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High Occurrence Rate of Multidrug-Resistant ESBL-Producing *E. coli* Recovered from Table Eggs in District Peshawar, Pakistan

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Authors' Contribution
SUR designed and conceived the project. SUR, TU and TA performed the lab work. SA, AU, HM, IA, AK and IK helped in sample collection and processing and contributed in reagent. SUR, TA, US and HK wrote the manuscript.

Key words
Food-producing animals, *E. coli*, Extended spectrum β lactamase, Carbapenemase, Table eggs.

ABSTRACT

Whilst food-producing-animals could be a pool of resistance-conferring elements in the existing animal production system in Pakistan, the issue has not yet judiciously been highlighted. This study was conducted to determine incidence of extended spectrum β lactamase (ESBL) - producing *Escherichia coli* in table eggs and human with history of close association with table eggs. For this purpose, a total of 200 table eggs and 50 stool samples from human were analyzed. Results showed that out of 80 *E. coli* isolates recovered from eggs, 20 (25%) were found to be ESBL-producers, while, of the 17 human-isolates, 4 (23.5%) were ESBL producers. PCR revealed that bla_{CTXM} (bla_{CTXM-1}=15 and bla_{CTXM-9}=4) was carried by all 22 (91.6%) ESBL-producers with additional bla_{SHV2} (n=12) and bla_{NDM-1} (n=3), but no bla_{TEM} was identified. A predominant combination of bla_{CTXM} + bla_{SHV2} (n=12) followed by bla_{CTXM} + bla_{NDM-1} (n=3) was determined. All these isolates (phylogroup D=14/24, A= 6/24 and B2=4/24) were found to be multidrug resistant displaying resistance against at least three different classes of antibiotics. Class 1 integron was carried by (21/24) followed by additional class 2 integron (15/24). A total of 8 isolates were harboring insertion sequence common region 1 (IS*CR1*), which was found to be linked with bla_{CTXM} in 50% (4/8) isolates. Results of the current study indicate contamination of eggs with ESBL-producing *E. coli* suggesting to improve hygienic process for end consumer during- egg production.

INTRODUCTION

Food-producing animals-associated antimicrobial resistance (FAAMR) contributes to a greater incidence of clinical infections and hence regarded as public health issue. Especially, spread of microorganisms resistant to β-lactams-and carbapenems-the most efficient and safe drugs when other drugs apparently fail to work-through food-producing animals (FPAs) and retail meat is alarming. ESBLs can inactivate antibiotics including third- and fourth-generation cephalosporins and monobactams, however, could not inactivate cephamycins and carbapenems, while carbapenemases inactivate carbapenem drugs (ur Rahman *et al.,* 2018a). ESBLs and carbapenemases are predominantly produced by *Enterobacteriaceae* including *Escherichia coli* and are being implied as crucial mechanisms of resistance to cephalosporins and carbapenems, respectively (Queenan and Bush 2007; ur Rahman *et al.,* 2018a; Younas *et al.,* 2019).

ESBL-encoding genes have been categorized into three major classes - bla_{CTXM}, bla_{SHV} and bla_{TEM}- of which, bla_{CTXM} has been reported as the most successful and widespread genotype in Asia (Ali *et al.,* 2017, 2016). The bla_{CTXM} has been has been further divided into

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five subgroups (bla_{CTXM-1}, bla_{CTXM-2}, bla_{CTXM-8}, bla_{CTXM-9}, $bla_{CTXM-25}$). The families of each of these three categories of bla_{CTXM}, bla_{SHV} and bla_{TEM} have been expanded widely with more than 170 variants have been described for each ESBL genotype (http://www.lahey.org/studies) which have been reported worldwide from community as well as FPAs (Ali *et al.,* 2016, 2017). It has been shown that ESLB-producing *E. coli* spread through contaminated food chain or water utilizing mobile genetic elements such as integron, insertion sequence common region 1 (IS*CR1*) and conjugative plasmids (Ali *et al.,* 2016; Geser, 2015).

Carbapenemases are highly versatile family of β-lactamases recognizing almost all hydrolysable β-lactams, and inactivate carbapenem drugs (Nordmann and Poirel, 2002). Genes responsible for carbapenemase production are quite often acquired from other related bacteria or environment when present on mobile genetic elements such as conjugative plasmids. Some commonly identified carbapenemase enzymes are NDM-, OXA-48- and KPC- type of enzymes whose presence has been reported from various parts of the world (Queenan and Bush, 2007). *E. coli* expressing NDM- and OXA-48-type of carbapenemases has been increasingly reported from Pakistan largely from the clinical settings. Use of antibiotics in agriculture and FPAs is not strictly regulated in Pakistan, and the excessive use of antibiotics in poultry production may likely be the prime stimulus for the emergence of FAAMR (Kumar *et al.,* 2009). Despite this, literature regarding ESBL-producing *E. coli* in FPAs in Pakistan is rare making it an ignored issue. For the very first time, we report on the incidence of ESBL -producing *E. coli* isolated from shells of table eggs and human dealing with these eggs in district Peshawar, Pakistan.

MATERIALS AND METHODS

Study area and types of samples

This study was conducted during March 2016 and December 2016 in district Peshawar, Pakistan. Table eggs were obtained from farms, small grocery shops and supermarkets. Stool samples of those human who had history of constant physical contact with table eggs, such as shop keepers, workers at farms, drivers and helper loading and unloading table eggs for transport were collected from a collection at Khyber teaching hospital, Peshawar.

Ethics

The study was approved by the local institutional ethical committee and all work described here was performed according to local and institutional guidelines. A proper written consent for publication of the resultant data was obtained from donors and owners of the shops.

Sample collection

A total of 200 table eggs and 50 stools samples from human beings analyzed. Eggs were collected from small grocery shops (n=7), supermarkets (n=5) and poultry farms (n=3). Each point was visited two times approximately two weeks apart. A total of 50 human stool samples were obtained from collection of diagnostic lab of Khyber Teaching Hospital, Peshawar.

Isolation of E. coli

Isolation of *E. coli* from egg shells was performed as described earlier (Musgrove *et al.,* 2005). About 2 g of human faecal samples were suspended in 5 ml of saline (0.9%) and 100 µl of each sample was plated directly on MacConkey agar (Abdallah *et al.,* 2017) containing cefotaxime (1 µg/ml) and meropenum (0.5 µg/ml) for screening of ESBL and carbapenemase *E. coli* producers as advised by the clinical & laboratory standard institute (CLSI) (CLSI, 2014). Our previously confirmed *E. coli* producing CTXM (Ali *et al.,* 2016) was used as positive control while *E. coli* ATCC 25922 was implied as susceptible control strain.

Identification of E. coli

Presumptive lactose fermenting pink colour colonies were picked up for further specie identification by API 20E kits as per manufacturer's instruction (bioMérieux, Marcy I'Etoile, France) and specie specific PCR as mentioned elsewhere (Tantawiwat *et al.,* 2005). Confirmed *E. coli* isolates were stored in brain heart infusion broth (BHI; Sigma-Aldrich) containing 30% glycerol at -80°C.

Confirmation of ESBL producers

Presumptive ESBL- and carbapenemase-producers were subjected to double disc synergy test as per recommendation of CLSI (2014). ESBL producers were confirmed by 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 Dickison, Sparks, MD USA). The test was regarded positive when the zone of inhibition of cefotaxime plus clavulanic acid or ceftazidime plus clavulanic acid was ≥5mm larger than their respective single discs. *E. coli* ATCC 25922 (ESBL-negative) and *Klebsiella pneumoniae* ATCC 700603 (ESBL-positive) while quality control strain.

Genotypic screening for ESBL and carbapenemase encoding genes

Conventional PCR was performed on ESBL and carbapenemase positive isolates for the presence of bla_{CTX-M}, bla_{SHV-2}, and bla_{NDM-1} (Table I). Template DNA was prepared by conventional boiling method (Ali *et al*., 2016).

Table I.- Primers and target genes used in this study.

Primer name	Target gene	Sequence (5´-3´)	Size (bp)	Reference
β -lactamases				
CTX-M –F CTX-M –R	bla_{CTXM}	ATGGTTAAAAAATCACTGCG AAACCGTTGGTGACGAT	~873	(Paauw *et al.,* 2006)
CTX-M9-F CTX-M9-R	bla_{CTXM9}	TGGTGACAAAGAGAGTGCAACG TCACAGCCCTTCGGCGAT	~875	(Paauw *et al.,* 2006)
SHV –F SHV -R	bla_{SHV}	GGG TTA TTC TTA TTT GTC GC TTAGCGTTGCCAAGTGCTC	~567	(Chang *et al.,* 2001)
CTXM1-F[1] CTXM1-R	bla_{CTXM-1}	GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG	~490	(Shibata *et al.,* 2006)
TEM-F TEM-R	Bla_{TEM}	ATA AAA TTC TTG AAG ACG AAA GAC AGT TAC CAA TGC TTA ATC	~1086	(Yao *et al.,* 2007)
NDM-F NDM-R	Bla_{NDM1}	AGCTGAGCACCGCATTAG CGGAATGGCTCATCACGATC	~720	(Poirel *et al.,* 2011)
Integrons and integron variable region				
intI1-F intI1-R	intI1	CCT CCC GCA CGA TGA TC TCC ACG CAT CGT CAG GC	~280	(Dillon *et al.,* 2005)
intI2-F intI2-R	intI2	AAA TCT TTA ACC CGC AAA CGC ATG TCT AAC AGT CCA TTT TTA AAT TCT A	~439	(Dillon *et al.,* 2005)
intI3-F intI3-R	intI3	AGT GGG TGG CGA ATG AGT G TGT TCT TGT ATC GGC AGG TG	599	(Dillon *et al.,* 2005)
intI1-VR-F ntI1-VR-R	intI1vari-able region	TCA TGG CTT GTT ATG ACT GT GTA GGG CTT ATT ATG CAC GC	variable	(White *et al.,* 2000)
Specific to *E. coli*				
UAL UAR	uidA	TGG TAA TTA CCG ACG AAA ACG GC ACG CGT GGT TAC AGT CTT GCG	~147	(Tantawiwat *et al.,* 2005)
***E. coli* phylogrouping**				
ChuA-F ChuA-R	ChuA	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	~279	(Clermont *et al.,* 2000)
YjaA-F YjaA-R	YjaA	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211-bp	(Clermont *et al.,* 2000)
TspE4C2-F TspE4C2-R	TspE4C2	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152-bp	(Clermont *et al.,* 2000)
ISCR1	IS*CR1*	CGC CCA CTC AAA CAA ACG GAG GCT TTG GTG TAA CCG	469-bp	(Ali *et al.,* 2016)

F, forward; R, reverse.

PCR assays was also performed separately on the isolated-purified plasmid of random isolates using plasmid isolation kit TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions, in order to show that the ESBL or carbapenemase encoding genes are located on the plasmids.

Primers were synthesized by Sunbiotech, Beijing. All PCR amplification reactions were carried out in thermo-cycler (Bio Rad T100) and products were resolved on 1% agarose gel. *Klebsiella pneumoniae* ATCC 700603 (ESBLs-positive strain), DNA from a previously confirmed NDM-1 producer were used positive controls, respectively, in all related PCR assays.

Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing was performed by Kirby–Bauer disk diffusion method and interpreted as per CLSI guidelines (CLSI, 2014). The following antibiotic disks were used, ampicillin (10 µg), cephalexin (30 µg), amoxicillin/clavulanic acid (20/10 µg), ceftazidime/clavulanate (30/10 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), meropenem gentamicin (10 µg), norfloxacin (10 µg), gentamicin (10µg), tetracycline (30µg) (Oxoid, UK). *E. coli* ATCC 25922 (ESBL-negative) and *Klebsiella pneumoniae* ATCC 700603 (ESBL-positive) were used as quality control strains. ESBL *E. coli* were declared as

multidrug resistant (MDR) when found resistant to more than two categories of antimicrobial drugs (Magiorakos *et al.,* 2012).

Phylogenetic analysis

A triplex PCR was applied to categorize *E. coli* into one of the four phylogenetic groups (A, B1, B2 and D) as described earlier (Clermont 2000).

Integron, variable region and insertion sequence ISCR1

PCR was used to identify one of the three types (Type 1-III) of integrons (Dillon *et al.,* 2005), variable region of the *intII* positive isolates (Ali *et al.,* 2016; White *et al.,* 2000) and association of IS*CR1* with ESBL/ carbapenemase-encoding genes as we described earlier (Ali *et al.,* 2016).

Statistical analysis

Data obtained were stated in absolute values, and percentages for which Microsoft excell was mainly used. Degrees of antimicrobial resistance and genotypes of ESBLs found in *E. coli* recovered from table eggs and human specimens were compared by chi-square test at P≤0.01 probability level using SPSS 16.0 analysis software.

RESULTS

Isolation rate of E. coli *and occurrence of ESBL and carbapenemase producers*

Isolation rate and occurrence of ESBL producers is mentioned in Table II. A total of 40% (80/200) of table egg samples were found positive for *E. coli*, while a total of 17 (34%) human samples were declared positive for *E. coli* growth. Table eggs were found significantly highly contaminated (P ≤ 0.01) as compared to human samples**.** A total of 20 isolates (25%) from table eggs were ESBL producers, while 4 (23.5%) ESBL-producers were recovered from human (Table II).

Genotyping of ESBL producers

All 24 ESBL producers were further assayed for the presence of genes encoding ESBL and few commonly reported carbapenemase encoding genes. Our results indicated that 91.6% (22/24) isolates were harbouring bla_{CTXM} gene (Table III). Further subtyping indicated that a total of 15 isolates were carrying bla_{CTXM-1} and 4 were

Table II.- Isolation frequency of ESBL and Carbapenemase producers.

Sample nature	Isolation rate	ESBL producers	MDR
Table eggs	80/200 (40%)	20/80 (25%)	24/80 (30%)
Human specimens	17/50 (34%)	4/17 (23.5%)	06/17 (35.3%)
Total	97/250 (38.8%)	24/97 (24.7%)	30/97 (30.9%)

Table III.- Genotyping of ESBL producers (n=24).

Genotypes	Number	Frequency
CTXM	22	91.6
CTXM-1	15	62.5
CTXM-9	4	16.6
SHV2	12	50.0
TEM	0	0.00
NDM-1	3	12.5
CTXM+SHV2	12	50.0
CTXM+NDM-1	3	12.5

Table IV.- Antibiotic susceptibility test of ESBL-(n=24).

Antibiotics	Abbr.	µg/ml	Resistant isolates (number) %		Intermediate isolates (number)%		Sensitive isolates (number)%	
			Eggs	Human	Eggs	Human	Eggs	Human
Cephalexin	CLR	30	(24)100	(4)100	(0) 0.0	(0) 0.0	(0) 0.0	(0) 0.0
Ampicillin	AM	10	(20) 83.3	(3) 75	(4) 16.6	(1) 25	(0) 0.0	(0) 0.0
Cefotaxime	CTX	30	(21) 87.5	(3) 75	(2) 8.3	(1) 25	(1) 4.3	(0) 0.0
Ceftazidime	CZA	30	(22) 91.7	(3) 75	(2) 8.4	(1) 25	(0) 0.0	(0) 0.0
Cefepime	FEP	30	(17) 70.83	(3) 75	(4) 16.6	(1) 25	(3)12.5	(0) 0.0
Cefoxitin	FOX	30	(3) 12.5	(3)75	(1) 25	(1) 16.7	(18) 75.0	(0) 0.0
Aztreonam	AZT	30	(17) 70.83	(2) 50	(4) 16.6	(1) 25	(3) 12.5	(1) 25.0
Meropenem	MPN	10	(2) 8.4	(3) 75	(1) 25	(0) 0.0	(21) 87.5	(0) 0.0
Norfloxacin	NOR	10	(7) 29	(1) 25.0	(13) 54.1	(2) 50.0	(4) 16.5	(1) 25.0
Gentamicin	G	10	(8) 33.4	(3) 75.0	(8) 33.4	(1) 25.0	(8) 33.4%	(0) 0.0
Tetracycline	TE	30	(18) 75.0	(3) 75.0	(6) 25	(1) 25.0	(0)0.00	(0) 0.0

harbouring bla_{CTXM-9}. A total of 2 isolates were harbouring both bla_{CTXM-1} and bla_{CTXM-9} in combination. A total of 12 isolates were carrying bla_{SHV2}, while 3 isolates were also carrying bla_{NDM-1}. Other combinations such as *blaCTXM-*$_{1+}bla_{SHV2}$ (n=12) and bla_{CTXM} + bla_{NDM-1} (n=3) was also noticed (Tables III, IV). No bla_{TEM} type was however amplified from any of the isolates.

Antibiotic susceptibility profile

All 24 under study isolates were tested further against a panel of drugs containing first-2nd and 3rd generation cephalosporins as well as non-β lactam drugs. All isolates were found resistant to first generation cephalosporin (Cephalexin). Majority, both of human and table eggs, of our isolates were found resistant to third- (ceftazidime, 91.7%, cefotaxime, 87.5%), and fourth (cefepime 70.83%)-generation cephalosporin, however, high susceptibility was detected towards carbapenem (meropenem,87.5%), cephamycins (cefoxitin, 75%); Table IV). Overall, most of the isolates were found to be MDR displaying resistance against more than two classes of antibiotics tested.

Integron, variable regions and insertion sequence ISCR1

Of the 24 isolates, 21 were carrying integron 1, while 15 were carrying additional integron 2. However, none of the isolates was found carrying integron 3. Variable region could be PCR amplified from 14 isolates, while insertion sequence common region 1 (IS*CR1*) was found in total of 8 isolates. Of these, IS*CR1* was found linked with bla_{CTXM} among 4 isolates (50%) (Table V).

Table V.- Integrons, variable regions and insertion sequence of ESBL and carbapenemase producers.

No.	ID	Origin	Genotype	Int. 1	Int. 2	Int. 3	VR	ISCR1	ISCR1+ESBL	PG
1	110	Eggs-PF	CTXM1, SHV-2	+	+	_	+	_	_	D
2	78	Eggs-SM	CTXM1, SHV-2	+	+	_	+	+	+	D
3	76	Eggs-SM	CTXM1, SHV-2	+	+	_	+	+	+	D
4	90	Eggs-PF	CTXM1, SHV-2	+	+	_	+	+	+	D
5	150	Eggs-GS	CTXM1, CTXM-9, SHV-2, NDM-1	+	+	_	+	+	+	D
6	135	Eggs-SM	CTXM1	+	+	_	+	+	_	D
7	33	Eggs-PF	CTXM-9, SHV-2	+	+	_	+	_	_	D
8	20	Eggs-SM	-	+	_	_	_	_	_	A
9	21	Eggs-SM	CTXM	+	+	_	_	_	_	A
10	15	Eggs-SM	CTXM	+	_	_	_	_	_	A
11	25	Eggs-GS	CTXM	+	+	_	+	_	_	D
12	62	Eggs-SM	-	+	+	_	_	+	_	D
13	16	Eggs-GS	CTXM	-	_	_	+	_	_	D
14	70	Eggs-PF	CTXM1, SHV-2	+	_	_	_	_	_	D
15	67	Eggs-GS	CTXM1, SHV-2	+	_	_	_	_	_	D
16	19	Eggs-SM	CTXM1, SHV-2	-	_	_	_	_	_	A
17	69	Eggs-SM	CTXM1, SHV-2	+	_	_	_	_	_	A
18	61	Eggs-SM	CTXM1, SHV-2	+	+	_	+	_	_	D
19	THU4	Hn-St	CTXM1, CTXM-9	+	-	_	+	+	_	B2
20	THI1	Hn-St	CTXM, NDM-1	+	+	_	+	+	_	B2
21	THU3	Hn-St	CTXM, NDM-1	+	+	_	+	_	_	B2
22	THI2	Hn-St	CTXM1, CTXM-9, SHV-2	+	+	_	+	_	_	B2
23	78	Eggs-PF	CTX-M1, SHV-2	+	+	_	+	_	_	D
24	19	Eggs-GS	CTX-M	_	_	_	_	_	_	A

Eggs-PF, egg samples taken directly from poultry farms; Eggs-SM, egg samples taken from super market; Eggs-GS, egg samples taken from ordinary grocery shop; Hn-St, human stool samples; Int.1, integron 1; Int. 2, integron 2; VR, variable region; P.G, phylogenetic group.

DISCUSSION

ESBL-producing *E. coli* is becoming a huge challenge in clinical settings due to its ability to resistance β-lactams and carbapenems- the most effective drugs. The current increasing reports on ESBL- producing *E. coli* particularly from FPAs has major public health consequences in addition to compromising animal welfare (Adnan *et al.,* 2017; Ali *et al.,* 2016, 2017; ur Rahman *et al.,* 2018b). Quite often, ESBL-producing isolates exhibit multidrug resistant phenotypes and could easily transfer these features to other bacteria through horizontal transfer by means of conjugative plasmids and mobile elements (Nordmann and Poirel, 2002). This phenomenon of emergence of drug resistance corroborates with that of toxin production in response to signals present in the surroundings of microbes (ur Rahman and van Ulsen, 2013).

Poultry industry is one of the most successful and dynamic industry contributing a lion share of 28.0% of total meat produced in the country and contributes 1.3% to national GDP (Economic Survey of Pakistan, 2016). In addition, poultry sector provides the cheapest sources of quality protein in the form of meat and eggs. However, unfortunately, use of antimicrobials in poultry sector is not strictly regulated in Pakistan encouraging blind and excessive use of antibiotics (Naeem *et al.,* 2006). Consistent and excessive use of antimicrobial provides selective pressure for emergence of antimicrobial resistance. These resistant microbes would find its way to disseminate among the community or would enable to pass on its resistance-encoding elements to their surrounding ecosystem. Eggs laying hen are normally kept in open or semi-mechanized poultry sheds in Pakistan with abundant supply of antibiotics. In such scenario, laid eggs would have chances to get contaminated by bacteria carrying resistance elements (Huneau-Salaün *et al.,* 2010). Besides environment, people working at such farms, transporters of eggs or those selling eggs at shops would also be at risk upon physical contact. Hence to protect consumers, various countries have implied various tools to sanitize eggs such as ultraviolet radiation and gaseous ozone. Our study revealed an overall 24.8% ESBL producing *E. coli* isolated mainly from shells of table eggs. Our results are in agreement with previous findings from Spain with 22.3% of egg shells were reportedly found contaminated with ESBL- *E. coli* (Grande-Burgos *et al.,* 2016). The higher incidence rate of ESBL-producing *E. coli* is possibly due to overuse of antibiotics during poultry production. ESBL-producing-*E. coli* has been widely reported from human patients that were hospitalized in Pakistan and community (Abrar *et al.,* 2017; Ullah *et al.,* 2017) suggesting widespread colonization of ESBL producers. However, results of our study cannot be generalized as we analyzed limited number of samples from a single district Peshawar of Khyber Pakhtunkhwa province-Pakistan. Due to limited resources, we could not extend our study further to investigate the occurrence and trend of ESBL producing *E. coli* in eggs of backyards and different mechanized and open shed poultry farms in Khyber Pakhtunkhwa province. We speculate that the prevalence of ESBL will be higher than we have observed in the current study. We are currently extending our study to other districts, and our preliminary results support this notion.

As observed earlier, our study indicates that bla_{CTXM} remained the predominant ESBL genotype in Pakistan (Khan *et al.,* 2010) and other neighboring countries like China (Ali *et al.,* 2016, 2017). This goes along with higher occurrence of SHV genotype in our study and as reported by others (Habeeb *et al.,* 2013; Chang *et al.,* 2001). Overall, our results suggest a higher incidence of ESBL producing *E. coli* in table eggs suggesting to improve hygienic process along the chain of egg production and discourage overuse of antibiotics.

CONCLUSION

We report on high occurrence of ESBL-producing *E. coli* recovered from table eggs carrying additional carbapenemase-encoding genes suggesting indiscriminate use of antibiotics. Hence, strict use of antibiotics and effective antimicrobial resistance surveillance program is highly needed for intervention.

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Statement of conflict of interest

The authors declare no conflict of interest.

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