



Isolation, Pathotyping and Drug Susceptibility Pattern of *Escherichia coli* Isolates from Diarrheic Piglets

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ABSTRACT

Background: Multidrug resistant strains of *Escherichia coli* have been causing worldwide outbreaks of food borne diseases in recent years. The emergence of antibiotic-resistant strains of *E. coli* and the occurrence of transmissible drug resistance among the organism has amplified the complications associated with its control and treatment measures. Data associated with the prevalence, virulence factors and antimicrobial susceptibility of *E. coli* isolates are needed to be studied in order to develop an effective control strategy for colibacillosis in piggeries. The goal of the investigation was to study the antimicrobial susceptibility pattern and pathotyping of *E. coli* isolates from diarrhoeic piglets.

Methods: 102 rectal swab samples were collected from diarrhoeic piglets of ICAR-AICRP/MSP on Pig, College of Veterinary Science, Assam Agricultural University, Khanapara, ICAR-National Research Centre on Pig, Rani, Guwahati, Assam and from different unorganized farms of the state. These samples were then inoculated in Luria Bertani (LB) broth and incubated aerobically at 37°C for 24 hours followed by sub culturing them in MacConkey's lactose agar (MLA) and Eosin Methylene Blue (EMB) agar for purification of the suspected *E. coli* isolates. The isolates were characterized by biochemical tests and motility tests and were pathotyped based on detection of specific virulence genes by multiplex Polymerase Chain Reaction (PCR).

Result: Out of 102 rectal swab samples examined, 92 (90.2%) yielded *E. coli* which included 41 (91.12%) of 45 samples collected from ICAR-AICRP/MSP on Pig, College of Veterinary Science, Assam Agricultural University, Khanapara and 32 (88.89%) of 36 samples from ICAR-National Research Centre on Pig, Rani, Guwahati, Assam and 19 (90.48%) of 21 samples from different unorganized farms. All the 92 *E. coli* isolates were examined for the presence of *stx1*, *est1*, *elt1* and *eaeA* genes using specific primers. Among these isolates, 25 (27.17%) were positive for *stx1* gene, 18(19.56%) for *est1* gene, 6(6.52%) for *elt1* gene, 3 (3.26%) for both genes *est1* and *elt1* and 12 (13.04%) for *eaeA* gene. Antimicrobial susceptibility pattern of the isolates revealed that the highest percentage (71.74%) of the isolates were resistant to tetracycline and the least (4.34%) to imipenem.

Key words: AMR, Antibiotic sensitivity, Diarrheic piglets, *E. coli*, Multi-drug resistance, Multiplex PCR.

INTRODUCTION

E. coli is one of the most important and pervasive pathogen that pose a serious threat to global public health (Ahmed and Shimamoto, 2014). Infections with *E. coli* account for several cases of diarrhoea in animals as well as human worldwide and are frequently encountered in the younger age groups. Colibacillosis is an important disease from zoonotic point of view (Dorn, 1988; Rice *et al.*, 1996).

Many *E. coli* strains have been identified as an important cause of several diseases in pigs worldwide including neonatal diarrhoea, neonatal septicaemia, post-weaning diarrhoea, oedema disease (bowel oedema or gut oedema), cystitis, septicaemia, polyserositis, coliform mastitis, urinary tract infections and can also colonise existing lesions elsewhere in the body. These diseases have a significant economic impact on the pig industry due to their association with high morbidity and mortality, as well as decreased weight gain in pigs (Fairbrother and Gyles, 2012; Taylor, 2013).

The commonest form of neonatal *E. coli* infections in piglets is diarrhoea without bacteraemia or any morphological evidence of enteritis (Smith and Jones, 1963; Moon *et al.*, 1966).

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The broad classes of *E. coli* causing diarrhoea in pigs include shigatoxigenic *E. coli* (STEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and extra-intestinal pathogenic *E. coli* (ExPEC). The major characteristics associated with the pathogenic *E. coli* infections are presence of specific fimbriae and production of enterotoxins, especially by the enterotoxigenic *E. coli* (ETEC) and shiga toxin by shiga toxin-producing *E. coli* (STEC). Other toxins are Enterotoxigenic heat-stable toxin 1 (EAST1), cytotoxins, cytolethal distending toxin, hemolysin; outer membrane proteins (intimin) and adhesin involved in diffuse adherence. In addition, study has shown that F18 was the main colonization factor for STEC and ETEC with F18ab and F18ac as subgroups (Cheng *et al.*, 2005).

In recent years, there has been a serious concern on the multidrug resistant strains of *E. coli* causing multiple food borne disease outbreaks worldwide (Kemper, 2011; Yamasaki *et al.*, 2015). *E. coli* can exchange antibiotic-resistance genes between strains of the same species as well as other bacteria (Li *et al.*, 2020). The emergence of antibiotic-resistant strains leads to failure of treatments which greatly complicates the control of infections (Amarillas *et al.*, 2017). The magnitude of this problem has been further amplified by the occurrence of transmissible drug resistance among *E. coli* (Koh and Kok, 1984; Popovic and Popara, 1987; Raghav *et al.*, 2022). Pathogenic bacteria have evolved and become more diverse as a result of the acquisition of virulence factors through successive horizontal gene transfer (Ogura *et al.*, 2009; Ji *et al.*, 2020; Kumar *et al.*, 2022). Data on the prevalence of virulence factors and the antimicrobial susceptibility of *E. coli* isolates are needed in order to effectively develop control strategies for colibacillosis in piggeries.

MATERIALS AND METHODS

Collection of samples

The research was conducted in the Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati from January 2019 to March 2020. 102 rectal swab samples were collected from diarrhoeic piglets of ICAR-AICRP/MSP on Pig, College of Veterinary Science, Assam Agricultural University, Khanapara, ICAR-National Research Centre on Pig, Rani, Guwahati, Assam and from different unorganized farms of the state and were inoculated in Luria Bertani (LB) broth and incubated aerobically at 37°C for 24 hours. Subsequent subcultures were made in MacConkey's lactose agar (MLA) and Eosin Methylene Blue (EMB) agar for purification of the suspected *E. coli* isolates. The isolates were characterized based on morphology, colony characteristics and biochemical tests like IMViC and motility tests as per the method of Cruickshank *et al.* (1975).

Pathotyping of *E. coli* isolates

The *E. coli* isolates were pathotyped based on detection of specific virulence genes by multiplex Polymerase Chain Reaction (PCR) as per the method described by Rajkhowa

Table 1: Primer sequences.

Primer	Forward	Amplicon size (bp)
<i>est1</i>	F: CATGACGGGAGGTAACATGA R: GCACAGGCAGGATTACAACA	222
<i>elt1</i>	F: CCCTCACCCATATGAACAGG R: TGCTCAGATTCTGGGTCTCC	308
<i>eaeA</i>	F: CAACATGACCGATGACAAGG R: ACGGATATCGAAGCCATTG	535
<i>stx1</i>	F: GCGTGAGGATGTCAAGAAT R: CCTTCGCCACCACATTAAT	127

Table 2: Multiplex PCR cycling conditions.

Steps	Temperature	Time	No of cycles
Initial	95°C	5 min.	1
Denaturation	95°C	30 sec.	
Annealing	55.2°C	45 sec.	35
Extension	72°C	45 sec.	
Final extension	72°C	5 min.	1
Hold	4°C	∞	1

et al. (2015). Template DNA was prepared from each isolate for detection of *stx1*, *elt1*, *est1* and *eaeA* genes by multiplex PCR. Pure colonies from EMB agar plates were enriched in LB broth (2 ml) by incubating aerobically at 37°C for 16-18 hours following which the broth culture was centrifuged at 3000 rpm for 10 minutes. Then the pellet was washed with 250 µl of Tris EDTA buffer by centrifuging at 3000 rpm for 10 minutes and a suspension was made in 100 µl of Nuclease Free Water and heated for 10 minutes followed by immediate snap cooling in ice for 10 minutes. Finally, the contents were centrifuged at 3000 rpm for 10 minutes and 80 µl of the supernatant was collected. The extracted DNA concentration was measured in Nanodrop Spectrophotometer (Thermo fisher scientific, USA) and stored at -20°C for further use.

Amplification of template DNA for detection of virulence genes by multiplex PCR

The detection of virulence genes, viz., *stx1*, *elt1*, *est1* and *eaeA* was done by PCR using standard strains of *E. coli* as positive control, reported primers (Table 1) for all the four genes (20 pmol for each primer) in a single final reaction volume of 25 µl containing 2 X DreamTaq Master mix (0.05 units/of Taq polymerase, 4.0 mM MgCl₂, 10 mM Tris- HCl, 50 mM KCl, 0.4 mM of each dNTPs) and cycling conditions mentioned in Table 2.

The amplicons were separated by electrophoresis using 2% agarose gel containing 0.5 µl/ml ethidium bromide in 1X TAE buffer and the gel was visualized in the gel documentation system and images were captured by ImageLab software.

Antimicrobial susceptibility test

All the *E. coli* isolates were tested for *in-vitro* susceptibility to 28 different antimicrobial agents (HiMedia Laboratories)

Table 3: Isolation of *E. coli* from diarrheic piglets.

Source of sample	No. of samples	No. of positive samples	Percentage of positive samples (%)
ICAR-AICRP/MSP on pig, College of Veterinary Science, Assam Agricultural University, Khanapara	45	41	91.12%
National Research Centre on Pig (ICAR), Rani, Guwahati, Assam	36	32	88.89%
Unorganized pig farms	21	19	90.48%
Total	102	92	90.2%

by disc diffusion technique as per method described by Bauer *et al.* (1966).

RESULTS AND DISCUSSION

Isolation of *E. coli* from rectal swabs of diarrhoeic piglets

In the present study a total of 102 samples of rectal swab from diarrhoeic piglets were examined for isolation of *E. coli* of which, 92 (90.2%) samples yielded *E. coli* showing characteristic morphology (Fig 1 and 2). In respect to source of samples, 41 (91.12%) out of 45 samples were collected from ICAR-AICRP/MSP on Pig, College of Veterinary Science, Assam Agricultural University, Khanapara and 32 (88.89%) out of 36 samples were collected from ICAR-National Research Centre on Pig, Rani, Guwahati, Assam while 19 (90.48%) out of 21 samples were collected from different unorganised farms (Table 3).

Biochemical characterization of *E. coli*

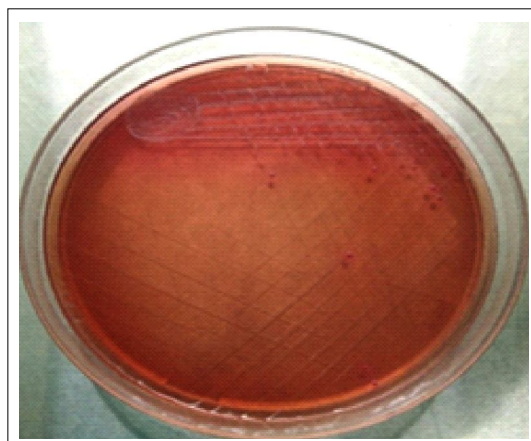
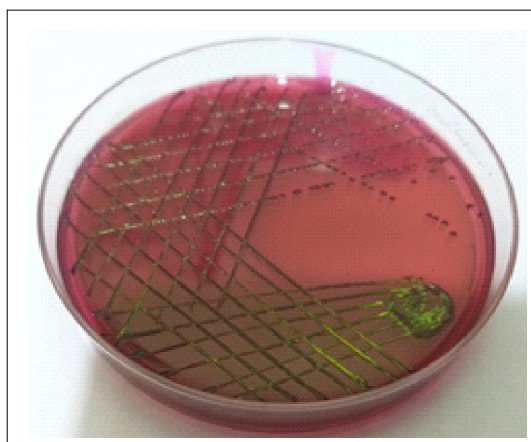
The isolates were subjected to biochemical tests like IMViC tests, catalase test and motility tests (Table 4). 92 isolates were positive for indole and methyl red tests, in which the isolates have produced red/pink colour ring (Fig 3) and red colour respectively (Fig 4). None of the samples were positive for Voges-Proskauer (Fig 5) and citrate utilization tests (Fig 6). Catalase (Fig 7) and motility tests (Fig 8) showed positive reaction for 92 isolates. All the biochemical reactions confirmed the presence of *E. coli*.

Pathotyping of *E. coli* isolates

Nucleic acid amplification by Polymerase Chain Reaction is recognised as one of the most important scientific developments to come along during the last two centuries for detection of genes encoding virulence factors of pathogenic organisms (Rahman, 2002; Moon *et al.*, 2003). Multiplex PCR is a useful adoption in which several PCR primers are combined to detect virulent genes in a single reaction. In the present study, a oligonucleotide primer set was used which encodes the genes typically found in pathotypes of *E. coli* prevalent in piglet diarrhoea viz. Enterotoxigenic or ETEC (*est1*, *elt1*), Shigatoxigenic or STEC (*stx1*) and Enteropathogenic or EPEC (*eaeA*) *E. coli*. Primers were selected on the basis of published nucleotide sequence of the 127 bp for *stx1*, 222 bp for *est1*, 308 bp for *elt1* and 535 bp for *eaeA* genes (Rajkhowa *et al.*, 2015).

Table 4: Results of the biochemical tests for *E. coli* isolates.

Biochemical tests	Number of screened samples	Number of positive samples
Indole test	102	92
Methyl red test	102	92
Voges-Proskauer test	102	0
Citrate utilization test	102	0
Catalase test	102	92
Motility test	102	92

**Fig 1:** Pink colonies of *E. coli* on MLA plate.**Fig 2:** Metallic sheen of *E. coli* on EMB plate.

A total of 92 isolates of *E. coli* were examined for the presence of *stx1*, *est1*, *elt1* and *eaeA* gene by multiplex PCR using standard primers. The study revealed that 25 isolates were positive for *stx1* gene (27.17%), 18 for *est1* gene (19.56%), 6 for *elt1* genes (6.52%), 3 for presence of both genes *est1* and *elt1* (3.26%) and 12 for *eaeA* genes (13.04%), which corroborates with the findings of Bessone *et al.* (2017) and Fratomico *et al.* (2008).

This suggests that the *E. coli* isolates were capable of producing heat stable (ST) and heat labile (LT) toxins. Prevalence of ETEC in *E. coli* isolates were also reported by some workers (García-Meniño *et al.*, 2018, Yang *et al.*, 2019, Li *et al.*, 2020).

stx1 gene encodes for Shiga toxin 1 which is a characteristic cytotoxin released by verocytotoxigenic *E. coli* (VTEC) or Shigatoxigenic *E. coli* (STEC). Shiga toxin-

Table 5: Detection of virulence genes in the *E. coli* samples isolated from diarrheic piglets.

Source of samples	No. of isolates tested	No. of isolates positive for virulence genes					
		Total	<i>stx1</i>	<i>est1</i>	<i>elt1</i>	<i>est1</i> and <i>elt1</i>	<i>eaeA</i>
ICAR-AICRP/MSP	41	33	16	6	4	2	5
ICAR-NRC on pig	32	23	7	10	1	1	4
Unorganized pig farms	19	8	2	2	1	0	3
Total	92	64	25	18	6	3	12

Table 6: Pathotyping of *E. coli* isolates from diarrhoeic piglets.

Virulence genes	Pathotypes of <i>E. coli</i>	Source of samples			Total
		ICAR-AICRP/MSP	ICAR-NRC on pig	Unorganized pig farms	
<i>stx1</i>	STEC	16	7	2	25 (27.17%)
<i>est1</i>	ETEC	6	10	2	18 (19.56%)
<i>elt1</i>		4	1	1	6 (6.52%)
<i>est1</i> and <i>elt1</i>		2	1	0	3 (3.26%)
<i>eaeA</i>	Atypical EPEC	5	4	3	12 (13.04%)



Fig 3: Indole test.

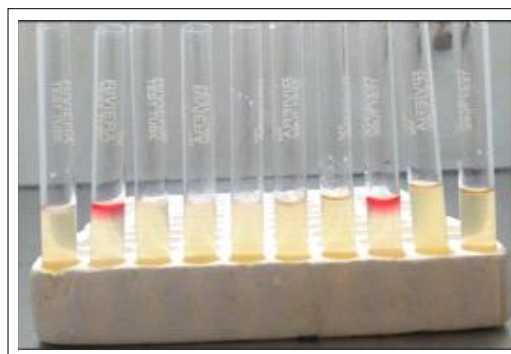


Fig 5: Voges-Proskauer test.

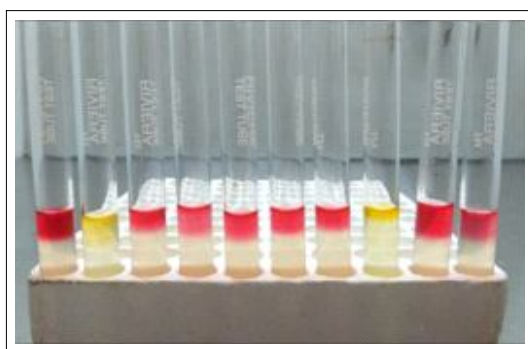


Fig 4: Methyl red test.

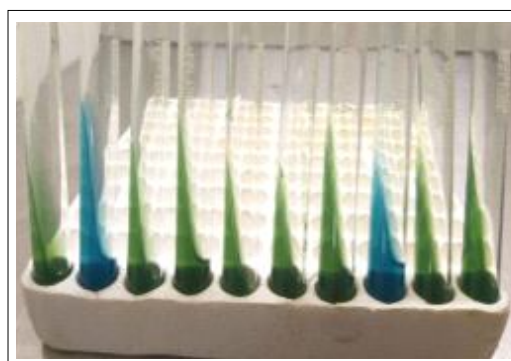


Fig 6: Citrate test.



Fig 7: Catalase test.

producing *E. coli* (STEC) are a group of highly pathogenic foodborne zoonotic pathogens, associated with diarrhoea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. As a matter of fact, STEC have been isolated from pigs and pork products, in some cases associated with episodes of HC and HUS in humans (Baranzoni *et al.*, 2016; Honish *et al.*, 2017). This is an important finding from public health concern as these strains can be cross transferred to human via food chain.

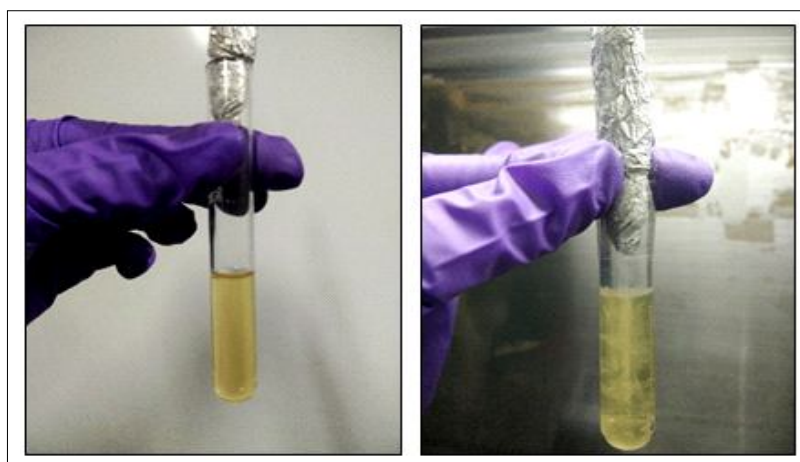


Fig 8: Motility test before and after inoculation of sample.

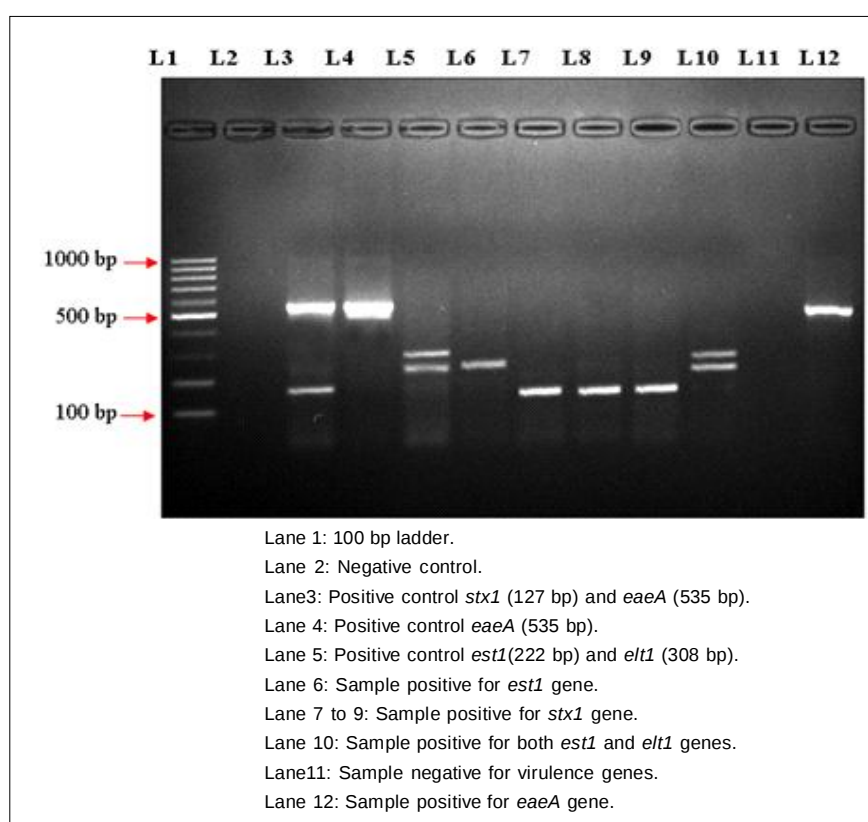


Fig 9: Agarose (2%) gel showing band patterns of *E. coli* isolates in multiplex PCR for detection of virulence genes in *E. coli* from diarrhoeic piglets.

Overall, pathotyping of the *E. coli* isolates by detection of the virulent genes revealed that 25 (27.17%) isolates belonged to STEC, 27(29.34%) to ETEC and 12(13.04%) to EPEC as depicted in Table 5, 6 and Fig 9, 10.

Antimicrobial resistance pattern of the isolates

The widespread use of antibiotics in livestock has led to the emergence of multidrug-resistant (MDR) *E. coli*, posing a threat to animal and human health. In the present study, *E. coli* isolates resistant to more than 3 drugs were considered MDR Sahm *et al.* (2001) and 50% of *E. coli* isolates were found to be MDR, exhibiting resistance to three or more antimicrobials. In all, 46 (50%) of the 92 *E. coli* isolates were found to be MDR. Table 7 and Fig 11 shows that the *E. coli* isolates tested for antimicrobial susceptibility showed maximum resistance towards tetracycline (71.74%), nalidixic acid (70.65%), framycetin (59.78%), cefazolin (54.35%), ofloxacin (51.09%), erythromycin (50%), gatifloxacin (48.91%) and ampicillin (47.83%), while moderate resistance was observed against nitrofurantoin (43.48%), co-trimoxazole (41.30%), cefixime (41.30%), neomycin (41.30%) ciprofloxacin (40.22%) and streptomycin (35.87%). Moderate susceptibility was recorded to piperacillin/ tazobactam (33.69%), cefuroxime (33.69%) cephalothin (32.61%), chloramphenicol (29.35%) and levofloxacin (29.34%), while maximum susceptibility was observed for meropenem (21.74%), norfloxacin (21.74%), ceftazidime (21.74%), cefepime (16.30%), gentamicin (11.95%), ceftazidime (10.87%), amikacin (8.69%), polymyxin- B (6.52%) and imipenem (4.34%). Fig 12-15 depicts the antimicrobial sensitivity and resistance patterns of *E. coli* isolates on Mueller Hinton agar.

High resistance rates were observed for tetracycline (71.74%), nalidixic acid (70.65%), framycetin (59.78%), cefazolin (54.35%) and several other commonly used drugs. Moderate resistance was noted for nitrofurantoin, co-trimoxazole and ciprofloxacin, among others. The frequent use of these antibiotics likely drives resistance development. The occurrence of MDR *E. coli* with resistance to up to 20 antimicrobials suggests plasmid-mediated resistance and highlights the urgent need for prudent antibiotic use in livestock to mitigate the spread of resistance (Chakravarti *et al.*, 1990). Conversely, higher susceptibility to carbapenems (meropenem, imipenem), polymyxin B, and certain cephalosporins indicates limited use of these drugs in veterinary practice, preserving their effectiveness.

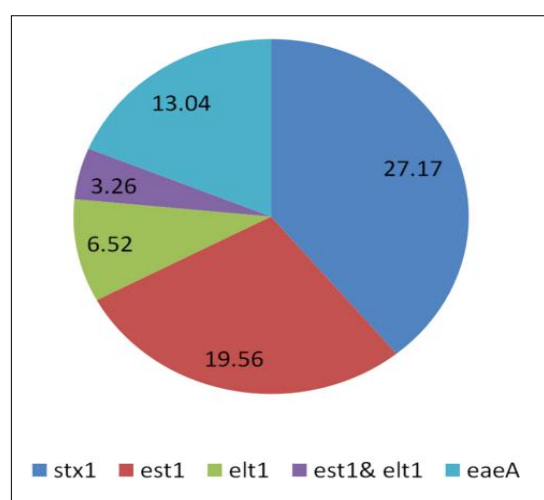


Fig 10: Different pathotypes of *E. coli* isolated from diarrhoeic piglets.

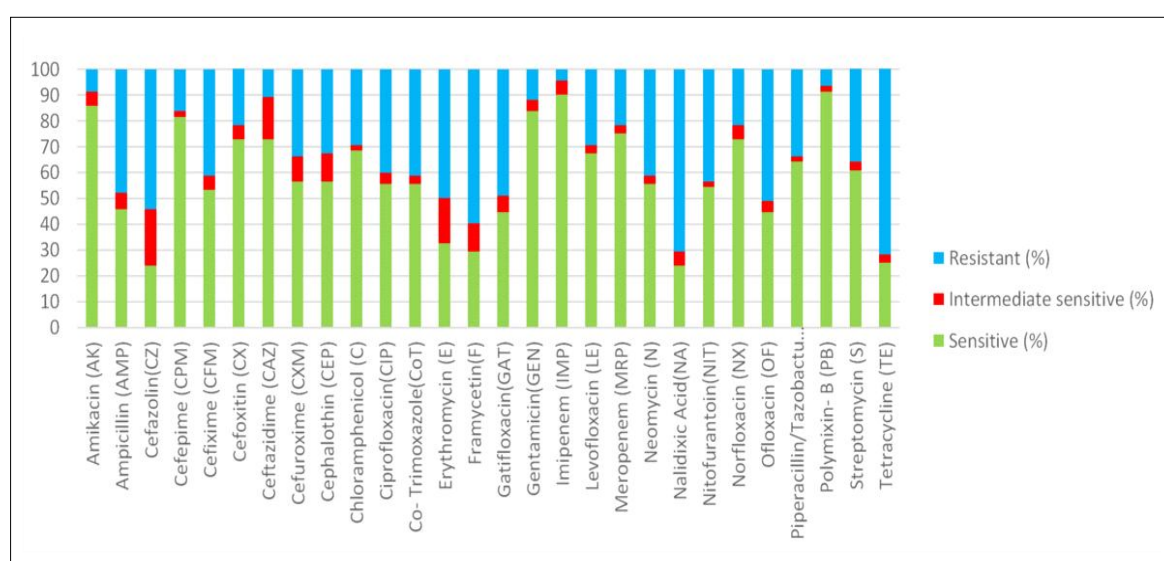
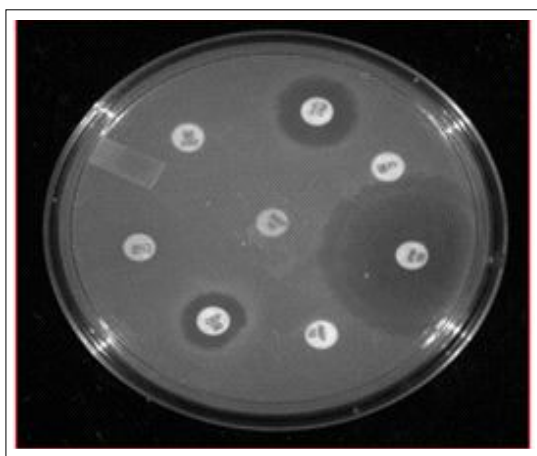
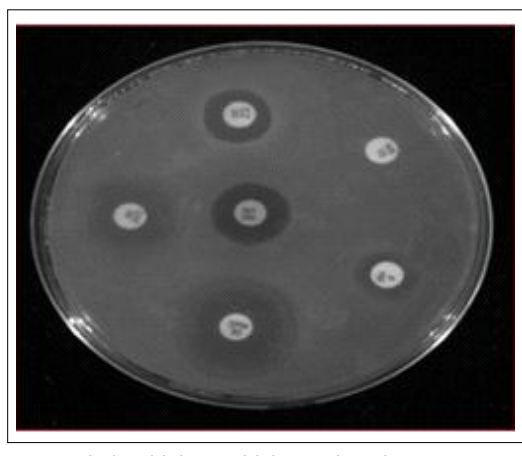


Fig 11: Antimicrobial susceptibility patterns of *E. coli* isolates from different sources.

Table 7: Antimicrobial susceptibility patterns of *E. coli* isolates from different sources.

Antimicrobial agents and disc concentration	Pattern of antibiogram		
	Sensitive (%)	Intermediate sensitive (%)	Resistant (%)
Amikacin (AK) (30 mcg)	79 (85.87%)	5 (5.44%)	8 (8.69%)
Ampicillin (AMP) (10 mcg)	42 (45.65%)	6 (6.52%)	44 (47.83%)
Cefazolin (CZ) (30 mcg)	22 (23.91%)	20 (21.74%)	50 (54.35%)
Cefepime (CPM) (30 mcg)	75 (81.52%)	2 (2.17%)	15 (16.30%)
Cefixime (CFM) (5 mcg)	49 (53.26%)	5 (5.44%)	38 (41.30%)
Cefoxitin (CX) (30 mcg)	67 (72.83%)	5 (5.44%)	20 (21.74%)
Ceftazidime (CAZ) (30 mcg)	67 (72.83%)	15 (16.30%)	10 (10.87%)
Cefuroxime (CXM) (30 mcg)	52 (56.52%)	9 (9.78%)	31 (33.69%)
Cephalothin (CEP) (30 mcg)	52 (56.52%)	10 (10.87%)	30 (32.61%)
Chloramphenicol (C) (30 mcg)	63 (68.48%)	2 (2.17%)	27 (29.35%)
Ciprofloxacin (CIP) (5 mcg)	51 (55.43%)	4 (4.34%)	37 (40.22%)
Co-trimoxazole (CoT) (25 mcg)	51 (55.43%)	3 (3.26%)	38 (41.30%)
Erythromycin (E) (15 mcg)	30 (32.61%)	16 (17.39%)	46 (50%)
Framycetin (F) (100 mcg)	27 (29.35%)	10 (10.87%)	55 (59.78%)
Gatifloxacin (GAT) (5 mcg)	41 (44.56%)	6 (6.52%)	45 (48.91%)
Gentamicin (GEN) (10 mcg)	77 (83.69%)	4 (4.34%)	11 (11.95%)
Imipenem (IMP) (10 mcg)	83 (90.22%)	5 (5.43%)	4 (4.34%)
Levofloxacin (LE) (5 mcg)	62 (67.39%)	3 (3.26%)	27 (29.34%)
Meropenem (MRP) (10 mcg)	69 (75%)	3 (3.26%)	20 (21.74%)
Neomycin (N) (30 mcg)	51 (55.43%)	3 (3.26%)	38 (41.30%)
Nalidixic Acid (NA) (30 mcg)	22 (23.91%)	5 (5.44%)	65 (70.65%)
Nitrofurantoin (NIT) (100 mcg)	50 (54.35%)	2 (2.17%)	40 (43.48%)
Norfloxacin (NX) (10 mcg)	67 (72.83%)	5 (5.44%)	20 (21.74%)
Ofloxacin (OF) (5 mcg)	41 (44.56%)	4 (4.34%)	47 (51.09%)
Piperacillin/Tazobactam (PIT) (30 mcg)	59 (64.13%)	2 (2.17%)	31 (33.69%)
Polymixin- B (PB) (300 mcg)	84 (91.30%)	2 (2.17%)	6 (6.52%)
Streptomycin (S) (300 mcg)	56 (60.87%)	3 (3.36%)	33 (35.87%)
Tetracycline (TE) (30 mcg)	23 (25%)	3 (3.36%)	66 (71.74%)

**Fig 12:** Antimicrobial sensitivity and resistance patterns of *E. coli* isolates on Mueller Hinton agar plate (AMP, C, CEP, COT, IPM, MRP, PIT and TE).**Fig 13:** Antimicrobial sensitivity and resistance patterns of *E. coli* isolates on Mueller Hinton agar plate (CAZ, CFM, CZ, F, NIT and NX).

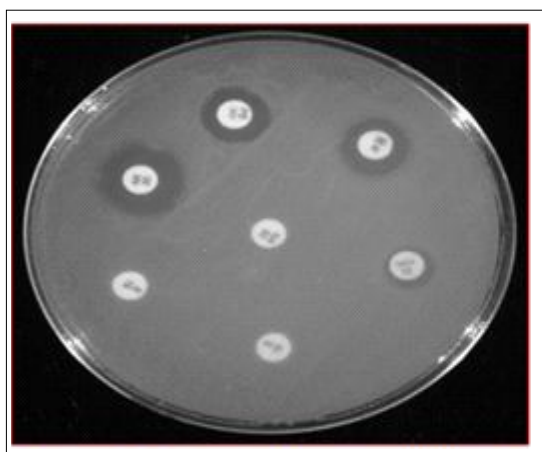


Fig 14: Antimicrobial sensitivity and resistance patterns of *E. coli* isolates on Mueller Hinton agar plate (CIP, CXM, CPM, CX, GAT, LE and OF).

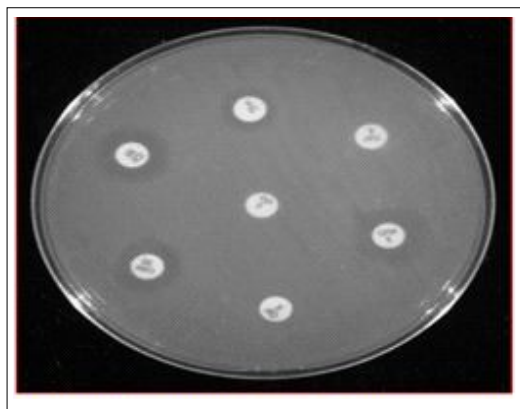


Fig 15: Antimicrobial sensitivity and resistance patterns of *E. coli* isolates on Mueller Hinton agar plate (AK, E, GEN, N, NA, PB and S).

CONCLUSION

Findings of the present study indicated a high prevalence of VTEC/STEC *E. coli* isolates among diarrhoeic piglets which suggested *E. coli* as a leading cause of piglet diarrhoea. Furthermore, high level of resistance against the commonly used antimicrobial agents and presence of ETEC and STEC among the isolates suggested the possibility of cross-transfer of drug-resistant strains from pigs to human through the food chain.

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Conflict of interest

The authors declare no conflict of interests.

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