RESEARCH ARTICLE

Molecular Detection of Antimicrobial Resistant genes with special reference to ESBL in *E. coli* from Diarrheic Piglets

Kushal Grakh, Dinesh Mittal, Anand Prakash*, Pallavi Moudgil, Pankaj Kumar, Ramesh Kumar

Abstract

Antimicrobial resistance (AMR) has been identified as a global threat. *E. coli* is known to carry different AMR genes and responsible for huge economic loss owing to AMR. In the present study 14 previously confirmed *E. coli* isolates associated with piglet diarrhea were analyzed for pathogenicity and hemolysin production traits, molecular detection of AMR genes, screening for extended-spectrum β-lactamases (ESBLs) by phenotypic and molecular methods. These isolates were previously identified as *E. coli* by Vitek 2 Compact system and revealed antimicrobial resistance using the disc diffusion method. After re-confirmation using PCR amplification of *usp*A (universal stress protein) gene, these isolates were characterized for pathogenic traits. AMR genes for penicillins, cephalosporins, tetracyclines, and sulfonamide drugs were probed in 14 isolates. Phenotypic ESBL detection method revealed 5/14 (37.5%) of the isolates as ESBL positive. Molecular methods using ESBL genes found 6/14 (42.8%) isolates as ESBL positive. We also investigated the presence of sulfa and tetracyclines AMR genes in these isolates. Out of 14 isolates, the sul2 gene in five isolates, sul1 gene in seven isolates and tetB gene in two isolates, was detected out of 14 isolates. None of the isolates were found to carry *tetA, tetM,* and *dfrA1* genes. The present study finds swine *E. coli* isolates carrying *blaTEM*, *blaSHV,* and *blaCTX-M* genes along with other antimicrobial resistance genes viz., *sul1*, *sul2,* and *tetB*. Thus, this study suggests further surveillance for the detection of resistance genes among *E. coli* isolates of swine-origin as these genes can be transferred to human *E. coli* isolates.

Keywords: *E. coli*, Antimicrobial-resistant genes, ESBL, Swine, PCR

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Introduction

Escherichia coli (*E. coli*) is a gram-negative, facultatively anaerobic bacterium that is a normal inhabitant of the gastro-intestinal tract of animals and humans. Because of its pathogenic nature, *E. coli* is also responsible for a wide spectrum of diseases in animals and humans. Various genes are responsible for *E. coli* 's pathogenicity and disease potential. *E. coli* possess six universal stress protein (*usp*) genes different environmental stressors control namely A, C, D, E, F and G and the expression of these genes. The *uspA* gene is essential for cellular growth, adhesion, and motility in *E. coli* (Mishra *et al*., 2017). Diarrhea is a primary disease affecting the porcine industry, particularly piglets. Post-weaning diarrhea (PWD) in pigs is a multifactorial disease which involve interaction among infectious agents, sow, piglet, environment and managemental conditions (Rhouma *et al*., 2017). PWD due to *E. coli* is an economically important disease of piglets worldwide. PWD affects piglets during the first 2 weeks after weaning. The disease is characterized by sudden death, diarrhea, dehydration, and growth retardation in surviving piglets (Fairbrother *et al*., 2005). The ETEC (Enterotoxigenic *E. coli*) and to a lesser extent EPEC (Enteropathogenic *E. coli*) are associated with bacterial PWD.

Though antimicrobial usage has contributed to livestock health and production, it has also accelerated the evolution of antimicrobial resistance (AMR) strains of bacteria. The food animals such as poultry and pigs are the major reservoirs for human antimicrobial-resistant microbes (Argudin *et al*., 2017). These food animals spread antimicrobial-resistant *E. coli* to humans because of their proximity with humans and the transport of these animals as they carry resistant bacteria in the gut (Muloi *et al*., 2018). The spread of microbes from food animals to humans and vice-versa is a serious public health concern and responsibility for AMR development. India is the fourth-largest consumer of antibiotics in food animals, mainly poultry, pigs, and cattle, but AMR's burden in livestock and food animals has been poorly documented in India (Gandra *et al*., 2017). A limited number of studies were conducted on food animals related to AMR in India (Gandra

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et al., 2017). Fewer studies pertaining to molecular detection of antimicrobial resistance has been done in India.

Multiple mechanisms responsible for AMR exist in *E. coli* (Nelson *et al*., 2019). The extended-spectrum beta-lactamases (ESBLs) are derived from the amino acid substitutions of their parent enzymes TEM and SHV beta-lactamases. These enzymes have expanded their beta lactamases activity to hydrolyze all penicillins, cephalosporins, and aztreonam (Naas *et al*., 2008). Among these ESBLs, TEM, SHV and CTX-M predominate in gram-negative bacteria like *E. coli* whose production is mediated by *blaTEM, blaSHV* and *blaCTX-M* genes, respectively (Ehlers *et al*., 2009). Gram-negative bacteria including *E. coli* acquire tetracycline resistance by energy-dependent efflux pump system and ribosomal protection. Of 28 efflux pump systems identified so far, *tetA* and *tetB* genes are the most frequently occurring tetracycline resistance determinants. These efflux pumps eject out tetracycline molecules from the cell against a concentration gradient (Nguyen and Sperandio, 2012). Ribosomal protection mechanism involves proteins like TetM, TetO etc are paralogs of translational EF-G, which removes tetracyclines from the ribosomes (Li *et al*., 2013). Sulfonamides inhibit dihydrofolic acid production in bacteria by competitively binding to dihydropteroate synthase (DHPS), a catalytic enzyme needed for folic acid synthesis (Skold, 2000). *Escherichia coli* can acquire resistance to sulfonamides either by mutations in the chromosomal DHPS gene (*folP*) or by the acquisition of an alternative DHPS gene (*sul*), whose product has a lower affinity for sulfonamides (Perreten and Boerlin, 2003). Three sulfonamide resistance DHPS genes (*sul1*, *sul2, and sul3*) in *E. coli* have been described (Arabi *et al*., 2015). Resistance to trimethoprim is caused by modifications of *dfr* gene-encoded enzyme dihydrofolate reductase (Seputiene *et al*., 2010). These are the primary mechanisms of AMR in *E. coli.*

E. coli is known to carry different AMR genes and responsible for huge economic loss owing to AMR. This prompts us to study AMR in *E. coli*. In the present study 14 previously confirmed *E. coli* isolates associated with piglet diarrhea were analyzed for pathogenicity, hemolysin production traits, molecular detection of AMR genes and screening for extended-spectrum β-lactamases (ESBLs) by phenotypic and molecular methods.

Materials and Methods

Collection of Samples and Processing & Isolation and Characterization of Bacteria

These isolates had been recovered from a total of 25 fecal samples/rectal swabs collected from piglets from pig farms from Uttar Pradesh and Haryana, with clinical signs of diarrhea, dehydration, and decreased weight gain. The samples were collected with sterile swabs under aseptic conditions, transported to the laboratory on ice, and streaked on MacConkey Lactose Agar (MLA) for the isolation of members of the Enterobacteriaceae family. The isolated colonies were subjected to Gram's staining. The lactose fermenter and lactose non-fermenter colonies were further sub-cultured to obtain pure colonies. The purified lactose fermenter colonies were then streaked on eosin methylene blue (EMB) agar and lactose non-fermenter colonies on brilliant green agar (BGA) for further characterization. The isolates were confirmed as *E. coli* on the basis of Vitek 2 compact system (Mittal *et al*., 2018).

PCR amplification for *uspA* gene and AMR genes

The genomic DNA for PCR amplification was extracted using heat lysis/snap chill method (Englen and Kelley, 2000) with some modifications. Briefly, freshly cultured loopful of bacterial colonies were suspended in 50 µl of TE buffer and then vortexed to get a uniform suspension. Bacterial cells were lysed by heating at 99°C for 10 minutes and snap chilled at 4°C. Cellular debris were settled by centrifugation at 12,000 rpm for 2 minutes, and the supernatant was used as DNA template for PCR. The quality and concentration of DNA was assessed by A260/280 ratio using a spectrophotometer (Eppendorf BioPhotometer, Germany). The concentration of each reagent in a PCR mixture included 100 ng of DNA template, 2X PCR buffer (Top Taq Qiagen, Netherlands) containing 250 µM of each dNTP, 1 U of *Taq* polymerase, 0.5 µM of each *E. coli* specific *usp*A primers (Mishra *et al*., 2017) with nuclease-free water to make a final volume of 25 µl. The DNA from ATCC 25922 was used as a positive control for *E. coli*. PCR amplifications were done in a thermal cycler (Applied Biosystems, USA) with initial DNA denaturation step at 95 °C for 2 min followed by 30 cycles beginning with 30 secs of denaturation at 94 °C, annealing at 58°C for 1 min and extension at 72 °C for 1-minute. The final extension step was performed at 72 °C for 5 min. The amplified products were separated in 1.5% agarose gel. The gel was visualized by staining with ethidium bromide (0.5 mg/mL). A 1000 bp and 100 bp ladder molecular weight markers (Thermofisher, USA) were used to detect amplified products. The images of ethidium bromide-stained DNA bands were digitized using a gel documentation system (Alpha imager TM 3400, USA).

An antimicrobial sensitivity test has already been done on the isolates by the disc diffusion method (Mittal *et al*., 2018). All 14 isolates were tested for the presence of ESBL and AMR genes using primers described previously. The extracted DNA was used as a template for PCR. Each of the isolates were examined for the presence of ESBL genes viz., *blaTEM* (Sharma *et al*., 2010)*, blaSHV* (Aleisa *et al*., 2013) and *blaCTX-M* genes (Edelstein *et al*., 2003), tetracycline resistance genes *tetA, tetB* (Aminov *et al*., 2002)*, tetM* (Jones *et al*., 2006), sulphonamide resistant genes *sul*1, *sul*2 and trimethoprim resistance gene *dfr*A1 (Arabi *et al*., 2015) by PCR amplification. The PCR amplification was carried out

in a thermal cycler (Applied Biosystems, USA) with initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at a specific temperature as in literature for 30 sec, extension at 72°C for 2 minutes and final extension at 72°C for 5 minutes.

Hemolysis on Sheep Blood Agar

E. coli isolates were subjected to 5% sheep blood agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for assessing the hemolysis ability of the isolates.

Double Disc Synergy Test (DDST) for Phenotypic Detection of ESBL

E. coli isolates were inoculated in Brain Heart Infusion (BHI) broth and incubated at 37°C overnight. The turbidity of the bacterial culture was adjusted to 0.5 McFarland standard, and a lawn of culture was streaked on Mueller Hinton agar (MHA) plates using sterile swabs. The discs containing ceftazidime (30 µg), ceftazidime/clavulanic acid (30 µg/10 µg) were placed 30 mm apart (center to center) from each other for detection of TEM/SHV production. The discs containing cefotaxime (30 µg), cefotaxime/clavulanic acid (30 µg/10 µg) were placed 30 mm apart (center to center) from each other for detection of CTX-M production as described earlier (Samanta *et al*., 2015). A ≥ 5 mm increase in zone diameter for either antibiotic - clavulanic acid combination versus antibiotic alone confirms an ESBL producing *E. coli* isolate (CLSI, 2015). All the antibiotic discs used in the study were procured from HiMedia Laboratories Pvt, Ltd., Mumbai, India.

Results and Discussion

In our study, all the 14 *E. coli* isolates were found positive for the presence of *usp*A gene, thus confirming them as *E. coli* isolates (Fig. 1). None of the isolates were hemolytic on blood agar (Fig. 2). Based on the result of DDST for phenotypic detection of ESBL on MHA plates, 5/14 (35.2 %) isolates were found to be ESBL positive (Fig. 3). We found that three isolates were positive for TEM/SHV and CTX-M and one isolate was positive for TEM/SHV and CTX-M. However, when the isolates were tested with PCR-based amplification of AMR genes (Fig. 4, 5, 6 and Table 1) using specific primers, a total of six out of 14 isolates (42.8%) were found to harbor ESBL genes. Of these six, two isolates had *blaTEM* gene, one each carried

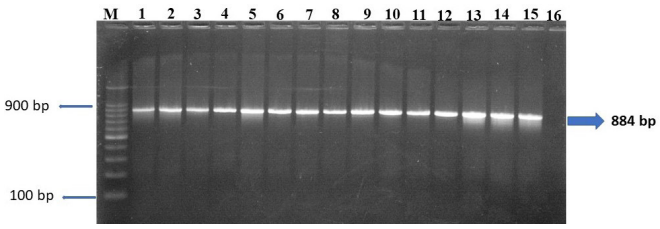

Fig. 1: Identification of *E. coli* using PCR amplification. Lane M: 100 bp DNA ladder; Lane 1 to 14 = *uspA* positive amplicons (884 bp) of *E. coli* isolates; Lane 15: PCR product of Positive Control (ATCC 25922); Lane 16: negative control

Fig. 2: *E. coli* on Blood Agar. Bacteria exhibit no hemolysis on blood agar.

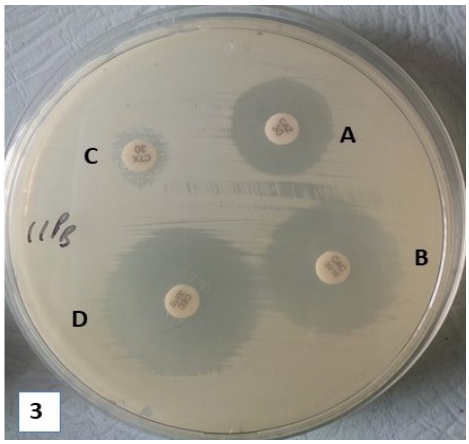

Fig. 3: ESBL DDST Test**.** A: ceftazidime (30 µg); B: ceftazidime/ clavulanic acid (30 µg/10 µg); C: cefotaxime (30 µg); D: cefotaxime/ clavulanic acid (30 µg/10 µg). The zone of inhibition between A and B, C and D is greater than 5mm.

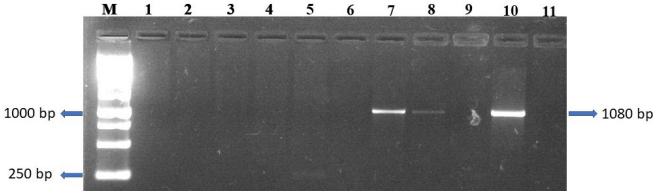

Fig. 4: PCR amplification of representative *E. coli* isolates carrying *blaTEM* gene. Lane M: 1000 bp DNA ladder; Lane 1 to 6 and 9: *blaTEM* negative *E. coli* isolates, Lane 7,8 and 10: *blaTEM* positive amplicons (1080 bp) of *E. coli*; Lane 11: negative control

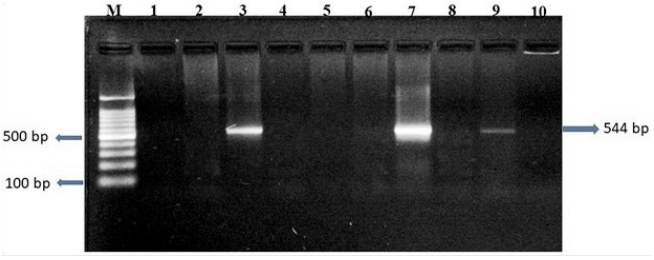

Fig. 5: PCR amplification of representative *E. coli* isolates carrying *blaCTX-M* gene. Lane M: 100 bp DNA ladder; Lane 1 and 2, 4, 5, 6 and 8: *blaCTX-M* negative *E. coli* isolates, Lane 3, 7 and 9 *blaCTX-M* positive amplicons (544 bp) of *E. coli*; Lane 10: negative control

Table 1: Distribution of AMR genes among *E. coli* isolates

Isolate no.	AMR Genes					
	ESBL GENE			OTHERS		
	bla TEM	bla SHV	bla CTX-M	tet B	sul 1	sul 2
2Y	+	-	-	-	-	-
4Y	-	-	-	-	-	-
5P	-	-	-	-	-	-
6P	+	-	-	-	-	+
7P	+	+	+	-	+	+
8P	-	-	+	-	+	+
9P	-	-	-	-	-	-
10P	-	-	-	-	+	+
11PB	-	-	-	+	+	-
12P	+	+	+	-	+	+
13P	-	-	-	+	-	-
14P	-	-	-	-	+	-
16P	-	+	-	-	-	-
21P	-	-	-	-	+	-

Table 2. Comparison of DDST and PCR method for detection of ESBL positive isolates

Isolate	DDST ESBL		PCR ESBL		
	TEM/SHV	CTX-M	blaTEM	blaSHV	blaCTX-M
2Y	+	+	+	-	-
6P	-	-	+	-	-
7P	-	+	+	+	+
8P	-	-	-	-	+
12P	+	+	+	+	+
16P	+	-	-	+	-
11PB	+	+	-	-	-

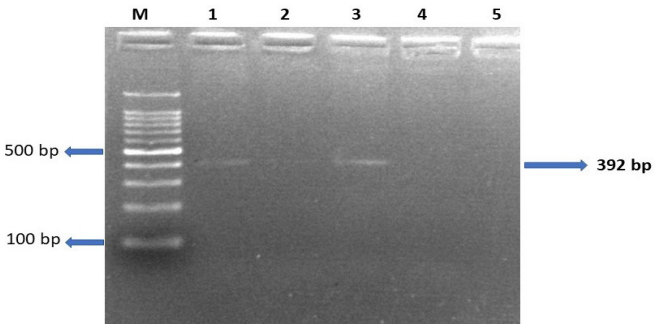

Fig. 6: PCR amplification of *E. coli* isolates carrying *blaSHV* gene. Lane M: 100 bp DNA ladder; Lane 1, 2 and 3: *blaSHV* positive amplicons (392 bp) of *E. coli*; Lane 4: negative control

blaCTX-M and *blaSHV* genes and two isolates had all three ESBL genes. Out of total five isolates confirmed to be ESBL positive by the phenotypic method, only four were found to be ESBL positive by PCR based detection. Phenotypic test however failed to detect two of ESBL confirmed isolates by PCR as positive (Table 2). Two isolates were positive for *tet*B gene (Fig. 7 and Table 1). The *sul2* gene was amplified from

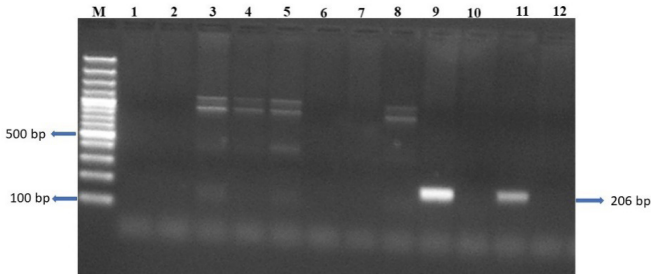

Fig. 7: PCR amplification of *E. coli* isolates carrying *tetB* gene. Lane M: 100 bp DNA ladder; Lane 1 to 8 and 10: *tetB* negative amplicons of *E. coli*; Lane 9 and 11: *tetB* positive amplicons (206 bp) of *E. coli*; Lane 12: negative control

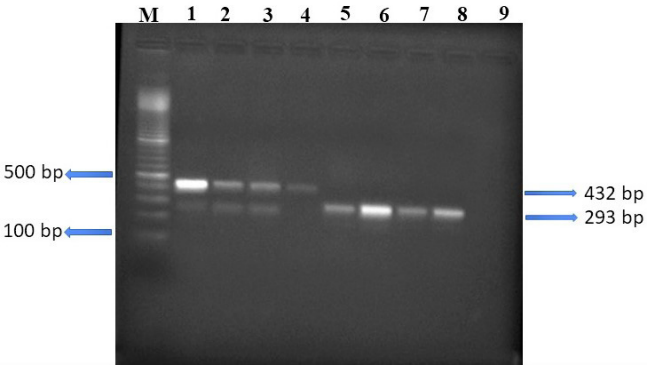

Fig. 8: PCR amplification of representative *E. coli* isolates carrying *sul1* and *sul2* genes**.** Lane M: 100 bp DNA ladder; Lane 1 to 4: *sul1* (432 bp) positive amplicons of *E. coli*; Lane 5 to 8: *sul2* (293 bp) positive amplicons of *E. coli*; Lane 9: negative control

five *E. coli* isolates and *sul1* gene from seven *E. coli* isolates (Fig. 8 and Table 1). None of the isolates were found to harbor *tetA*, *tetM,* and *dfrA1* genes in this study.

We had previously characterized *E. coli* isolates using VITEK 2 Compact system (Mittal *et al*., 2018) and in this study, we used PCR-based *usp*A gene to re-confirm those isolates. We found that both VITEK 2 and PCR amplification using *uspA* gene were equally efficient in identification of *E. coli*. The appearance of hemolytic colonies of *E. coli* in PWD is often used as a rapid tool for the diagnosis of ETEC diarrhea (Luppi *et al*., 2016). Generally, ETEC isolated from cases of PWD are mostly hemolytic. However, non-hemolytic *E. coli* strains can also be observed (Luppi *et al*., 2016). The mechanism by which ETEC causes diarrhea does not rely on the hemolytic nature of *E. coli* isolates but is due to toxin produced by ETEC strains (Francis, 2016). In our study, all the isolates were non-hemolytic on sheep blood agar. Non-hemolytic ETEC have also been previously reported from cases of PWD (Luppi *et al*., 2016). Screening of the isolates for ESBL by a phenotypic method is easy to perform and is a good method to decide on the antibiotics to treat and control the disease. It can be easily performed in labs at the field level. Based on the interpretation of the inhibition zone of isolates on MHA plates with antibiotic discs used, five isolates out of 14 (35.2 %) were found to be ESBL positive. We found that three isolates were positive for TEM/SHV and CTX-M and one isolate was

positive for TEM/SHV and CTX-M. Lower (25.29) % isolates were confirmed as ESBL producers by a similar method by Mandakini *et al*., (2015) from diarrheic piglet samples. Data from the Tigecycline Evaluation and Surveillance Trial (TEST), a global surveillance database, shows that the rate of ESBL production among *E. coli* isolates varies from 7.5 to 44.0 % (Falagas and Karageorgopoulos, 2009). Even though we have fewer samples, still our data lies in range provided by TEST.

PCR amplification to characterize isolates carrying ESBL using specific AMR gene was carried out and we found six isolates (42.8%) to harbor ESBL genes. There are a number of instances by which the phenotypic confirmatory tests for ESBL detection may be falsely positive or negative (Poulou *et al*., 2014). Furthermore, a high prevalence of ESBL-positive *E. coli* isolated from PWD piglets has been reported (Xu *et al*., 2015). ESBL genes are located on the plasmid that can be easily transferred between and within bacterial species. The high prevalence rate of ESBL-positive *E. coli* in diarrhea piglets may be explained by the fact that the third-generation cephalosporins are used more commonly in swine for treatment of neonatal diarrhea and PWD diseases (Xu *et al*., 2015).

The *sul1* gene was identified from seven *E. coli* isolates, and *sul2* gene was found in five *E. coli* isolates, which can be attributed to more use of sulpha drugs at farms for treatment or prophylaxis. The findings of the present study are nearly in consensus with Hammerum *et al*., 2006 in terms of *sul*1 and *sul*2 gene frequency among *E. coli* isolated from Diarrheic piglets. Sulpha drugs in combination with trimethoprim are extensively used in human medicine as standard treatment for UTI infections and to treat diarrhea and other infections in intensive animal husbandry (Soufi *et al*., 2011). Resistance against sulpha drugs in *E. coli* of swine-origin is a serious concern as strains from food-producing animals can contaminate meat products during slaughter and enter the food chain (Delsol *et al*., 2010). Two isolates were *tet*B gene positive. Kallau *et al*. (2018) do not find any of fecal *E. coli* isolate to harbor *tetB* gene, whereas Bryan *et al*., (2004) have reported higher frequency of *tetB* in *E. coli* from pig feces as compared to our study. Tetracyclines due to its broad spectrum and low cost, and side effects is widely used class of antibiotic in human as well as animal husbandry (Berniac *et al*., 2011). Thus, resistance to this drug raises concerns as resistant strains of *E. coli* of animal origin can colonize or cause infections in humans (Manges and Johnson, 2012). None of the isolates were found to harbor *tetA*, *tetM* and *dfrA1* genes in this study. The commensal *E. coli* of food animals can also acquire AMR determinants from pathogenic *E. coli* (Samanta *et al*., 2015). In India, various activities related to animals and meat production and slaughter are unhygienic and inadequately supervised, which provide a portal for transfer of *E. coli* from animals to humans. The transfer of antibiotic resistance to humans by *E. coli* may complicate the treatment in case of infections as similar antibiotics families are in use in human medicine. Higher frequency of ESBL genes, *sul* genes, and possible transmission to the human population warrants further surveillance of resistance patterns of various commonly used antibiotics in animal populations. Thus, the present study finds a higher prevalence of ESBL and other antimicrobial resistance genes among *E. coli* isolates of swine-origin, which poses a serious public health concern. In order to curb the higher AMR prevalence, rationale use of antimicrobials should be adopted by using antimicrobial susceptibility testing. Detailed surveillance for the detection of antimicrobial resistance is needed to establish the prevalence of resistance genes among *E. coli* isolates of swine origin.

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