Antibiogram, Phenotypic and Genotypic Detection of Extended-Spectrum β-lactamase Producing and Fluoroquinolone Resistant *Escherichia coli* Isolated from Pigs

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Author's contribution

The author confirms sole responsibility for this study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

Original Research Article

ABSTRACT

Antimicrobial resistance in food animals is a global issue. Particularly, the dissemination of Extended Spectrum Beta-Lactamase and fluoroquinolone resistant *Escherichia coli* in food-producing animals posed a public health threat to the efficacy of various antibiotics. Therefore, this study aimed to detect the presence of multidrug-resistant *Escherichia coli* from fecal samples of pigs in Puducherry. In total, 16 faecal samples from apparently healthy pigs of different age groups

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were chosen for this study and subjected to detection of ESBL production and FQ resistance. *E. coli* was isolated from all the faecal samples (100%) and confirmed by biochemical tests and Polymerase Chain Reaction. All the *E. coli* isolates were found to be positive for ESBL production and fluoroquinolone resistance by phenotypic method. Out of 16 *E. coli* isolates, 11 (68.75%) isolates were positive for the presence of ESBL-producing genes, in which, 2 harbored *bla* TEM and 9 harboured *bla* SHV genes. In total 13/16 (81.2%) *E. coli* isolates harbored PMQR genes, in which 11 isolates harbored the *qnr*S gene and 11 isolates harbored the *qnr*B gene. The results showed that 9 isolates harbored both *qnr*S and *qnr*B genes in combination. Antimicrobial susceptibility test showed that the majority of the isolates exhibited a high level of resistance against Amoxycillin/clavulanic acid (100%), Cefpodoxime (100%), Cefotaxime (80%), Aztreonam (70%), Ceftazidime (70%), Enrofloxacin (70%) and Ceftriaxone (40%). The present study showed the presence of ESBL-producing and FQ-resistant *E. coli* in swine in the Puducherry region and their antibiogram pattern.

1. INTRODUCTION

"Food animals, including pigs, are the important source for the development of antimicrobial-resistant (AMR) bacteria through the usage of antibiotics at a sub-therapeutic concentration in the form of feed additives and growth promoters in the farms" (Aarestrup FM, 2004). "This leads to the rapid emergence of one of the most important resistance mechanisms extended spectrum β-lactamases (ESBLs), which has a serious impact on the treatment or therapeutic strategy of various infections mainly caused by the enteric bacteria, namely *Escherichia coli, Salmonella* spp., and *Klebsiella pneumonia"* (Hamilton WL and Wenlock R). "The close association between animals and humans and the interaction between commensal and pathogenic bacteria present in the animal gut possibly trigger such infection in human patients" (Karesh WB et al, 2012). "Among the enteric bacteria, *Escherichia coli* is a commensal bacterium in the gut of animals and humans. The pathogenic strains can cause both intestinal and extra-intestinal infections in animals and humans" (Samanta I et al, 2014). "The commensal *E. coli* present in food animals can also acquire antibiotic resistance genes through mobile genetic elements either from the animal itself, where they are maintained under various antibiotic selection pressures, or from the environment" (González-Zorn B and Escudero JA, 2012). "*E. coli* is transmitted to humans through the food chain during slaughter, improper cooking, and unhygienic handling of food" (Frye JG and Jackson CR, 2013). "The rapid emergence of extended-spectrum β-lactamases in livestock and companion animals has been recorded and published worldwide" (Tseng CH et al., 2023).

"The primary cause of resistance to β-lactam antibiotics is the production of β-lactamases. Extended-spectrum β-lactamases are a group of β-lactamase enzymes produced by Gram-negative bacteria, which can hydrolyze all cephalosporins and aztreonam but their activity is inhibited by clavulanic acid" (Puii L et al, 2019). "Another group of important drugs were quinolones are broad-spectrum antibiotics and widely used in clinical practice. It plays an important role in the treatment of coliform infections and is also used in the treatment and prevention of diseases in pigs worldwide" (Michael JP and Jason AW, 2014, Tian G et al, 2009). Due to overuse, resistance to quinolone antibiotics is increasingly common and spreading in some developing countries (Hart CA and Kariuki S, 1998).

"In food animal production, pig farming is a significant component of the livestock sector worldwide and pork is considered a major traded commodity globally. As per 20th livestock, the total pig population in the country is 9.06 million which accounts for 1.7% of the total livestock population. There is a growing market demand for pork products in India, driven by changing dietary preferences, urbanization, and a rising middle-class income. Although there are sporadic reports on AMR enteric bacteria of animal origin from different parts of India available, so far, very little is known about the AMR bacteria in pigs of India" (Mandakini R et al, 2019). There are not many reports on antimicrobial-resistant *E. coli* in pigs that have been carried out in India. Therefore, this study aimed to determine the antimicrobial resistance pattern of ESBL-producing and fluoroquinolone resistance *E. coli* isolated from fecal samples of pigs in Puducherry.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Samples

Pigs of different age groups were reared under swill feeding to minimize the input costs were chosen for this study and a total of 16 fecal samples from pigs were collected, irrespective of their sex, age, or breed from two unorganized pig farms in Puducherry. The collected samples were processed at the Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry.

2.2 Isolation and Identification of *E. coli*

Each fecal sample was individually inoculated into sterile test tubes containing Luria broth and then incubated for 18 hours at 37°C. A loopful of culture was streaked onto MacConkey's agar plate and incubated at 37°C for 24 hours. The pink lactose-fermenting colonies were chosen for Gram staining. The gram-negative bacteria were identified as *E. coli* based on their morphology, cultural characteristics on MacConkey's agar and EMB agar, and biochemical reactions as per Bergey's Manual of Systematic Bacteriology (1984). The isolates biochemically characterized as *E. coli* were further confirmed by PCR, followed by detection of antimicrobial resistance.

2.3 Detection of *E. coli* using Polymerase Chain Reaction

Preparation of template DNA from *E. coli* strains was carried out as described by Kim et al., (2012) and Zhang et al., (2014) with minor modifications. PCR was performed for genotypic confirmation of *E. coli* by utilizing primers targeting the *alr* gene (Yokoigawa K et al, 1999). PCR amplification was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany), as per PCR conditions described by Yokoigawa et al. (1999). The resulting PCR products were analysed through 1.5% agarose gel electrophoresis under a UV transilluminator.

2.4 Antibiotic Susceptibility Testing

Antimicrobial susceptibility test was done on the Mueller-Hinton agar (HiMedia, Mumbai) plate as per the recommendation of the Clinical Laboratory Standard Institute (2019) using the commercially available antimicrobial discs (HiMedia) includes Amoxycillin/clavulanic acid (30μg), Cefpodoxime (10μg), Cefotaxime (30μg), Aztreonam (30μg), Ceftazidime (30μg), Enrofloxacin (30μg), and Ceftriaxone (30μg).

2.5 Phenotypic Identification of ESBL Production and Fluoro-Quinolone Resistance

"All the *E. coli* isolates were screened for resistance against five indicator antimicrobial agents: Cefotaxime (30μg), ceftazidime (30μg), cefpodoxime (10μg), ceftriaxone (30μg), and aztreonam (30μg). Resistance to at least one indicator antibiotic was considered a "positive" screening test for ESBL production. The isolates positive by phenotypic screening were further confirmed by the combination method by using cefotaxime (30μg), cefotaxime plus clavulanic acid (30/10μg) discs (Hi-Media) on Mueller–Hinton agar plates by Kirby-Bauer disc diffusion method following CLSI guidelines" (Clinical and Laboratory Standards institute 2019).

Table 1. Primers details used for PCR in this study and their amplicon size

Target	Primer sequence (5'-3')	Annealing Temperature	Size (bp)	Reference
alr gene	CTGGAAGAGGCTAGCCTGGACGAG AAAATCGGCACCGGTGGAGCGATC	72°C	366	(Yokoigawa et al., 1999)
bla TEM	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	56°C	867	(Rasheed JK et al, 1997)
bla SHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	56°C	393	(Colom K et al, 2003)
bla CTX-M	CAATGTGCAGCACCAAGTAA CGCGATATCGTTGGTGGTG	59°C	540	(Dutta TK et al, 2003)
*qnr*S	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	52°C	428	(Cattoir V et al, 2017)
*qnr*B	GGMATHGAAATTCGCCACTG TTTGCYGYYCGCCAGTCGAA	50°C	264	(Cattoir V et al, 2017)

The isolate was confirmed as an ESBL producer when the inhibition zone diameter around the combination disc was ≥ 5 mm when compared to a disc containing cephalosporin alone" (Clinical and Laboratory Standards institute 2019). Similarly, phenotypic confirmation of fluoroquinolone resistance (FQ) was carried out.

The samples were streaked onto Mac Conkey agar supplemented with ciprofloxacin antibiotic (1mg/L) as per Bhardwaj et al. (2021).

2.6 Genotypic Detection of AMR Genes

The isolates were further subjected to PCR for the detection of both ESBLs and fluoroquinolone-resistant genes and analysed.

3. RESULTS AND DISCUSSION

In this present study, *E. coli* was isolated from all 16 fecal samples of swine (100%) and identified phenotypically using cultural and biochemical characterization followed by genotypic confirmation by PCR using the primer targeting the *alr* gene specific for *Escherichia coli* (366 bp) (Fig. 3). All the *E. coli* isolates showed characteristic pink colored colonies in MacConkey agar (Fig.1), metallic sheen in EMB agar (Fig.2), gram-negative bacilli on microscopic examination, and biochemically they were catalase positive, oxidase negative, Indole test positive, Methyl red test positive, Voges Proskauer test negative, Citrate utilization test negative and urease test negative. The study on *E. coli* isolation from healthy pigs of organized farms showed higher occurrence (97%) in the Netherlands (Nijsten R et al, 1996) and varying prevalence rate (49%) in Portugal (Ramos S et al, 2013) and 37% in Germany (Von Salviati et al, 2014). In north-eastern India, 100% occurrence of *E. coli* in healthy and diarrhoeic pigs were reported (Rajkhowa et al, 2014). A lower occurrence (2 - 3%) was observed in rectal swabs of healthy pigs in Hong Kong (Duan RS et al, 2006) and Japan (Hiroi M et al, 2012).


Fig. 1. The lactose fermenting colonies of *E. coli* on MacConkey agar

Fig. 2. The characteristic metallic sheen appearance of *E. coli* on EMB agar
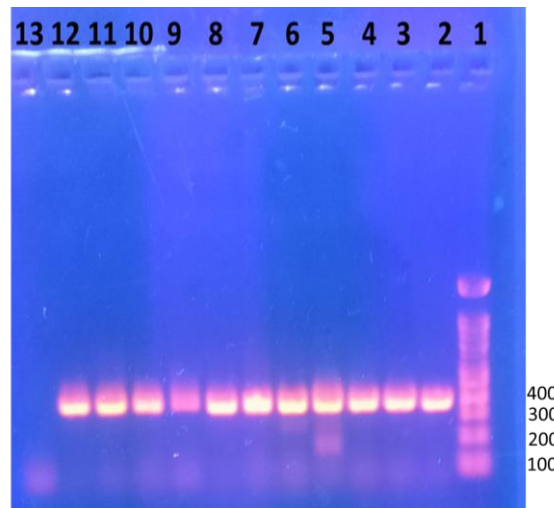
Fig. 3. Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 366 bp for the *alr* gene in *E. coli* isolates. Lane 1: 100 bp ladder; Lane 2 to 11: Field isolates positive for *alr* gene in *E. coli*; Lane 12: Positive control; Lane 13: Negative control
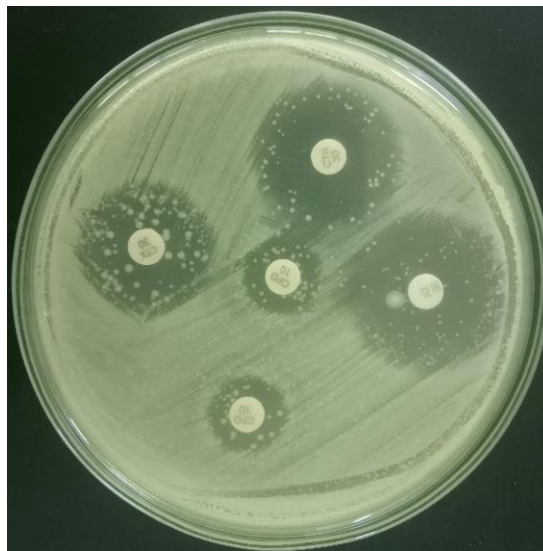

Fig. 4. Phenotypic detection of *E. coli* isolates by indicator antibiotic discs

In this study, all the *E. coli* isolates were detected to be ESBL producers by the indicator discs method (Fig.4) and further confirmed by the combination disc method. Previous studies have reported the varying occurrence rate of ESBL-producing *E. coli* (6 to 70%) in India (Lalzampuia H et al, 2013, Tamta S et al, 2020, Mandakini R et al, 2020, Lalruatdiki A et al, 2018).

In the present study, out of 16 isolates, 11 (68.75%) isolates were found positive for ESBL-producing genes by PCR, in which 2 (18.18%) isolates harbored *bla* TEM (Fig. 6) and 9 (81.81%) isolates harbored *bla*

SHV gene (Fig. 5). Two isolates harbored both *bla* TEM and *bla* SHV. None of the isolates were positive for *bla* CTX-M gene in this study. However, previous reports from other parts of the world indicated a comparatively higher prevalence of *bla* CTX-M among *E. coli* isolates from healthy pigs (15%) in Switzerland (Geser N et al, 2012), 2-10% in China (Tian G et al, 2009) and 21% from South Korea (Tamang MD et al, 2013). Previous studies reported that the *bla* SHV gene (6.25%) alone was detected in *E. coli* from healthy pigs (Bardoň J et al, 2013) and a lower prevalence (5.7%) of *bla* SHV was observed in organized pigs earlier (Ramos S et al, 2013).
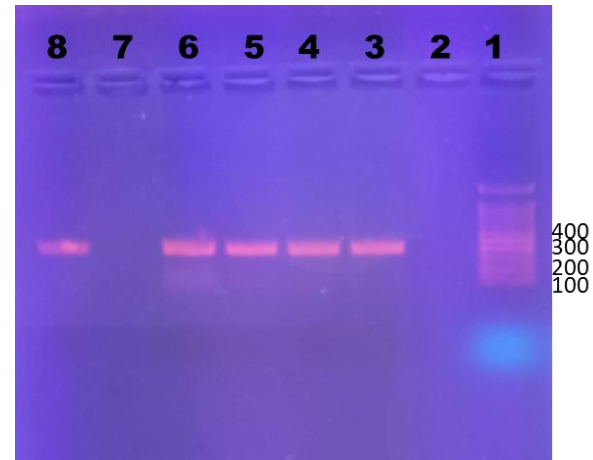
Fig. 5. Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 393 bp for the *bla* SHV gene in *E. coli* isolates. Lane 1: 100 bp ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4,5,6,7,8,9: Field isolates for *bla* SHV gene in *E. coli*;
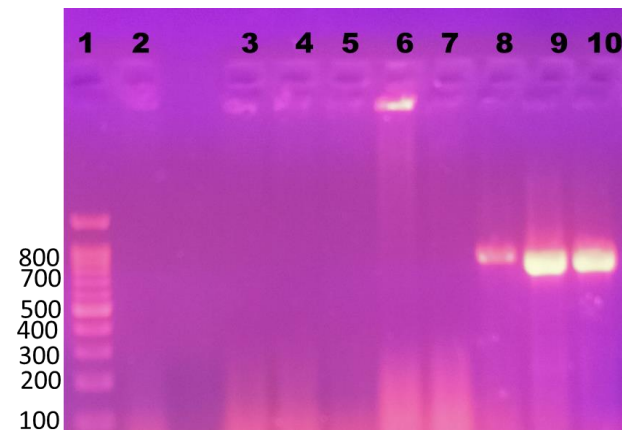
Fig. 6. Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 867 bp for the *bla* TEM gene in *E. coli* isolates. Lane 1: 100 bp ladder; Lane 3,4,5,6: Field isolates for *bla* TEM gene in *E. coli*; Lane 8: Positive control; Lane 2: Negative control
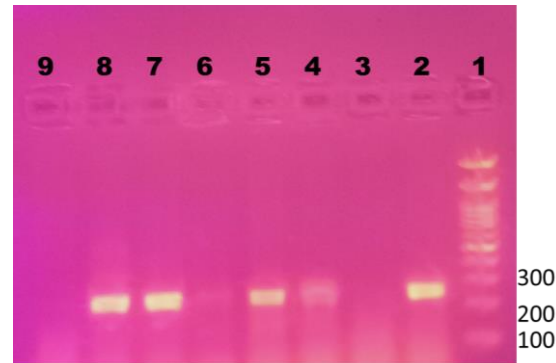
Fig. 7. Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 264 bp for the *qnr*B gene in *E. coli* isolates. Lane 1: 100 bp ladder; Lane 2&3,4,5: Field isolates for *qnr*B gene in *E. coli*; Lane 6: Positive control; Lane 7: Negative control (Seo K et al, 2022)
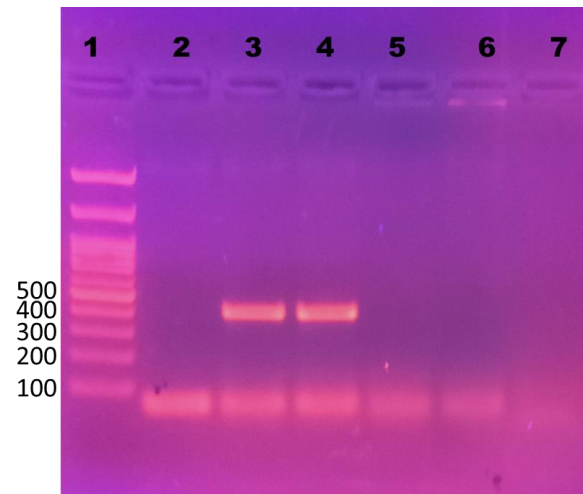

Fig. 8. Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 428 bp for the *qnr*S gene in *E. coli* isolates. Lane 1: 100 bp ladder; Lane 4,5,6,7: Field isolates for *qnr*S gene in *E. coli*; Lane 2: Negative control; Lane 3: Positive control

In this study about 13 (81.2%) samples were phenotypically positive for fluoroquinolone resistance. In total 13/16 (81.2%) *E. coli* isolates harbored FQ-resistant genes, in which 11 isolates harbored the *qnr*B gene (Fig. 7) and 11 isolates harbored the *qnr*S gene (Fig. 8). The results showed that 9 isolates harbored both *qnr*S and *qnr*B genes in combination. The resistance rate against fluoroquinolones was in the range of 74.4–82.6%, which is consistent with previous studies (Gao L et al, 2015, Zhu S et al, 2011, Xiao YH et al, 2011). The presence of both ESBL genes (*bla* CTX-M, *bla* TEM, and *bla* SHV) and fluoroquinolone resistance genes (qnrS and qnrB) among quails have been reported in Puducherry (Nehru BS and OP, 2024). Differences in geographical location, sampling pattern, and other factors could have influenced the occurrence rate in the present study.

In this study, antimicrobial susceptibility test showed that the majority of the isolates exhibited a high level of resistance against Amoxycillin/clavulanic acid (100%), Cefpodoxime (100%), Cefotaxime (80%) followed by Aztreonam (70%), Ceftazidime (70%), Enrofloxacin (70%) and least resistant to Ceftriaxone (40%). In the Dutch samples, a high prevalence of resistance was observed in *E. coli* for three commonly used antibiotics in pig medicine, amoxycillin (70-94%), oxytetracycline (78-98%), and trimethoprim (62-96%) (Van Den Bogaard, A. E. et al, 2000). Sasirekha (Sasirekha B and Shivakumar S et al, 2011) reported a similar resistance rate to Cefotaxime (84%), Ceftazidime (85%), and Ceftriaxone (75%) which is a higher resistant result when compared with the present study, among the *E. coli* isolates (Bangalore). All FQ-resistant *E. coli* isolates of suckling piglets showed MDR with high levels of resistance to aminoglycosides (100.0%), penicillins (100.0%), tetracyclines (97.7%), and phenicols (90.7%) (Seo K et al, 2022). *E. coli* isolates from unorganized farms of pigs in Mizoram showed maximum resistance recorded against cefalexin (73.91%) and sulfafurazole/sulfisoxazole (73.91%), followed by ampicillin (72.46%), and trimethoprim (64.49%) (Mandakini R et al, 2020). Similarly, phenotypic and genotypic detection of extended-spectrum beta-lactamase Producing and Fluoroquinolone Resistant Escherichia coli Isolated from Pigs.

4. CONCLUSION

The present study revealed the presence of *E. coli* carrying ESBL and fluoroquinolone-resistant genes in swine in the Puducherry region. The study indicates that the feces of swine can be a source of AMR and could spread in contact with humans and the external environment, resulting in a potential threat to public health and the environment.

5. RECOMMENDATIONS BASED ON STUDY

1. The findings of this study underscore need for further AMR surveillance in pigs and other food producing animals to monitor the prevalence of ESBL and FQ *E. coli*.
2. Public health awareness about the risk of AMR through food chain and promote safe food handling.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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