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Prevalence of Extended-spectrum Beta-lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae* among Pigs and Pig Handlers

Asiya Mushtaq[1], Tejinder Singh Rai[1], Anil Kumar Arora[1]
Mudit Chandra[1], Jasbir Singh Bedi[2], Jaswinder Singh[3]

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ABSTRACT

Background: The extensive use of beta-lactam antibiotics in food animals and humans has aggravated the issue of antimicrobial resistance (AMR) threatening global health. The rapid spread of extended-spectrum beta-lactamase (ESBLs) producing resistant bacteria mainly in Enterobacteriaceae has raised alarm worldwide.

Methods: In the present study, we investigated the prevalence, antimicrobial resistance pattern and genotypic detection of AMR genes of ESBL-positive *Escherichia coli* (E-EC) and *Klebsiella pneumoniae* (E-KP) isolated from pigs and animal handlers. Overall, 140 faecal samples from pigs and 33 hand swabs from pig handlers were screened for ESBL production. Among these samples, 70 (65.42%) E-EC strains were identified from pigs whereas only one E-KP and E-EC from pigs and handlers, respectively.

Result: The present study revealed a high level of antibiotic resistance dominated by ceftazidime (81.94%) and cotrimoxazole (80.55%). The presence of multidrug resistance (MDR) was observed in 70 ESBL isolates and the multiple antibiotic resistance (MAR) index of all the isolates was more than 0.21. The genotypic detection of ESBL genes by PCR revealed the dominance of the *bla*TEM gene (98.59%) in ESBL-*E. coli* isolates. On analyzing the genotypic results of various tetracycline (*tet*) and quinolone (*qnr*) resistance antibiotic genes, *tet*-A accounted for a significant proportion of 95.77% while *qnr*-S was detected at a proportion of 21.12%. The findings in our study highlight that the pig farms can act as potential carriers of ESBLs and multidrug-resistant *E. coli* which eventually culminates in a higher concentration of AMR genes in the ecosystem.

Key words: Antimicrobial resistance, ESBL, MAR, MDR, PCR, Pigs.

INTRODUCTION

An alarming surge in antimicrobial resistance owing to the advent of extended-spectrum beta-lactamase (ESBL) enzymes has become a major health issue worldwide (Himani *et al*., 2025; Lade *et al*., 2025; Maslikowska *et al*., 2016). ESBLs can inactivate oxyimino -lactam antibiotics like third-generation cephalosporins and aztreonam (Liu *et al*., 2015). The location of the ESBL genes is generally in the chromosomes and plasmids of the members of the Enterobacteriaceae family. Their presence has led to resistance in Enterobacteriaceae members, especially *E. coli* and *Klebsiella* species (Paterson and Bonomo, 2005).

In pig farms, there are frequent cases of neonatal infections and piglet diarrhoea, that are treated with antibiotics causing the emergence of resistant strains of bacteria (Hampson, 1994). A result of this irrational antibiotic use in animal farms to improve productivity gives ample opportunities to bacteria and their genetic determinants to reach the environment through animal faeces (Boerlin *et al*., 2001). Animal handlers working in farms and slaughterhouses are at high risk of acquiring infections with resistant strains of bacteria through daily contact with infected animals (Lerminiaux and Cameron, 2019).

In this article, we report on the antimicrobial resistance profiles of multidrug-resistant ESBL-producing *E. coli* (E-EC) and ESBL-producing *K. pneumoniae* (E-KP) isolated from pig faecal samples and hand swabs collected from the farm workers.

[1]Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.
[2]Centre for One Health, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.
[3]Department of Veterinary and Animal Husbandry Extension, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.

Corresponding Author: Asiya Mushtaq, Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India. Email: drasiya.mushtaq29@gmail.com
ORCIDs: 0000-0001-8620-6509, 0000-0002-4918-4977, 0000-0002-2175-2960, 0000-0003-1513-2340, 0000-0003-3132-7471, 0000-0002-6001-2164

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MATERIALS AND METHODS

Place of work

The present study was conducted at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India in the year 2022.

Sample collection

In the present study, samples were collected from 7 pig farms in three districts of Punjab, India. In each farm, faecal samples from pigs (number depending upon herd size) and hand swabs of pig handlers were obtained. We collected 140 faecal samples from pigs and 33 hand swabs from the handlers.

Bacterial Identification by MALDI-TOF

The faecal and hand swabs were directly streaked on Brain Heart Infusion agar (BHI) (Himedia, India) for primary isolation. After overnight incubation at 37 c for 24h, bacteria grown on the plates were subjected to MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) analysis using MALDI Biotyper Sirius system.

Phenotypic ESBL Identification

To check the ESBL production from *E. coli* and *K. pneumoniae* isolates, HiCrome ESBL agar (Himedia, India) was used for presumptive screening. The appearance of pink/ purple color was indicative of ESBL production for *E. coli* isolates and blue color for *Klebsiella* species.

The phenotypic confirmation of ESBL production was done using the double disc-synergy test (DDST) and the method described by Andrade *et al*., (2019) was followed. The formation of 'keyhole' in the direction of clavulanate disc were considered as positive for ESBL production.

Antimicrobial susceptibility pattern of ESBL isolates

The antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method as per CLSI guidelines 2020 (Bauer *et al*., 1966; CLSI, 2020) and the data obtained after an overnight incubation of the MHA plates was classified as susceptible, intermediate or resistant for each ESBL isolate.

Determination of multidrug resistance (MDR) and multiple antibiotic resistance (MAR) index

MDR was taken when an isolate was found resistant to more than three classes of antibiotics tested (Magiorakos *et al*., 2012). MAR index was calculated for each ESBL isolate by noting the number of antibiotics to which each isolate was resistant and dividing it by the total number of antibiotics used during the study (Krumperman, 1983).

Detection of antimicrobial resistance genes (AMR) by PCR

The DNA was extracted from E-EC and E-KP isolates using the Hot-Cold Lysis method screened for various AMR genes using PCR. The PCR reaction mixtures (10 µl) comprised of 5 µl of 1X Go Taq® Green Master Mix (Promega, USA), 1 µl of forward and reverse primers (0.5 µl of each), 2 µl of template DNA and 2 µl of nuclease-free water. The oligonucleotide primer sequences were synthesized by BioServe Biotechnologies (India) Pvt. Ltd. The amplified PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel containing 0.5g/ml of ethidium bromide for separation. DNA bands were observed and captured in a gel documentation system (Syngene, USA).

RESULTS AND DISCUSSION

Detection of ESBL-producing *E. coli* and *K. pneumoniae* from pigs and animal handlers

Out of 140 bacterial isolates subjected to MALDI-TOF analysis, 107 (76.42%) *E. coli* and 4 (3%) *K. pneumoniae* were identified. A large proportion of 70 (65.42%) of the *E. coli* isolates were confirmed as ESBL producers based on DDST and only one *K. pneumoniae* isolate was found to be an ESBL producer (Fig 1; Fig 2; Fig 3). Out of thirty-three hand swabs collected from animal handlers, 4 (12.12%) *E. coli* were identified using MALDI-TOF. A single *E. coli* isolate was confirmed as an ESBL producer based on DDST.

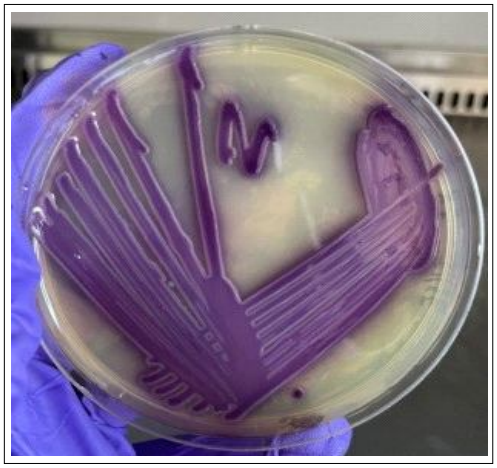

Fig 1: ESBL producing *E. coli* isolate on HiCrome ESBL agar.

Fig 2: ESBL producing *Klebsiella pneumoniae* isolate on HiCrome ESBL agar.

Antibiotic susceptibility test of ESBL isolates

The resistance profile of ESBL producing isolates (n=72) to different antibiotics was examined and converted into percentages (Table 1 and Fig 4). The majority of the ESBL isolates (70-82%) were resistant to ceftazidime, cotrimoxazole and nitrofurantoin. A considerable proportion (50-70%) of the isolates were resistant to ampicillin, cefepime, cefotaxime, aztreonam, piperacillin and tetracycline. High proportions (70-88%) of the isolates were sensitive to imipenem, ertapenem and cefoxitin. Nearly half the proportion (50-60%) of the isolates were sensitive to amikacin, gentamicin, cefuroxime and ciprofloxacin.

MDR

A total of 68 E-EC isolates recovered from pigs were labelled multidrug resistant with a maximum (24) number of isolates resistant to 4 classes of antimicrobials (Fig 5). The sole E-KP and E-EC isolated from a pig and an animal handler, respectively showed MDR to 6 classes of antimicrobial drugs.

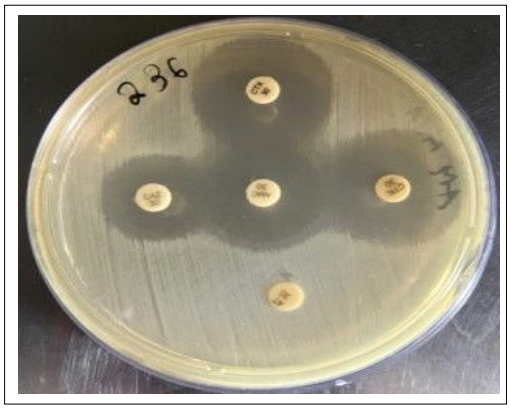

Fig 3: Double-Disc Synergy Test on MHA.

MAR index

All the ESBL-producing isolates had a MAR index greater than 0.2 (Fig 6).

Molecular detection of AMR genes in ESBL isolates

PCR analysis comprised of three different classes of resistance genes like ESBL gene determinants (*bla*CMY-2, *bla*CTXM, *bla*CTXM-1, *bla*CTXM-3, *bla*CTXM-15, *bla*CTXM-25, *bla*SHV and *bla*TEM), quinolones (*qnr-A*, *qnr*-B and *qnr*-S) and tetracyclines (*tet*-A, *tet*-B and *tet*-C). Among all the ESBL genes investigated in this study, *bla*TEM was the predominant (98.59%) gene fragment detected in E-EC isolates. The sole E-KP isolated from a pig did not harbor this gene. The *bla*CTXM and *bla*CTXM-3 genes were detected at proportions of 28.16% and 21.12%, respectively among the E-EC isolates while the E-KP isolate harboured only the *bla*CTXM gene. The *bla*CTXM-1 and *bla*CTXM-15 genes were detected at meagre proportions of 14.08% and 9.85%, respectively among E-EC isolates. None of the isolates harboured other ESBL gene determinants examined in this study including *bla*CMY-2, *bla*CTXM-25 and *bla*SHV. The *qnr*-S gene was detected in 21.12% of the E-EC isolates among the quinolone genes targeted in this study. In contrast, *qnr*-B and *qnr*-A were least detected at proportions of 7.04% and 4.22%, respectively. Among all the tetracycline gene determinants studied, the *tet*-A gene was dominant (95.77%) in the E-EC isolates followed by *tet*-C (35.21%) and *tet*-B gene (21.12%).

Table 1: AST pattern of ESBL producing isolates (n=72) from pigs and an animal handler.

Antibiotic class	Antibiotic	No. of isolates (%)		
		Sensitive	Intermediate	Resistant
Aminoglycosides	Amikacin	41(56.94)	08 (11.11)	23 (31.94)
	Gentamicin	41(56.94)	07 (09.72)	24 (33.33)
Beta-lactams	Amoxyclav	50(69.44)	02(02.77)	20(27.77)
	Ampicillin	21(29.16)	08(11.11)	43(59.72)
	Ampicillin/sulbactum (A/S)	47(65.27)	09(12.50)	16(22.22)
	Cefazolin	29(40.27)	08(11.11)	35(48.61)
	Cefepime	17(23.61)	09(12.50)	46(63.88)
	Cefotaxime	18(25.00)	09(12.50)	45(62.50)
	Cefoxitin	52(72.22)	05(06.94)	15(20.83)
	Ceftazidime	08(11.11)	05(06.94)	59(81.94)
	Ceftriaoxone	30(41.66)	07(09.72)	35(48.61)
	Cefuroxime	37(51.38)	08(11.11)	27(37.50)
	Piperacillin	17(23.61)	06(08.33)	49(68.05)
Carbepenems	Aztreonam	25(34.72)	08(11.11)	39(54.16)
	Ertapenem	60(83.33)	04(05.55)	08(11.11)
	Imipenem	63(87.50)	02(02.77)	07(09.72)
Fluoroquinolones	Ciprofloxacin	42(58.33)	02(02.77)	28(38.88)
Nitrofurans	Nitrofurantoin	12(16.66)	07(09.72)	53(73.61)
Sulphonamides	Cotrimoxazole	05(06.94)	09(12.50)	58(80.55)
Tetracyclines	Tetracycline	28(38.88)	03(04.16)	41(56.94)

The entire world is fighting against the growing menace of antimicrobial resistance often referred to as a silent pandemic. The occurrence of AMR has been attributed to the enduring effort of microorganisms modifying their genetic information on exposure to antimicrobials (WHO, 2023). Among all the antibiotics used globally, -lactams account for a high proportion of 60% by weight and their intensive usage in veterinary and human medicine has resulted in the surge of ESBL-producing resistant microbes (Peirano and Pitout, 2019; Livermore and Woodford, 2006). *Escherichia coli* exists as a part of gut microbiota found in normal animals and humans therefore making it a potential vector for the ESBL spread (O'Brien, 2002).

In the present study, high proportions (65.42%) of E-EC isolates detected in pigs may be the result of frequent usage of antibiotics mainly the -lactams and cephalosporins. Other possible reasons include the common practice of administering antimicrobials as a preventive measure to decrease the risk of infectious diseases during stressful periods (gestation, farrowing and weaning) (Kim *et al*., 2013; Dewey *et al*., 1999). The high occurrence of E-EC in pig farms can function as reservoirs of ESBL-producing resistant bacteria. Reports from other studies support the high occurrence of E-EC isolated in pig farms (Zhang *et al*., 2016; Hammerum *et al*., 2014). In the present study, we could isolate only one E-KP from pigs which is quite low in comparison to the findings from the previous studies (Founou *et al*., 2019; Samanta *et al*., 2018).

Animal handlers working on farms stay in close association with pigs and may get infected with the resistant strains of bacteria contributing to AMR transmission (Lerminiaux and Cameron, 2019). In this study, only one sample collected from an animal handler was positive for E-EC. Although there has been a high prevalence of E-EC isolated from the hands of animal handlers documented in earlier studies (Egbule *et al*., 2020; Founou *et al*., 2019). Low detection rates of ESBLs from hand swabs in our study may be an indication of good hygiene practices followed by the farm workers. Additionally, the microbiological tests conducted on hand swabs only provide a brief idea about the transmission events without depicting the entire episodes of human-animal encounters (Schmitt *et al*., 2021).

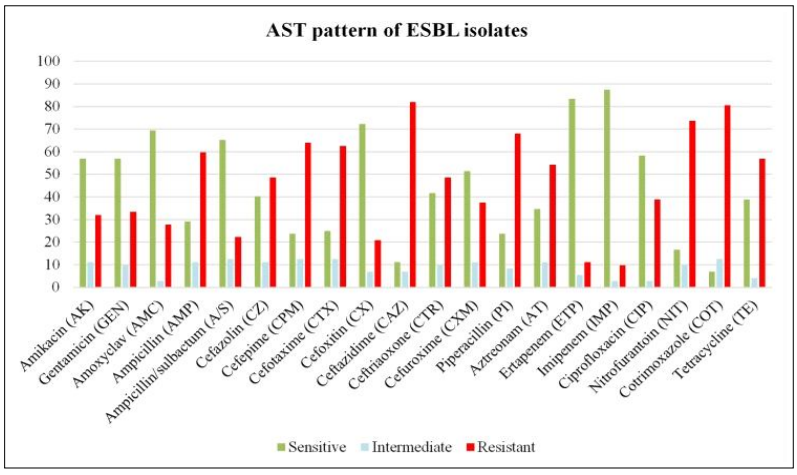

Fig 4: AST pattern of ESBL-producing *E. coli* isolates from pigs and an animal handler.

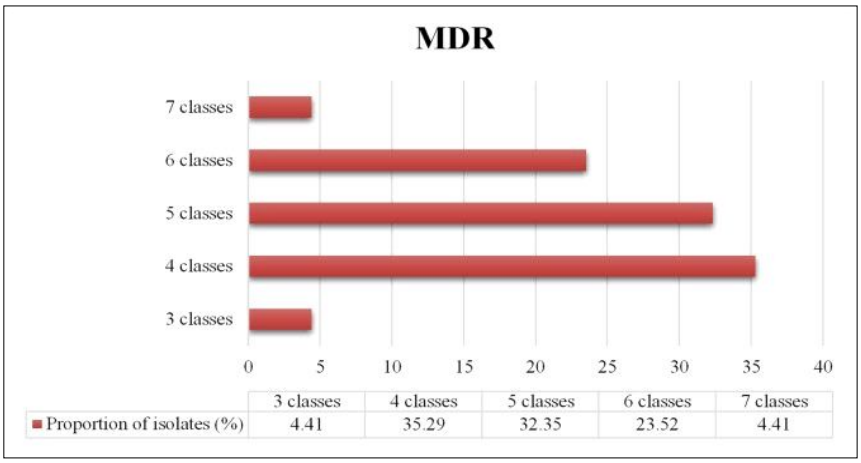

Fig 5: MDR of ESBL producing *E. coli* isolates from pigs.

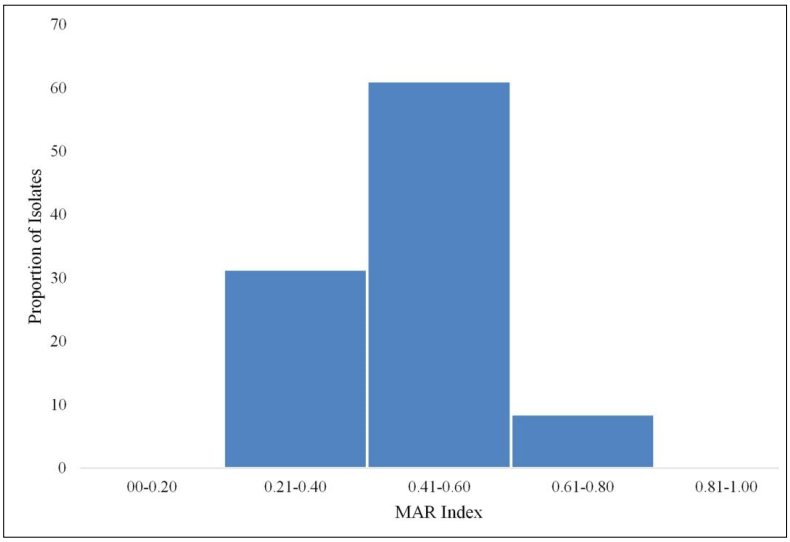

Fig 6: MAR indices of ESBL- producing isolates.

The antibiotic resistance pattern of the ESBL-positive isolates in this study revealed higher rates of resistance to one -lactam (ceftazidime, 81.94%) and two non-lactam antibiotics (cotrimoxazole, 80.55% and nitrofurantoin, 73.61%). Such observations suggest that the mobile ESBL-encoding genes can carry resistance genes to various unrelated classes of antimicrobials, including fluoroquinolones and co-trimoxazole (Liu *et al*., 2018; Wang *et al*., 2014). Fortunately, the majority of the isolates (70-88%) were found sensitive to imipenem, ertapenem and cefoxitin. The findings in this study are in line with earlier published reports (Yadav *et al*., 2022; Tamta *et al*., 2020; Zhang *et al*., 2016). Seventy (97.22%) ESBL isolates in the present study were considered multidrug-resistant strains (explained as isolates resistant to 3 or more categories of antimicrobials) and were consistent with other studies (Song *et al*., 2020; Liu *et al*., 2018). The significant prevalence of MDR in ESBL isolates could be because of tremendous selection pressure from antibiotic overuse in pig farms.

Extra-chromosomal plasmids are associated with multiple antibiotic resistance (MAR) as they bear resistance genes that are transferred between the same and different bacterial species (Osundiya *et al*., 2013). Based on our results, all the ESBL-positive isolates had a MAR index greater than 0.21 which affirms frequent use of antibiotics and high selective pressure in these areas. The findings of earlier studies reveal a similar range of MAR index (>0.2) in pigs (Vinodhkumar *et al*., 2019; Abdalla *et al*., 2021).

The substantial increase in AMR is attributed to the transmission of resistance genes through several genetic processes. The ESBL gene determinants most widely encountered are the *bla*CTXM, *bla*TEM, *bla*SHV and *bla*OXA genes that reside in the enteric bacteria increasing their potential for developing -lactam resistance (Ejaz *et al*., 2021). In the present study, TEM was the dominant (98.59%) type of -lactamase detected in E-EC isolates which is similar to earlier reports (Mobasseri *et al*., 2019; Liu *et al*., 2018). The tetracycline (*tet*) resistant genes assist the bacterial cells in removing the tetracycline antibiotics from the cell by efflux pumps (Kallau *et al*., 2018). The main mechanism identified for the spread of *tet*-resistant genes to other bacterial types in the environment is the horizontal transfer of genes (Urumova, 2016). In this study, the prevalence of the *tet*-A gene was recorded in high proportions (95.77%) correlating with resistance to tetracycline antibiotics. The findings of this paper are supported by other studies reporting a high prevalence of the *tet*-A gene in pigs (Lanz *et al*., 2003; Kallau *et al*., 2018; Urumova, 2016).

CONCLUSION

The findings of the present study revealed a high prevalence of multidrug resistance *E. coli* in pig farms. All the ESBL-producing isolates in our study exhibited a MAR index greater than 0.2. Among the antibiotic resistance genes investigated, *bla*-TEM and *tet*-A were the most prevalent resistance genes found.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The experiment protocols involving animals were approved by the Institutional Biosafety Committee (IBSC/2021/1765-66, Dated: 13/10/2021) and Institutional Animal Ethics Committee, GADVASU, Ludhiana, India (GADVASU/2021/

IAEC/61/23, Dated: 19/10/2021). The experiment involving animal handlers was approved by the Institutional Ethics Committee of Dayanand Medical College and Hospital, Ludhiana, India (DMCH/RandD/2021/104, Dated:10/09/2021).

Conflict of interest

The authors declare that there are no conflicts of interest.

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