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RESEARCH ARTICLE

Multidrug Resistant Carbapenemase-Producing *Escherichia coli* from Chicken Meat Reveals Diversity and Co-Existence of Carbapenemase Encoding Genes

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A B S T R A C T

Literature regarding multidrug resistant (MDR) carbapenemase-producing *Escherichia coli* (CPE) recovered from food-producing animals is lacking from Pakistan. Here, we report on the isolation and characterization of MDR-CPE recovered from retail-poultry meat in Khyber Pakhtunkhwa Pakistan. A total of 101 retail poultry-meat samples were screened for *Escherichia coli* and tested against 17 different antibiotics through disc diffusion test to define MDRs. MDR isolates were genotyped by PCR for carbapenemase-encoding elements. Finally, integron typing, insertion sequence common region 1 (IS*CR1*) and its association with carbapenemase-encoding genes was established through PCR. Of the 33 *E. coli* isolates, 28 (Phylo-group B2=39.2% (11/28), A=32.1 % (9/28) and group D=28.5% (8/28) were MDR displaying resistance to cefotaxime, tetracycline, ampicillin and trimethoprim-sulfamethoxazole, while, 96.4, 92.8, 89.2 and 85.7% were also found resistant to colistin-sulphate, enrofloxacin, doxycycline and ciprofloxacin, respectively. The *bla*$_{VIM}$ was predominantly identified in 8 (28.5%) isolates followed by *bla*$_{NDM}$ 4/28 (14.2%) and 2 isolates were carrying a combination of *bla*$_{VIM}$+*bla*$_{NDM}$. Class-1 integron was detected in 21 (75%), class-2 and class-3 integrons were detected in 25% and 7.1%, respectively. IS*CR1* element was identified among 11 isolates (39.28%) that was found associated with *bla*$_{VIM}$ gene among 3 isolates (27.25%) suggesting involvement in its mobilization. We report the incidence of *E. coli* carrying carbapenemase-encoding genes located on the plasmids carrying integron 1 recovered from retail-poultry-meat in Mardan, Pakistan.

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INTRODUCTION

Antimicrobial resistance (AMR) is challenging our current progress in health. AMR is increasingly reported from all over the world (Nordmann *et al.,* 2011; Mohsin *et al.,* 2017; Khattak *et al.,* 2018; ur Rahman *et al.,* 2018a). The scenario seems even more challenging for developing countries mainly due to unrestricted use of antimicrobials and lack of surveillance programs to monitor emergence of drug resistance (Khan *et al.,* 2010; Mitema 2010; ur Rahman *et al.,* 2018a). Particularly, resistance to carbapenem drugs is quite worrisome as these drugs are thought to be the last resort against MDR bacteria (Meletis, 2016). Carbapenem drugs are very important for those who develop infections due to bacteria that can inactivate extended spectrum cephalosporins. Resistance to carbapenem drugs is presented by carbapenemase enzymes. At present more than hundred types of carbapenemase genes have been reported (Meletis, 2016). Ambler classification divides these enzymes into four major categories such as A, B, C and D. Class A carbapenemases contain KPC type, class B Metallo-β-Lactamases are comprised of VIM, New Delhi metallo-β-lactamase-1 (NDM-1) and IMP types *etc*., and class D is represented by OXA-48 etc. Of the above mentioned carbapenemases, particularly NDM, though initially reported from a Swedish patient who travelled to subcontinent (Yong *et al.,* 2009), has now been reported widely from many other countries (Nordmann *et al.,* 2011).

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Co-occurrence of different carabepenemases or combination of carbapenemase with other resistance conferring elements has been associated with potentially increased spectrum of resistance in *Enterobacteriaceae* (Sattar *et al.,* 2016) including increased minimum inhibition concentration levels against carbapenems (Meletis, 2016). Elements such as insertion sequence common region 1 (IS*CR1*), conjugative plasmids and integrons have been found involved in mobilization and fast dissemination of resistance conferring elements including carbapenemase encoding genes (Ali *et al.,* 2016).

Despite the fact that antimicrobials are not strictly regulated in Pakistan and generous availability of antibiotics cheering its misuse and pumping selective pressure for the emergence of antimicrobial resistance, literature data regarding overall antimicrobial resistance surveillance is totally missing in the country with random reports suggesting widespread and high level of resistance against cephalosporins, sulfonamides and carbapenem drugs (Perry *et al.,* 2011; Sattar *et al.,* 2016; Adnan *et al.,* 2017; ur Rahman *et al.,* 2018b). To the best of our knowledge, no signal report is published from Khyber Pakhtunkhwa, Pakistan regarding the status of carbapenemase-producing *Enterobacteriaceae* associated with poultry meat in Pakistan.

MATERIALS AND METHODS

Ethics: This work was reviewed and approved by the ethical committee of Abdul Wali Khan University Mardan Pakistan and work was carried out according to local institutional and national guidelines.

Collection of samples and location: Samples from poultry meat (n=101) were collected from butcher shops at live bird market of chicken sales points during December 2017 to March 2018. Different parts of chicken were collected including 50g of liver, whole spleen and heart. All samples were transported in a sterile bag in ice box and streaked immediately for culturing.

Isolation and screening of *E. coli:* Samples were streaked onto MacConkey agar (Difco™ Becton Dickinson, Sparks, MD USA) supplemented with meropenem 0.5mg/L and incubated for 24 hours at 37 ºC. Pink candidate colonies were further purified and streaked onto eosin methylene blue agar (EMB) and presumably *E. coli* colonies were Gram stained, and further confirmed by specie specific PCR assay as described earlier (Tantawiwat *et al.,* 2005).

Antibiotic susceptibility testing and MDR identification: Mueller-Hinton agar (Difco™) has been used for the antibiotic susceptibility against 17 different antibiotics following the standard Kirby-Bauer disk diffusion method and results were interpreted according to the guidelines of the CLSI (CLSI, 2014). Minimum inhibitory concentration (MIC) of selected antibiotic was performed by microdilution method and interpreted as per CLSI guidelines.

Genotypic screening of carbapenemase-encoding enzymes: Bacterial DNA from MDR *E. coli* was isolated by boiling method as described earlier (Ali *et al.,* 2016). PCR assay was used for detection of NDM-, KPC-, OXA-48-, VIM- and IMP-encoding genes as described previously using specific primers and cited in Sup. Table 1.

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

Detection of integrons and IS*CR1:* Integron typing was performed as described earlier (Dillon *et al.,* 2005a). A PCR assay was also performed to detect insertion sequence IS*CR1* and its association with carbapenemase enzymes as we optimized and reported earlier (Ali *et al.,* 2016).

RESULTS

Isolation, antibiotic susceptibility and MDR *E. coli* from poultry meat: Of the total 101 sample, a total of 33 *E. coli* isolates were randomly screened initially against a panel of 17 different antibiotics. Briefly, 17 different antimicrobials including β-lactams (ampicillin), cephalosporins (cefotaxime, ceftazidime, cefepime *etc.*), carbapenems (meropenem, imipenem), tetracycline (tetracycline, doxycycline), quinolones (ciprofloxacin), floroquinolones (ciprofloxacin, norfloxacin), aminoglycosides (gentamycin), monobactams (aztreonam), sulfonamides (Trimethoprim-sulfamethoxazole, fosfomycin), phenicol (chloramphenicol) and polymyxin B (Colistin). Our results indicated that most of the isolates were found to be MDR displaying resistance against two or more classes of antimicrobials tested (Table 2). All isolates were found resistant to cefotaxime, tetracycline, ampicillin and trimethoprim-sulfamethoxazole. Isolates (96.4%) were also found resistant against colistin-sulphate, 92.8% were resistant to enrofloxacin, 89.2% to doxycycline and 85.7% to ciprofloxacin. Isolates that were found resistant against at least two classes of antibiotics were termed as MDR and detail is listed in Table 2. MIC value for ampicillin and cefotaxime was >64µg/ml indicated highly resistant phenotypes. 6 isolates (21.4%) displayed a MIC of ≥4 µg/ml against meropenem suggesting resistance, while, 8 isolates (28.5%) showed a MIC of ≥4 µg/ml against imipenem showing resistance. Finally, 26 isolates (92.8%) isolates showed resistance against doxycline displaying a MIC of ≥16µg/ml.

Carbapenemase genotyping of *E. coli* from poultry meat: All 28 MDR isolates were further characterized for the presence of carbapenemase-encoding genes including bla_{NDM} and bla_{OXA-48}, bla_{VIM}, bla_{IMP} and bla_{KPC} and results are shown in Table 3. Of these, 11 were found to harbor at least one type of the carbapenemase-encoding gene with 3 isolates carrying more than one type of carbapenemase family. Briefly, our results revealed that bla_{VIM} was predominant and identified in 8 (28.5%) isolates. This was followed by bla_{NDM} carried by 4/28 (14.2%) isolates. Two isolates were carrying KPC while one was harboring OXA-48 gene. Of note, no IMP type of genotype could be confirmed among all 28 under study *E. coli* isolates.

Table 1: Antimicrobial resistance profile of *E. coli* isolates (n=28) recovered from poultry meat

S. No	Antimicrobial agent	Abbreviation	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	8/28(28.5)	12/28(42.8)	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	10µg	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	10 µg	0/28(0)	0/28(0)	28/28(100)

Table 2: Frequency of MDR *E. coli* isolates from retail poultry meat

Source of samples	Total samples	No of *E. coli* isolates (%)	MDR phenotypes
Spleen	43	21 (48.8)	19(90.5)
Liver	20	6(30.0)	3(50.0)
Heart	38	6(15.8)	6(100)
Total	101	33(33.0)	28(84.8)

Table 3: Frequency of carbapenemase genotypes from retail poultry meat

S.no	Genes	Number of samples positive			Frequency	Percentage
		liver	spleen	heart		
1	*blaVIM*	1	5	2	8/28	28.5
	blaNDM	1	3	0	4/28	14.2
2	*blaKPC*	1	1	0	2/28	7.1
3	*blaOXA-48*	0	0	1	1/28	3.5
4	*blaIMP*	0	0	0	0/28	00.0
Co-existence of multiple carbapenemase encoding genes						
6	*blaVIM+blaNDM*		2		2/24	8.3
7	*blaVIM+KPC*		1		1/24	4.1

Strikingly, 8 isolates were harboring singular type of the carbapenemase encoding family tested, while 3 were carrying more than one type (Table 3). The most predominant combination of co-occurrence was of bla_{VIM}+bla_{NDM} (8.3%). One isolate was also carrying a combination of bla_{VIM}+$_{KPC}$ (1/24=4.1%).

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).

Integron typing of *E. coli* isolates from poultry meat: Our results showed that class-1 integron was detected in 21 (75%) isolates of *E. coli*, while, class-2 and class-3 integrons were detected in 25 and 7.1%, respectively (Table 4). Class 1 integron was identified among *E. coli* isolated from all different sources suggesting random distribution.

Insertion sequence IS*CR1* and its association with carbapenemase encoding genes: A total of 11 samples (39.28%) were carrying IS*CR1*, of which 3 isolates (27.25%) were found associated with bla_{VIM} revealed by the PCR product size (Table 4). We could not amplify any desired size product from combination of ISCR1 and OXA-48 or NDM suggesting absence of association.

DISCUSSION

The current upraise in bacterial resistance against antimicrobials is worrisome. No country of the world could escape of bacterial pathogens offering resistance to- at least- commonly used antibiotics. Consistent and misuse of antibiotics is known to energize selective pressure for the emergence of antibiotic resistance just like when bacteria secretes its toxins in response to external signals in the form of molecules available in its surrounding (ur Rahman and van Ulsen 2013; ur Rahman *et al.,* 2014; van Ulsen *et al.,* 2014; Piet *et al.,* 2016). Most of the use of antimicrobials goes for agriculture and livestock sector, and that too, not for treatment, but mainly for prevention of diseases and as growth promoter. Situation gets worsen as use of antimicrobials in developing countries like Pakistan is not strictly monitored mainly in agriculture and livestock. Furthermore, absence of regular surveillance data also makes situation further complicated. Poultry industry in Pakistan is a dynamic industry that contributes to 28% of total meat produced in the country (Pakistan, 2016). Besides meat, table eggs of poultry layer birds also contribute in daily breakfast of majority people in Pakistan. However, use of antibiotics in poultry industry is also not strictly regulated (Mitema, 2010) raising concern of emergence of AMR. Antimicrobial resistant *E. coli* have been increasingly reported from food producing animals (Ali *et al.,* 2017; ur Rahman *et al.,* 2018a; ur Rahman *et al.,* 2018b). The current study reports on the CPE recovered from retail poultry meat raising concern of its dissemination.

Among 33 *E. coli* isolates, 28 displayed the phenotype of MDR. Among these, 11 were found to produce at least one carbapenemase encoding gene suggesting MDR phenotype is possibly due to carbapenemase enzymes. Although, literature study suggests that NDM has been increasingly reported from clinical (Perry *et al.,* 2011; Hussain 2015; Sattar *et al.,* 2016) and community settings in Pakistan (Sartor *et al.,* 2014), current data demonstrated that bla$_{VIM}$ is instead a predominant carbapenemase encoding gene followed by bla$_{NDM.}$ Identification of NDM in isolates of *E. coli* recovered from poultry meat is alarming suggesting that NDM has been equally widespread both among animal- as well as human. NDM gene is considered to be originated from Indian continent particularly India, Pakistan and

Table 4: Characterization of MDR *E. coli* isolated from retail poultry meat

#	ID	Phylo-group	Place of isolation	Carbapenemase genes	ISCR1	ISCR1 + NDM	ISCR1 + VIM	ISCR1 + KPC	ISCR1+ OXA-48	Integron typing Int.1	Int.2	Int.3	R/I Phenotypes to the Antibiotics
1	ss3c1	B2	Malakand chaok	VIM,NDM	-	-	-	-	-	+	-	-	CAZ,MEM,CIP,AMP,NOR,SXT,TE,ATM,DO, C,FEF
2	ss26sc1	B2	Gajju khan	KPC, VIM	+	-	-	-	-	+	-	-	CIP.AMP,SXT,TE,CN,ATM,DO,C, MEM, IPM
3	ls5sc1	D	Colleg chaok	-	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C
4	ss16sc1	D	Colleg chaok	KPC	-	-	-	-	-	+	-	-	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C, MEM, IPM
5	ss12sc2	B2	Shaikh Malton	-	+	-	+	-	-	+	-	-	CIP,AMP,NOR,SXT,TE, MEM ,ATM,DO, IPM , CAZ
6	ss3sc2	D	Malakand chaok	VIM	-	-	-	-	-	+	+	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,FEF
7	ss10sc1	A	Malakand chaok	-	+	-	-	-	-	+	-	-	CIP,AMP,NOR,SXT,TE,ATM,DO,C, IPM
8	Hs19sc1	A	Shaikh Malton	-	+	-	-	-	-	-	-	-	CAZ,CIP,AMP,SXT,TE,ATM, MEM , IPM ,DO
9	ss12sc1	D	Shaikh Malton	-	+	-	+	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM, MEM, IPM , DO
10	Hs9sc2	A	Malakand chaok	-	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,C
11	ss15sc1	A	Colleg chaok	-	-	-	-	-	-	+	+	-	CIP,AMP,NOR,SXT,TE,ATM,DO,C,FEF
12	Hs11sc1	B2	Colleg chaok	-	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO
13	ss30sc1	B2	Gajju khan	VIM	-	-	-	-	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C,FEF, IPM
14	ls5sc4	B2	Colleg chaok	NDM,VIM	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C, IPM
15	ss22sc1	B2	Gajju khan	VIM	+	-	+	-	-	+	+	-	CAZ,MEM,CIP,AMP,NOR,SXT,TE,CN,,DO,C
16	ss14sc1	B2	Colleg chaok	NDM	-	-	-	-	-	-	-	-	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C, MEM, IPM
17	Hs12scc1	B2	Shaikh Malton	-	+	-	-	-	-	-	-	-	CAZ,CIP,AMP,NOR,SXT,TE,CN,DO,C, IPM
18	Ss13sc1	A	Shaikh Malton	-	-	-	-	-	-	+	+	-	CAZ,CIP,AMP,NOR,SXT,TE,DO, IPM
19	ss26sc2	B2	Gajju khan	VIM	+	-	-	-	-	-	-	-	CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C
20	ss18sc1	D	Colleg chaok	-	+	-	-	-	-	-	-	-	CAZ,AMP,SXT,TE,ATM,DO, MEM, IPM
21	Hs13sc1	D	Shaikh Malton	-	-	-	-	-	-	+	+	-	CAZ,AMP,NOR,SXT,TE,ATM,DO,C
22	ss8sc4	D	Malakand chaok	VIM	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,MEM,CN,ATM,DO,IPM,C,FEF
23	ss21sc1	A	Gajju khan	-	-	-	-	-	-	+	-	+	CAZ,CIP,AMP,NOR,SXT,TE,CN,ATM,DO,C, FEF
24	ss25sc1	A	Shaikh Malton	-	-	-	-	-	-	+	+	-	CAZ,AMP,SXT,TE,ATM,DO,C,FEF, IPM
25	ls25sc1	A	Shaikh Malton	-	+	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C
26	Hs38sc1	A	Shaikh Malton	OXA-48	-	-	-	-	-	+	-	-	CAZ,CIP,AMP,NOR,SXT,TE,ATM,DO,C, IPM
27	ss36sc1	B2	Gajju khan	-	-	-	-	-	-	-	-	-	CIP,AMP,SXT,TE,CN,DO,C, MEM, IPM
28	ls26sc1	D	Gajju khan	-	+	-	-	-	-	+	+	+	CAZ,MEM,AMP,NOR,SXT,TE,CN,MEM,ATM

Supplementary Table 1: Primers, Targeted Genes and Amplicon size

Primer	Sequence (5′ to3′)	Target gene	Annealing temperature	Amplicon size	References
Carbapenemase encoding genes					
IMP-F	GGAATAGAGTGGCTTAAYTCTC	bla_{IMP}	55°C	232	(Poirel *et al.,* 2011)
IMP-R	GGTTTAAYAAAACAACCAC				
VIM-F	GATGGTGTTTGGTCGCATA	bla_{VIM}	55°C	390	
VIM-R	CGAATGCGCAGCACCAG				
OXA-48-F	GCGTGGTTAAGGATGAACAC	bla_{OXA-48}	55°C	438	
OXA-48-R	CATCAAGTTCAACCCAACCG				
NDM-F	GGTTTGGCGATCTGGTTTTC	bla_{NDM}	55°C	621	
NDM-R	CGGAATGGCTCATCACGATC				
KPC-F	CGTCTAGTTCTGCTGTCTTG	bla_{KPC}	55°C	798	
KPC-R	CTTGTCATCCTTGTTAGGCG				
Integrons					
intI1-F	CCT CCC GCA CGA TGA TC	*intI1*	54°C	280-bp	
intI1-R	TCC ACG CAT CGT CAG GC				Dillon *et al.*, 2005
intI2-F	AAA TCT TTA ACC CGC AAA CGC	*intI2*	54°C	439-bp	
intI2-R	ATG TCT AAC AGT CCA TTT TTA AAT TCT A				
intI3-F	AGT GGG TGG CGA ATG AGT G	*intI3*	54°C	599-bp	
intI3-R	TGT TCT TGT ATC GGC AGG TG				
E. coli-Specific					
UAL	TGG TAA TTA CCG ACG AAA ACG GC	*uidA*	62°C	147-bp	(Tantawiwat *et al.,* 2005)
UAR	ACG CGT GGT TAC AGT CTT GCG				
PHYLO-Group					
ChuA-F	GAC GAA CCA ACG GTC AGG AT	*ChuA*	55°C	279-bp	(Clermont, 2000)
ChuA-R	TGC CGC CAG TAC CAA AGA CA				
YjaA-F	TGA AGT GTC AGG AGA CGC TG	*YjaA*	55°C	211-bp	
YjaA-R	ATG GAG AAT GCG TTC CTC AAC				
TspE4C2-F	GAG TAA TGT CGG GGC ATT CA	*TspE4C2*	55°C	152-bp	
TspE4C2-R	CGC GCC AAC AAA GTA TTA CG				
IS*CR1*					
ISCR1-F	CGC CCA CTC AAA CAA ACG	IS*CR1*	55°C	469-bp	(Ali *et al.,* 2016)
ISCR1-R	GAG GCT TTG GTG TAA CCG				

F-Forward, R- reverse.

Bangladesh and spread to other parts of the world quite speedily (Poirel *et al.*, 2010; Habeeb *et al.*, 2013). Isolates that produce NDM have shown resistance to most of the cephalosporins and carbapenems. However, our results were quite interesting as few of the isolates were still found susceptible to meropenum raising further curiosity. Possibly, other co-expressing resistance encoding elements or promoter sequences might have role in such phenotypic characteristics.

Co-existence of multiple carbapenemase encoding genes been reported previously in Pakistan (Sattar *et al.*, 2016). Sattar et al reported the co-existence of KPC and NDM-1 encoding genes in clinical isolates of *Klebsiella pneumonia* isolates, while we identified VIM and NDM combination. Although, sources of samples of *E. coli* isolation were different, co-existence of similar combination of two different carbapenemase encoding genes is alarming. It would be interested to further investigate the genetic background of these genes, plasmid types, and plasmid sizes, and whether all these genes are carried on the same or different plasmids. Altogether, our data report on the presence MDR-CPE suggesting that poultry meat might be a source of these genes or CPE.

Conclusions: We report the occurrence of MDR-CPE recovered from poultry meat suggesting that poultry meat could be a source for the tested carbapenemase-encoding genes, which may further be disseminated to environment and human. An urgent intervention is required including awareness regarding prudent use of antimicrobials in poultry production systems. Furthermore, Sequence typing by whole genome sequencing of few candidate isolates should be carried out to analyze them further.

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Authors contribution: SUR designed and conceive the project. MY collected all the samples and processed them. MMS and IK helped in culturing and processing. SS and SUR wrote the draft. All authors approved the final manuscript.

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