla_{SHV}$ and $bla_{TEM}$, of ESBL-encoding genes have been described well so far with $bla_{CTXM}$ as the most prevailing genotype, which has been further divided into five subgroups ($bla_{CTXM-1}$, $bla_{CTXM-2}$, $bla_{CTXM-8}$, $bla_{CTXM-9}$, $bla_{CTXM-25}$). The families of each three categories have been expanded so much that almost more than 172 variants have been described for each type of ESBL (http://www.lahey.org/studies) (Chong *et al.* 2018). With obvious geographical variations among the ESBL subtypes, $bla_{CTXM-15}$ genotype has been reported frequently from Asia (D'Andrea *et al.* 2013), and has even frequently been isolated from food-producing-animals (Ali *et al.* 2016, Ali *et al.* 2017) as well as from community (Abrar *et al.* 2017, Brigante *et al.* 2005). *E. coli* carrying $bla_{CTXM-15}$ often carry many other antibiotic resistance genes and often causing community acquired infections (Chong *et al.* 2018). It has been shown that ESBL carrying *E. coli* spread through contaminated food chain or water thereby resulting spread of resistance elements harboring by these bacteria through the help of mobile elements on conjugative plasmids such as integron and insertion sequence common region 1 (IS*CR1*) (Ahmed *et al.* 2016, Ali *et al.* 2016, Huang *et al.* 2014).

Carbapenemases are highly versatile family of β-lactamases recognizing almost all hydrolysable β-lactams, and are resilient against almost all available commercial β-lactamase inhibitors (Lolans *et al.* 2005, Nordmann and Poirel 2002). Carbapenemase-encoding genes are not naturally found in *E. coli*, however, these genes have been acquired largely from other related or environmental bacteria when found associated with mobile genetic elements such as conjugative plasmids (Queenan and Bush 2007). The first carbapenemase producing *E. coli* was reported in 1993 encoded by NmcA (Naas and Nordmann 1994). Since then and particularly over the last decade, increasing number of carbapenemase encoding genes have been described in *Enterobacteriaceae,* enlisting them into 3 classes of β-lactamases such as the Ambler class A, B, and D β-lactamases (Queenan and Bush 2007). Moreover, rare chromosomally-encoded cephalosporinases- the Ambler class C have also been described with slight expanded-spectrum activity against carbapenems (Giske *et al.* 2008).

Infections due to β-lactamases-producing *Enterobacteriaceae* have serious implications both for public health and infection control strategies. Such kind of infections are difficult to treat increasing the risk of infection-related mortality by 5 fold (Kumar *et al.* 2009). Finally, the overwhelming ability of the β-lactamases-producing *Enterobacteriaceae* to persist and speedily disseminate with the help of mobile elements in different clinical- and community-settings, and their frequent association with MDR phenotypes presents a huge challenge (El Salabi *et al.* 2013, Ali *et al.* 2016, Nordmann and Poirel 2002). MDR *Escherichia coli* carrying ESBL and carbapenemases is increasingly disseminating all over the world. The situation is more alarming in the developing countries partly due to excessive use of antibiotics and absence of surveillance reports. The persistent and excessive use of antibiotics for treatment and as growth promoter in poultry industry in Pakistan is more likely providing impetus for the emergence of novel mechanisms of resistance including resistance to β-lactams in *Enterobacteriaceae*. Thus, this study was designed with the objectives to determine the occurrence of extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing *E. coli* in broiler and poultry farm environment in Peshawar-Pakistan and characterize these isolates for the types of ESBL genes and susceptibility common antimicrobials.

## Materials and methods

### Ethics

Proper and prior ethical approval was obtained from the supervisory and technical committee of the University of Agriculture Peshawar and Abdul Wali Khan University Mardan. All work described in here is carried out according to the local institutional and national guidelines and legislations for human and animal samples for research purpose. Prior consent was obtained from owners of the poultry farm/bird or human beings along with consent for publication of the resultant data.

### Study area and samples

Peshawar is the capital city of Khyber Pakhtunkhwa situated in the northwest of Pakistan with 34.0150° N, 71.5805° E coordinates. Samples for study were collected in September 2016 to April 2017. A total of 200 samples including 50 samples from healthy chickens, 50 from apparently looking sick chicken,

50 from poultry rearing environment within poultry farms and finally 50 samples from human beings dealing with poultry in the farms or purchase points were collected from Peshawar. Samples from sick broiler chickens were obtained from Poultry postmortem section of Veterinary Research Institute, Peshawar, where farmers from various cities including nearby peri-urban areas of Peshawar city bring sick birds for disease diagnosis or/and treatment. Samples from healthy broiler chicken were obtained from live bird market at Peshawar city at sales point, while environmental samples were obtained from open shed poultry farms of Peshawar district that provides broiler to these sales points. These samples included drinking water (n = 20), swabs from floor (n = 10), utensils (n = 10) and humid walls (n = 10). Fecal swab samples or swab samples from hands, nose and feet or blood samples of human beings (n = 50) dealing with these broiler at different spots such as butchers, live bird sellers, farmers and truck drivers who transport birds from farm to market were included in this study.

## Culturing, identification of *Escherichia coli*, screening and confirmation of ESBL production

Meat samples of healthy broiler chicken at retail market and sick broiler chicken from the postmortem room, were sealed in a sterile plastic bag and transported in icebox to Microbiology Laboratory of University of Agriculture or College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University and placed at 2-8 °C for maximum of 10 hr until further processed. Cotton swab samples from the environment of the broiler farms or humans were transported in transport medium in icebox and were processed immediately for culturing and isolation. Human fecal swab samples in the transport medium or a total of 10 ml blood were aseptically obtained from people.

Meat samples of broiler were processed for isolation of *E. coli* (Doy *et al.* 2010, Paterson *et al.* 2010). Briefly, approximately 25 mg of meat was used from each sample for culturing after mechanically mincing in 10 ml of Brain heart infusion (BHI; Sigma-Aldrich) media followed by enrichment at 37 °C overnight (Doy *et al.* 2010, Paterson *et al.* 2010). On the next day, 50-100 µL of this broth was streaked onto MacConkey agar for selective growth. In the case of swab samples a sterile cotton swab was streaked directly onto MacConkey agar.

Screening and confirmation of ESBL was performed as defined by Clinical and Laboratory Standards Institute (CLSI) (CLSI 2014). The media plates used for each type of sample included (i) MacConkey agar plate with no antibiotics which was control for *E. coli* growth, (ii) MacConkey agar media plate with having cefotaxime (2 µg/mL) which was used as selective media for ESBL-producing *E. coli* growth and (iii) MacConkey agar media plate with having meropenem (0.5 µg/mL) which was selective for growth of *E. coli* producing carbapenemase. The plates were incubated overnight at 37 °C after inoculation. Pink color colonies that grew on (ii) and (iii), and presented morphology related with *E. coli* were candidates for ESBL-producing and carbapenemase-producing phenotypes and further biochemical tests were performed for *E. coli* identification. *E. coli* species were confirmed by using API kits (bioMérieux, Marcy l'Etoile, France). Biochemically confirmed *E. coli* isolates were also genotyped by specific PCR as described earlier (Tantawiwat *et al.* 2005). Confirmed *E. coli* isolates and suspected ESBL producers were stored in BHI broth containing 30% glycerol at - 80 °C.

Presumptive ESBL-producing *E. coli* were confirmed phenotypically for ESBL production by double-disc synergy test in accordance with recommendations of the Clinical and Laboratory Standards Institute (CLSI 2014), using antimicrobial discs of cefotaxime (30 µg), cefotaxime plus clavulanic acid (30/10 µg), ceftazidime (30 µg), ceftazidime plus clavulanic acid (30/10 µg) (Becton Dickson, Sparks, MD USA) for ESBL production. The test was declared positive for ESBL production when the zone of inhibition of cefotaxime plus clavulanic acid or ceftazidime plus clavulanic acid was ≥ 5 mm larger than their respective single discs (CLSI 2014). For positive control, *Klebsiella pneumoniae* ATCC 700603 (ESBLs-positive strain) was used.

## ESBL and carbapenemase encoding genes identification

Identification of ESBL and carbapenemase encoding genes was achieved by regular PCR. Total DNA was extracted by using standard boiling method (Johnson and Woodford 1998) PCR was first performed for $bla_{CTX-M}$, $bla_{SHV}$, and $bla_{TEM}$ of ESBL-producing isolates (Table I). Variants, $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$, of $bla_{CTX-M}$ were also determined through PCR using specific primers as described in Table I. All ESBL producers were also subjected to PCR for carbapenemase-encoding genes like $bla_{OXA}$, and $bla_{NDM}$. The primers, amplicons expected size and references are described in Table I. The polymerase chain reaction was performed as described earlier (Ali *et al.* 2016, Sartor *et al.* 2014). Amplified PCR products were evaluated by 1% agarose gel electrophoresis.

## Antibiotic susceptibility testing

Mueller-Hinton agar (Difco™) was used to perform

antibiotic susceptibility of ESBL carrying isolates. A total of 9 different types of antibiotic discs (Becton Dickison, Sparks, MD USA) following standard Kirby-Bauer disk diffusion method according to recommendations of the CLSI (CLSI 2014) were tested. Both, *β*-lactam and non-*β*-lactam antibiotics, were tested such as ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), aztreonam (30 µg) and meropenem (10 µg), and tetracycline (30 µg), gentamicin (10 µg), tetracycline (20 µg), meropenem (10 µg) and norfloxacin (10 µg), respectively. *E. coli* ATCC 25922 (ESBL-negative) and *Klebsiella pneumoniae* ATCC 700603 (ESBL-positive) were used as quality control strains (CLSI. 2014). The ESBL *E. coli* were marked declared as extensively-drug resistant (XDR) if found resistant to all commonly used drugs, multi-drug resistant (MDR)-if found resistant against more than two different kinds of antibiotics tested (Magiorakos *et al.* 2012).

## Determination of phylogroups of *E. coli*

Phylogenetic analyses was performed on ESBL-producing-*E. coli* isolates to place them into one of the four phylogenetic groups: phylogeneticgroup A, group B1, group B2 and group D. For this purpose, a triplex PCR assay targeted *chuA*, *yjaA* genes and the DNA fragment TspE4 was used as described previously by Clermont and colleagues (Clermont *et al.* 2000).

**Table I.** *Primers used in this study.*

| Primer name | Target gene | Sequence (5′-3′) | Size -bp | Ref |
|---|---|---|---|---|
| | | **β -lactamases** | | |
| CTX-M –F | $bla_{CTXM}$ | ATGGTTAAAAAATCACTGCG | 873 | Paauw *et al.* 2006 |
| CTX-M –R | | AAACCGTTGGTGACGAT | | |
| CTX-M9-F | $bla_{CTXM}$ | TGGTGACAAAGAGAGTGCAACG | 875 | Paauw *et al.* 2006 |
| CTX-M9-R | | TCACAGCCCTTCGGCGAT | | |
| SHV –F | $bla_{SHV}$ | GGG TTA TTC TTA TTT GTC GC | 567 | Chang *et al.* 2001, Yao *et al.* 2007 |
| SHV -R | | TTAGCGTTGCCAAGTGCTC | | |
| CTXM1-F1 | $bla_{CTXM-1}$ | GCT GTT GTT AGG AAG TGT GC | 490 | Shibata *et al.* 2006 |
| CTXM1-R | | CCA TTG CCC GAG GTG AAG | | |
| TEM-F | $Bla_{TEM-1,-52,-71,-104-105,-138,-151,-152}$ | ATA AAA TTC TTG AAG ACG AAA | 1086 | Yao *et al.* 2007 |
| TEM-R | | GAC AGT TAC CAA TGC TTA ATC | | |
| NDM-F | $Bla_{NDM1}$ | AGCTGAGCACCGCATTAG | 720 | Poirel *et al.* 2011 |
| NDM-R | | CGGAATGGCTCATCACGATC | | |
| OXA-F | $Bla_{OXA-48}$ | GCGTGGTTAAGGATGAACAC | 438 | Poirel *et al.* 2011 |
| OXA -R | | GTTGTCATCCTTGTTAGGCG | | |
| | | **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 | | |
| intI1-VR-F | *intI1* variable region | TCA TGG CTT GTT ATG ACT GT | variable | White *et al.* 2000 |
| ntI1-VR-R | | GTA GGG CTT ATT ATG CAC GC | | |
| | | **Specific to *E. coli*** | | |
| UAL | *uidA* | TGG TAA TTA CCG ACG AAA ACG GC | 147-bp | Tantawiwat *et al.* 2005 |
| UAR | | ACG CGT GGT TAC AGT CTT GCG | | |
| | | ***E. coli* phylogrouping** | | |
| ChuA-F | *ChuA* | GAC GAA CCA ACG GTC AGG AT | 279-bp | Clermont *et al.* 2000 |
| ChuA-R | | TGC CGC CAG TAC CAA AGA CA | | |
| YjaA-F | *YjaA* | TGA AGT GTC AGG AGA CGC TG | 211-bp | Clermont *et al.* 2000 |
| YjaA-R | | ATG GAG AAT GCG TTC CTC AAC | | |
| TspE4C2-F | *TspE4C2* | GAG TAA TGT CGG GGC ATT CA | 152-bp | Clermont *et al.* 2000 |
| TspE4C2-R | | CGC GCC AAC AAA GTA TTA CG | | |
| ISCR1 | I*SCR1* | CGC CCA CTC AAA CAA ACG | 469-bp | Kiiru *et al.* 2013 |
| | | GAG GCT TTG GTG TAA CCG | | |

F = Forward; R = Reverse.

## Detection of integrons and variable region

For identification of the integrons (class 1-3) in all ESBL-producing *E. coli* isolates using integron-integrase gene specific primers, *intI1*, *intI2* and *intI3* as described (Dillon *et al.* 2005). The variable regions of class 1 integrons were detected in all *intI1* positive ESBL-producing *E. coli* using PCR amplification with primers targeting the flanking regions (White *et al.* 2000).

## Statistical analysis

Data was compiled with the help of Microsoft Excel and analysed through chi-square test at $P < 0.05$ probability level using SPSS 16.0 analysis software.

# Results

## *Escherichia coli* isolates and ESBL phenotypes

A total of 81 *E. coli* isolates were recovered from 200 samples tested. Frequency of sample positivity of *E. coli* was 60% (30/50) from disease broiler chicken, 42% (21/50) from healthy chicken, 36% (18/50) from the farming environment and 24% (12/50) from risk associated to humans. Chi-square test indicated a significantly ($P < 0.05$) higher *E. coli* positivity rate from sick chicken as compared to healthy chicken and between the human and environmental samples. Screening of ESBL phenotypes by double disc synergy test indicated a total of 36 (44.4%) presumptive ESBL-producing *E. coli* isolates, majority (50%; 15/30) of these recovered from diseased chicks followed by healthy chicken (47.6%; 10/21), chicken rearing environment (38.8%; 7/18) and human (16.6%; 2/12) (Table II).

**Table II.** *Occurrence of drug resistant* Escherichia coli *(n = 200).*

| Sample nature | Total no samples | Sample positivity n | ESBL producers n | MDR n | Chi- Sq |
|---|---|---|---|---|---|
| Sick chicken | 50 | 30 (60.0%) | 15/30 (50.0%) | 17/30 (56.7%) | 52.00* |
| Healthy chicken | 50 | 21 (42.0%) | 10/21 (47.6%) | 12/21 (57.1%) | |
| Environment | 50 | 18 (36.0%) | 7/18 (38.9%) | 9/18 (50.0%) | 1.000* |
| Human | 50 | 12 (24.0%) | 4/12 (33.3%) | 5/12 (41.7%) | |
| Total | 200 | 81 (40.5%) | 36/81 (44.4%) | 43/81 (53.1%) | |

* Indicates statistical significance difference in the diseased chicks vs healthy chicks and Environmental vs human sample.

## Distribution and diversity of ESBL-and carbapenemase-encoding genes

All ESBL- producing isolates were further assessed for the types of ESBL-encoding genes and results are presented in Table III. Our results indicated that $bla_{CTXM}$ was found to be the most prevalent (38.9%; 14/36) ESBL-encoding gene followed by SHV2 (25%; 9/36). Strikingly, no $bla_{TEM}$ and $bla_{CTXM9}$ genes could be amplified from any of the isolates under study. Interestingly, these all isolates were also screened for common carbapenemase-encoding genes which showed that $bla_{NDM1}$ was found to be the most prevalent gene (16.7%; 6/36) followed by $bla_{OXA-48}$ (13.9%; 5/36). Chi square test indicate significant association ($P < 0.05$) of $bla_{CTX-M}$, $bla_{SHV2}$ and $bla_{OXA-48}$ gene with diseased chicken and healthy chicken while between human being and environment significance association was observed in $bla_{CTX-M-1}$ and $bla_{SHV-2}$ genes. Interestingly, there were co-occurrence of multiple ESBL-encoding genes in a single isolate, and combination of $bla_{SHV2} + bla_{CTXM}$ was found as dominant combination with a frequency of 19.4%. Of note, $bla_{NDM1}$ -carrying isolates (n = 4) were also found positive for $bla_{CTX-M}$ indicating an alarming combination or favorable co-occurrence. Four isolates were carrying a combination of three different genes viz., $bla_{CTXM} + bla_{NDM1} + bla_{OXA-48}$, while a single isolate was carrying four different resistance genes ($bla_{CTXM} + bla_{SHV2} + bla_{NDM-1} + bla_{OXA-48}$) showing co-occurrence of different ESBL-encoding genes in a single isolate. Frequency of ESBL or carbapenemase encoding genes is mentioned in Table IV.

**Table III.** *Frequency prevalence of extended-spectrum β-lactamase encoding genes (n = 36).*

| Name of gene | Diseased chick | Healthy chick | Chi-sq | Environment | Human being | Chi-sq | Total n |
|---|---|---|---|---|---|---|---|
| CTX-M | 7/15 (46.7%) | 3/12 (25.0%) | 1.44* | 2/7 (28.6%) | 2/2 (100.0%) | 1.24 | 14/36 (38.9%) |
| CTX-M -1 | 1/15 (6.7%) | 1/12 (8.3%) | 1.100 | 2/7 (28.6%) | 1/2 (50.0%) | 1.110* | 5/36 (13.9%) |
| SHV-2 | 4/15 (26.7%) | 2/12 (16.7%) | 1.100* | 1/7 (14.3%) | 2/2 (100.0%) | 1.130* | 9/36 (25.0%) |
| OXA-48 | 2/15 (13.3%) | 1/12 (8.3%) | 78.00* | 1/7 (14.3%) | 1/2 (50.0%) | 68.00 | 5/36 (13.8%) |
| NDM-1 | 2/15 (13.3%) | 2/12 (16.7%) | 1.12 | 1/7 (14.3%) | 1/2 (50.0%) | 1.22 | 6/36 (16.7%) |
| TEM | 0/15 (0.0%) | 0/12 (0.0%) | 1.22 | 0/7 (0.0%) | 0/2 (0.0%) | 1.32 | 0/36 (0.0%) |

* Indicates statistical significance difference in the diseased chicks vs healty chicks and Environmental vs human sample.

**Table IV.** *Distribution of extended-spectrum β-lactamase-encoding genes (n = 36)*

| *β*-lactamase gene | Total number of isolates | Frequency (%) |
|---|---|---|
| *CTXM* | 14 | 38.9 |
| *CTXM-1* | 5 | 13.9 |
| *SHV2* | 9 | 25.0 |
| *TEM* | 0 | 0.0 |
| *NDM-1* | 6 | 16.7 |
| *OXA-48* | 5 | 13.9 |
| *CTXM + SHV2* | 7 | 19.4 |
| *CTXM + NDM-1* | 4 | 11.1 |
| *SHV2 + OXA48* | 2 | 5.6 |
| *SHV2 + NDM-1* | 3 | 8.3 |
| *OXA-48 + NDM-1* | 3 | 8.3 |
| *OXA-48 + CTXM* | 4 | 11.1 |
| *CTXM + SHV2 + NDM-1* | 2 | 5.6 |
| *CTXM+ NDM+ OXA-48* | 4 | 11.1 |
| *CTXM + SHV2 + OXA-48 + NDM-1* | 1 | 2.8 |

## Antimicrobial susceptibility patterns

ESBL- producing isolates were subjected to drug susceptibility testing by standard disc diffusion method and results are presented in Table V. Results indicated that almost all isolates under study were found resistant to first generation cephalosporins (Cefalexin), while resistance to third generation cephalosporins, cefotaxime and ceftazidime was noted to be 100% and 79.4%, respectively. Of note, 82.4% isolates were resistant against aztreonam, 58.8% against gentamicin and 11.8% against meropenum. Moreover, 29.4% were found resistant to norfloxacin and 52.9% were resistant against tetracycline. Altogether, all of the isolates were found MDR by showing resistance against at least two classes of antimicrobials tested.

## Phylogenetic analysis

A triplex PCR based phylogenetic analysis of the ESBL-producing *E. coli* was carried out and results are depicted in Table VI. Our results showed that the commensal phylogroup A was found to be the most prevalent (50.0%), followed by virulent extra-intestinal group D (33.3%) and phylogroup B2 (16.7%), respectively.

## Integron, variable region and insertion sequence IS*CR1*

All ESBL-producing isolates were further characterized for the type of integrons, presence of variable regions and insertion sequence common region 1 (IS*CR1*) and results are presented in Table VI. Overall, our results showed that integron 2 was present in a total of 17 isolates (51.5%) followed by integron 1, which was found in 16 (48.5%) isolates. Notably, integron 3 could not be amplified from any of the isolates. Interestingly, a combination of integron 1 and 2 was also observed in a total of 7 isolates (21.1%) that was found mainly in isolates recovered from sick chicken. Only 3 isolates recovered from healthy chicken carrying class 1 integron, while 2 isolates of them were carrying class 2. Of the 36 isolates, 14 were also found positive for the presence of variable region with amplicon size ranging from ~ 0.25 kb to 2 kb. Furthermore, insertion sequence IS*CR1* was also PCR-amplified from a total of 14 isolates (42.4%). Finally, association of the ESBL genes with the IS*CR1* was assessed by primer combination targeting IS*CR1* and an ESBL gene, indicating a total of 7 isolates produced an amplicon of the expected size showing association with the insertion sequence and more likely role in dissemination of these resistance encoding genes. Overall, our results showed ESBL producing multidrug resistant *E. coli* carrying diverse ESBL and carbapenemase encoding genes with CTX-M in dominance followed by SHV-2.

**Table V.** *Antibiotic sensitivity profile of* E. coli *isolates (n =36).*

| Drug | Concentration (µg) | Resistant % | Intermediates % | Susceptible % |
|---|---|---|---|---|
| CLR | 30 | 94.1 | 5.9 | 0.0 |
| AM | 10 | 88.2 | 11.8 | 0.0 |
| CTX | 30 | 100.0 | 0.0 | 0.0 |
| CAZ | 30 | 79.4 | 8.8 | 11.8 |
| AZT | 30 | 82.4 | 11.8 | 5.9 |
| GM | 10 | 58.8 | 29.4 | 11.8 |
| TE | 20 | 52.9 | 14.7 | 32.4 |
| MPN | 10 | 11.8 | 14.7 | 73.5 |
| NOR | 10 | 29.4 | 32.4 | 38.2 |

CLR = Cefalexin; AM = Ampicillin; CTX = Cefotaxime; CAZ = Ceftazidime; AZT = Aztreonam; GM = Gentamicin; TE = Tetracycline; MPN = Meropenem; NOR = Norfloxacin.

# *Discussion*

Over the last couple of years, ESBL- and carbapenemase-producing *E. coli* have been frequently reported from food-producing animals presenting a global challenge for public health and food security (Ali *et al.* 2016, Ali *et al.* 2017, Ali *et al.* 2018, Seiffert *et al.* 2013). Quite often, these isolates exhibiting MDR phenotypes further complicating its eradication during a disease condition by reducing the number of choices of drugs available against them (ur Rahman *et al.* 2018, Nordmann and Poirel 2002). Antimicrobial resistance (AMR) is even more serious in developing countries like Pakistan where usage of antimicrobials are not strictly regulated,

**Table VI.** *Frequency prevalence of extended-spectrum β-lactamase encoding genes (n = 36).*

| | ID | Source | P.G | ESBL genotype | | | | Carbapenemase | | Integron typing | | | VR | ISCR1 | ISR1 + ESBL | R/I phenotypes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | CTXM | CTXM1 | SHV2 | TEM | Oxa-48 | NDM-1 | Int.1 | Int.2 | Int.3 | | | | |
| 1 | PD 20 | DC | D | - | - | + | - | + | - | + | + | - | + | + | + | CLR, AM,GM,TE, NOR |
| 2 | PD 30 | DC | D | + | + | - | - | + | - | - | + | - | - | - | - | CLR, AM,GM, C,TE |
| 3 | PH 3 | HC | A | + | + | + | - | - | + | + | - | - | + | - | - | CLR,GM,TE, C, AM, NOR |
| 4 | 0 | HC | D | - | - | + | - | - | - | + | - | - | + | - | - | CLR,TE, AM |
| 5 | PE 32 | E | A | - | + | - | - | - | - | + | + | - | - | + | - | CLR, AM |
| 6 | PD 21 | DC | D | + | - | + | - | - | - | - | - | - | - | + | + | CLR, AM, GM,TE, NOR |
| 7 | PD 46 | DC | A | + | - | - | - | - | - | - | + | - | + | - | - | CLR, AM,TE |
| 8 | B | HC | A | - | - | - | - | - | - | - | - | - | - | - | - | CLR |
| 9 | PD 33 | DC | D | + | - | + | - | - | - | + | + | - | + | - | - | CLR, AM,GM,TE |
| 10 | PH 4 | HC | A | + | - | - | - | + | - | - | - | - | - | - | - | CLR, GM, C, |
| 11 | PD 32 | DC | D | + | - | - | - | - | + | + | - | - | + | + | - | CLR, AM, C,TE |
| 12 | PD 28 | DC | D | - | - | - | - | - | - | + | + | - | + | - | - | - |
| 13 | PE 33 | E | A | + | + | + | - | - | - | - | - | - | - | - | - | CLR,TE,C |
| 14 | PD 25 | DC | D | - | - | - | - | - | - | + | + | - | - | + | + | CLR |
| 15 | PE 43 | E | A | - | - | - | - | - | - | - | + | - | - | - | - | - |
| 16 | PD 24 | DC | D | - | - | - | - | - | - | + | - | - | + | - | - | CLR, AM |
| 17 | PD 34 | DC | D | - | - | - | - | - | - | + | - | - | + | + | + | CLR |
| 18 | PD 1 | DC | A | - | - | - | - | - | - | - | + | - | - | - | - | CLR ,TE |
| 19 | PD 31 | HC | A | - | - | - | - | - | - | - | + | - | - | - | - | CLR |
| 20 | PE 31 | E | B2 | + | - | - | - | + | - | + | + | - | + | + | + | CLR,GM,TE |
| 21 | PD 41 | DC | A | - | - | + | - | - | - | - | - | - | - | + | - | CLR,TE |
| 22 | PD 5 | DC | A | - | - | - | - | - | - | - | + | - | - | - | - | CLR, AM |
| 23 | Phh 44 | HC | A | + | - | - | - | - | - | - | + | - | - | + | - | CLR, AM |
| 24 | HL 13 | HC | A | - | - | - | - | - | + | + | - | - | + | + | - | CLR, AM, GM,TE, C |
| 25 | PE 47 | E | A | - | - | - | - | - | - | - | - | - | + | + | - | CLR |
| 26 | PE 5 | E | D | - | - | - | - | - | - | - | + | - | - | - | - | CLR |
| 27 | PD 39 | DC | A | + | - | - | - | - | + | + | - | - | + | + | + | CLR, MPN,AM,GM, NOR, C,TE |
| 28 | PH 45 | HC | A | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 29 | PD 29 | DC | A | - | - | - | - | - | - | - | + | - | - | - | - | CLR, AM |
| 30 | Phb 3 | Human | B2 | + | - | + | - | + | + | - | + | - | - | + | - | CLR,TE, AM,NOR,C |
| 31 | Phb 17 | E | B2 | + | - | - | - | - | + | + | - | - | - | - | - | CLR,TE, NOR,C |
| 32 | Phh 40 | HC | B2 | - | - | - | - | - | - | + | - | - | + | + | - | CLR |
| 33 | Phb 11 | Human | B2 | + | + | + | - | - | - | + | + | - | + | - | - | CLR,TE,GM, MPN,NOR,C |
| 34 | Phb 37 | HC | A | - | - | - | - | - | - | - | + | - | - | - | | CLR,TE |
| 35 | Phb 25 | Human | B2 | - | - | - | - | - | - | - | - | - | - | - | - | CLR,TE |
| 36 | Phb 20 | Human | D | - | - | - | - | - | - | - | - | - | - | - | - | CLR,TE, NOR, C |

DC = Diseased chicken; HC = Healthy chicken; E = Environment; + = Present; - = Absent; Int.1 = Integron class 1; Int.2 = Integron class 2; Int.3 = Integron class 3; VR = Variable region; P.G = Phylogenetic groups; CLR = Cephalexin; C = Chloramphenicol; GM = Gentamicin; TE = Tetracycline.

particularly in animals, triggering emergence of AMR. This phenomenon corroborates with bacterial response of toxin production in response to signals in the surroundings of microbes (ur Rahman and van Ulsen 2013, ur Rahman *et al.* 2014, van Ulsen *et al.* 2014, Piet *et al.* 2016). Furthermore, lack of surveillance and monitoring data regarding usage of antimicrobials and emergence of drug resistance further complicates the scenario in developing countries. Poultry industry in Pakistan is one of the biggest and dynamic industry with over 200 billion Pak. Rs. (2 Bill. $) investment, and this sector provides a lion share of 28% of total meat produced in the country (Pakistan 2016). Unfortunately, the use of antibiotics in poultry industry is not strictly regulated (Mitema 2010), raising concerns about the possible emergence of antimicrobial-resistant microorganisms (Naeem *et al.* 2006, Shahid *et al.* 2007). We designed this study to identify the prevalence of ESBL- and carbapenemase-producing *E. coli* isolated from poultry meat and risk-associated human beings.

The results showed a prevalence of 41.9% of ESBL-

producing *E. coli*. This is a higher incidence rate of ESBL producing *E. coli* recovered from poultry as compared to previously published report of 32% in Peshawar Pakistan (Ahmad *et al.* 2018), 30% in poultry in Bangladesh (Hasan *et al.* 2012) and 10.7% in France (Girlich *et al.* 2007). The higher incidence rate of ESBL-producing *E. coli* is possibly due to over use or consistent usage of antibiotics during poultry production. Contrary to our findings, however, even higher incidence rate of ESBL-producing *E. coli* have been reported from other parts of the world such as 93% in Spain (Egea *et al.* 2012) and in 81% in the Netherlands (Blaak *et al.* 2015). ESBL-producing *E. coli* has been widely reported from human patients that were hospitalized (Abrar *et al.* 2017, Rahman *et al.* 2016, Ullah *et al.* 2017) as well as from community and environment in Pakistan (Ullah *et al.* 2009). However, results of our study cannot be generalized as we analyzed a limited number of samples from a single district, *i.e.* Peshawar of Khyber Pakhtunkhwa province, Pakistan. Due to limited resources, we did not extend our study further to investigate the occurrence and trend of ESBL-producing *E. coli* in different mechanized and open shed poultry farms in the whole district. Also, we did not investigate the occurrence of ESBL- and carbapenemase-production in bacteria other than *E. coli*.

Our study indicates that $bla_{CTX-M}$ remained the predominant genotype in ESBL-producing *E. coli*. These results are in agreement with other contemporary findings from Pakistan (Khan *et al.* 2010, Mirza *et al.* 2006), China (Ali *et al.* 2016, Ali *et al.* 2017) and India (Upadhyay *et al.* 2015), etc. This strongly suggests that $bla_{CTX-M}$ is the most dominant genotype of ESBL. This goes along with higher occurrence of SHV genotype in our study and as reported by others (Habeeb *et al.* 2013). More worrisome is the combination of ESBL with carbapenemase encoding genes such $bla_{OXA-48}$ and $bla_{NDM-1}$ (Table III). Co-occurrence of ESBL and carbapenemase encoding genes have also been reported earlier from Pakistan (Ullah *et al.* 2017, ur Rahman *et al.* 2018a, Younas *et al.* 2019) from other districts from human patients and poultry. Isolation of *E. coli* harboring resistance elements encoding for ESBL and carbapenemase enzymes from poultry meat and its environment is in fact highly alarming posing threats to public health as these resistance elements can finally be acquired by ham pathogens. Combination of $bla_{CTX-M}$ + $bla_{SHV}$ + $bla_{OXA-48}$ and $bla_{NDM-1}$ is quite challenging. Such combinations of different resistance conferring-genetic elements would certainly have an impact on the severity of resistance to different antibiotics and play a role in the development of MDR. This is partly reflected by phenotypic resistance to most of the antibiotics tested in this study (Table V). Our results show that most of the isolates are found resistant to first generation cephalosporins and cefotaxime, while almost 50% of isolates are found resistant to other antibiotics tested, except carbapenems. This observed lower susceptibility of these isolates to majority of antibiotics tested is most probably indicative of consistent use of these antibiotics that might provide selective pressure for the resistant bacteria. Finally, the majority of these isolates were found to be MDR that belonged to commensal phylogroup A. Although resistance determinants were also found to be carried by virulent groups of *E. coli* (D/B2), however, acquiring these elements by commensal groups is highly worrisome given the increasing chances of dissemination of these resistant elements. This is suggesting the need for an improvement of the rationale use of antibiotics in poultry production.

Mobile elements such as integron and insertion sequences are considered crucial for the dissemination of resistance conferring elements and emergence of MDR bacteria. Previous reports suggested an overwhelming presence of clinical class 1 integron in ESBL-positive *E. coli* (Dillon *et al.* 2005, Gu *et al.* 2008). Integron of class 1 generally carries a variable region comprising a gene cassette arrays encoding different resistance elements. Our results of identification of class 1 integron in the majority of isolates along with variable regions (Table V) suggesting its involvement in dissemination of resistance elements. Strikingly, we determined that majority of $bla_{CTX-M}$ genes were associated with 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, Kiiru *et al.* 2013, Xu *et al.* 2015). Notably, our findings of the most pre-dominant ESBL genotype (CTX-M) and its strong association with the ISCR1 indicated that they are more likely mobilized by IS*CR1* elements. Conversely, only one carbapenemase encoding gene (OXA-48) was found linked to IS*CR1*.

## Conclusions

Our study concludes a high prevalence of ESBL- and carbapenemase-producing *E. coli* in the region. Taken together, the current high occurrence of multidrug resistant ESBL-producing *E. coli* carrying clinical class 1 integron and its association with IS*CR1* is quite worrisome. This calls for an efficient control policy with restriction on the consumption of extended-spectrum cephalosporins for long term use in poultry industry in the area.

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