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Phenotypic and multiplex PCR-based genotypic characterization of ESBL-producing *E. coli* (ESBL-Ec) isolates from commercial layer poultry

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Abstract

Poultry farming has greatly contributed to the GDP of developing countries like India through large-scale production in the recent past. This massive production of poultry is accompanied by many deteriorative consequences due to the indiscriminate use of antibiotics, resulting in the emergence of antibiotic resistance with one health implication. The current study describes the prevalence of ESBL-producing *Escherichia coli* among the commercial layer chickens in the Namakkal region by phenotypic identification followed by genetic characterization to identify the genes conferring the resistance. Cloacal swab samples from layer chickens were collected from 25 different farms around Namakkal by random sampling, and *E. coli* were isolated. Further, they were subjected to antimicrobial sensitivity tests against five antibiotics using the Kirby-Bauer disk diffusion method on Muller Hinton agar (MHA) as per CLSI guidelines. The isolates identified to be resistant to cefotaxime and ceftazidime were further screened for the ESBL production by the combination disk method using cefotaxime and ceftazidime in combination with clavulanic acid inhibitors, viz. Cefotaxime/Clavulanic acid (CEC-30/10mcg) and Ceftazidime/Clavulanic acid (CAC-30/10 mcg). Those isolates identified as ESBL producers by the phenotypic assays were subjected to multiplex PCR to determine the gene conferring ESBL resistance. The present study revealed that among the 25 samples screened, 24% (6/25), 20% (5/25), 36% (9/25), 8% (2/25), 32% (8/25) and 72% (18/25) were resistant to cefotaxime, ceftazidime, cotrimoxazole, amoxicillin, colistin and amikacin, respectively. About 28% (7/25) were found to be multidrug resistant. Interestingly, 24% (6/25) were found to be phenotypically ESBL-producing isolates using the combination disc method with Cefotaxime/Clavulanic acid (CEC-30/10mcg), Ceftazidime/Clavulanic acid (CAC-30/10mcg). Molecular characterization by multiplex PCR revealed that all six ESBL-producing isolates (100%) harboured the SHV gene, whereas TEM, OXA, and CTX-M genes were not detected. The predominance of SHV-mediated ESBL-producing *E. coli* among commercial layer chickens highlights poultry as a potential reservoir of clinically important antimicrobial-resistant bacteria. The high prevalence of amikacin resistance and multidrug-resistant isolates further emphasizes the urgent need for judicious antimicrobial use, continuous surveillance, and strengthened antimicrobial stewardship programmes in poultry production. These findings provide valuable baseline epidemiological data for the Namakkal poultry belt and reinforce the importance of integrated One Health strategies to curb the dissemination of ESBL-producing bacteria across animal, human, and environmental interfaces.

Keywords: ESBL, *E. coli*, antimicrobial resistance, multiplex PCR, SHV type, poultry

Introduction

Enterobacteriaceae is a family of Gram-negative, facultative anaerobic, non-spore-forming rods. Although they are part of the normal gut microflora, many members, particularly *E. coli*, are responsible for clinical conditions involving the gastrointestinal and urinary tract, and various systemic infections (Paterson *et al.*, 2006) [1]. Antibiotics, primarily beta-lactam groups, aminoglycosides, and fluoroquinolones are commonly used to treat the infections caused by these organisms. In recent times, there is an increasing threat of the emergence of antimicrobial resistance among animals due to the imprudent use of antibiotics in them. The indiscriminate and continuous use of beta-lactam antibiotics has led to the development of resistance to penicillin and lower-generation cephalosporins.

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Consequently, in the 1980s third-generation cephalosporins emerged as a solution to the increased resistance towards lower-generation drugs. But, the bacteria are smart enough to evolve and rapidly develop resistance even against the third generation cephalosporins. Hence, the effectiveness of this group is further enhanced by combining them with beta-lactam inhibitors such as clavulanic acid, tazobactam and sulbactam. However, the bacteria developed mechanisms to survive in the presence of beta-lactam inhibitors and render these drugs ineffective by inactivating the drugs with enzymes, modifying the antibiotic's target sites, or preventing the drug from reaching its target. The resistance to beta-lactam antibiotics in bacteria are conferred majorly by beta-lactamase enzymes including the Extended Spectrum Beta-Lactamases (ESBLs) encoded in transferable plasmids or chromosomes, which hydrolyse the beta-lactams, making them ineffective against penicillin, cephalosporins, and monobactams except for cephamycins and carbapenems (Smet *et al.*, 2008; Teklu *et al.*, 2019) [6, 4]. Additionally, the plasmid-mediated spread of ESBL resistance makes it easier to propagate resistance to other members of *Enterobacteriaceae* present in the intestinal microflora. *E. coli* is a primary driver in the dissemination of ESBL mediated resistance via horizontal gene transfer and high-risk bacterial clones as it is ubiquitous and resilient gut microbe in humans and animals and acts as a massive environmental reservoir. These resistant strains and their plasmids are continuously exchanged between humans, livestock/food products, and the environment (via wastewater), making the dissemination cycle widespread as a part of the "One Health" cycle (Marcantonio *et al.*, 2025) [14].

The most prevalent ESBL genes CTX-M, SHV, TEM and OXA variants exhibit enhanced catalytic activity against ceftazidime, cefotaxime, and ceftriaxone and are commonly found in *Enterobacteriaceae*, particularly *E. coli* (Carvalho *et al.*, 2019; Caneiras *et al.*, 2019) [12, 13]. CTX-M enzymes represent a growing family of class A ESBLs that preferentially hydrolyze cefotaxime over ceftazidime, both oxyimino cephalosporins, including more than 30 members organized in six groups (Bonnet *et al.*, 2004) [10].

The spread of ESBL producing *E. coli* from poultry to humans is unneglectable as poultry meat and eggs contribute a major part of the human diet. There have been numerous investigations regarding presence of ESBL producing bacteria in broilers (Saliu *et al.*, 2017; Kola *et al.*, 2012; Smet *et al.*, 2008) [2, 3, 6] and layers (Wibisono *et al.*, 2020; Olopade *et al.*, 2022) [5, 7] outside India, but minimal studies are available in India. Hence, it is important to assess the presence of ESBL-producing bacteria in poultry birds to formulate an effective mitigation and control strategies and prevent the spread of ESBL resistance to humans. The study area, Namakkal is popularly known as the poultry hub of south India as it houses a high density of layer birds. The present work aims to study the prevalence of ESBL-producing *Enterobacteriaceae* group of bacteria by phenotypic characterization as well as to determine the genes responsible for conferring resistance in layer birds around Namakkal.

Materials and Methods

Collection of sample

A total of 25 cloacal swabs from the live layer chickens at varying stages of laying were collected in a sterile container

from Namakkal region using random sampling during August to December 2022 and shipped to laboratory on ice.

Isolation of *E. coli* from cloacal swabs

The collected swabs were inoculated in nutrient broth and incubated overnight at 37 °C. The inoculum from the nutrient broth was streaked onto MacConkey agar and incubated at 37 °C overnight for isolation of Gram-negative bacteria from the samples. The pink colonies (lactose fermenters) from the MacConkey agar were randomly selected and inoculated into eosin methylene blue agar to confirm the isolates as *E. coli*. Simultaneously, the genus of the isolates was determined by the colony morphology, gram staining and biochemical tests using Hi24 Enterobacteriaceae Identification Kit (Cat. No. KB016). A single colony of each isolate was inoculated in nutrient broth and incubated at 37 °C overnight (Lupindu *et al.*, 2017) [8].

Phenotypic detection of ESBL production

The antibiotic sensitivity pattern of the isolates were determined in Mueller Hinton Agar (MHA) by Kirby Bauer disc diffusion method as per the CLSI standards using antibiotic discs such as amoxicillin/clavulanate (30 µg[20/10]), cefotaxime (30 µg), ceftazidime (30 µg), Cotrimazole (25 µg) and Amikacin (30 µg) (HiMedia India Pvt. Ltd) to determine the sensitivity patterns. The *E. coli* isolates which were not susceptible (either resistant or intermediate) to 3 or more antibiotics classes were identified as multidrug resistant (MDR) (Magiorakos *et al.*, 2012) [17]. The *E. coli* isolates found to be resistant to third generation cephalosporins such as cefotaxime (30 µg), ceftazidime (30 µg) were subjected to combination disc test to determine their ability to produce ESBL by inactivating clavulanic acid and to grow in the presence of clavulanic acid inhibitors. A disc of ceftazidime (30 µg), ceftazidime and clavulanic acid (30 µg/10 µg), cefotaxime (30 µg), cefotaxime and clavulanic acid (30 µg/10 µg) was placed at an appropriate distance on a MHA plate inoculated with a suspension of each resistant isolate equating 0.5 McFarland turbidity standards and incubated overnight (18-24 h) at 37 °C. The isolates showing a difference of ≥ 5 mm in the inhibition zone diameter around the respective combination disc versus cefotaxime (30 µg) or ceftazidime (30 µg) disc alone were identified phenotypically as ESBL producers (Fröding *et al.*, 2017) [18]. The data obtained was analysed in MS Excel and depicted in percentages.

Genotypic characterization of ESBL-producing isolates

The *E. coli* isolates phenotypically identified as ESBL producers were subjected to genotypic characterization targeting four common genes encoding the ESBL production (*bla*_{OXA}, *bla*_{SHV}, and *bla*_{TEM}) by multiplex PCR (Dallenne *et al.*, (2010) [19] and *bla*_{CTX-M} by uniplex PCR (Hasman *et al.*, 2005) [9]. The whole-cell DNA from the resistant bacterial isolates was prepared by suspending a colony of ESBL isolate in 100 µL of distilled water and boiling at 100 °C for 10 min, cooled on ice followed by brief centrifugation at 4,000 rpm for 10 s (Queipo-Ortuño *et al.*, 2008) [11] and the concentration of the DNA in the supernatant was determined in a Nano drop equipment. The primers and the amplifying conditions mentioned by Dallenne *et al.*, 2010 [19] & Hasman *et al.*, 2005 [9] were followed in this study (Table 1).

The PCR was subsequently performed in a 50 µL reaction mixture containing 25 µL of Taq DNA polymerase Master mix RED (Amplicon), 2 µL of the supernatant containing the DNA as template, 1 µL of forward and reverse primer each of bla_{OXA}, bla_{SHV}, and bla_{TEM} gene-specific primers and 17 µL nuclease free water. PCR amplification was performed at an initial temperature of 94 °C for 10 min, followed by 30 cycles of 94 °C for 40 sec, 60 °C for 40 sec,

and 72 °C for 1 min, and then a final extension of 72 °C for 7 min. The PCR amplification conditions for the bla_{CTX-M} gene were 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 60 °C for 15 sec, 72 °C for 30 sec, and 72 °C for 5 min. The amplified PCR products were analysed by 1.5% agarose gel electrophoresis (w/v) at 100 V for one hour, and viewed in a gel documentation apparatus. The amplicon size of products was compared alongside a 100 bp DNA ladder.

Table 1: List of Primers used to amplify the targeted β-lactamase genes and their product size.

Multiplex PCR				
β-lactamase gene targeted	Class of ESBL	Primer name	Primer Sequence 5'→3'	Amplicon Size (bp)
TEM variants including TEM-1 & TEM-2	A	Multi TSO-T-For	CATTTCCGTGTCGCCCTTATTC	800
		Multi TSO-T-Rev	CGTTCATCCATAGTTGCCTGAC	
SHV variants, including SHV-1	A	Multi TSO-S-For	AGCCGCTTGAGCAAATTAAC	713
		Multi TSO-S-Rev	ATCCCGCAGATAAATCACCAC	
OXA-1, OXA-4 & OXA-30	D	Multi TSO-O-For	GGCACCAGATTCAACTTTCAAG	564
		Multi TSO-O-Rev	GACCCCAAGTTTCCTGTAAGTG	
Uniplex PCR				
blaCTX-M	A	blaCTX-M F	ATGTGCAGYACCAGTAARGTKATGGC	593
		blaCTX-M R	TGGGTRAARTARGTSACCAGAAAYCAGCGG	

Results

E. coli could be successfully isolated from all the cloacal swabs collected from the layer chickens. The colonies were confirmed as *E. coli* by the characteristic pink colour colonies produced by the lactose fermenters in the

MacConkey agar (Fig.1A), and green metallic sheen colonies in the EMB agar (Fig.1B), as well as by Gram staining revealing the presence of uniform Gram-negative rods (Fig.1C), and biochemical characteristics like IMViC reaction (+ + --) (Fig.1D)

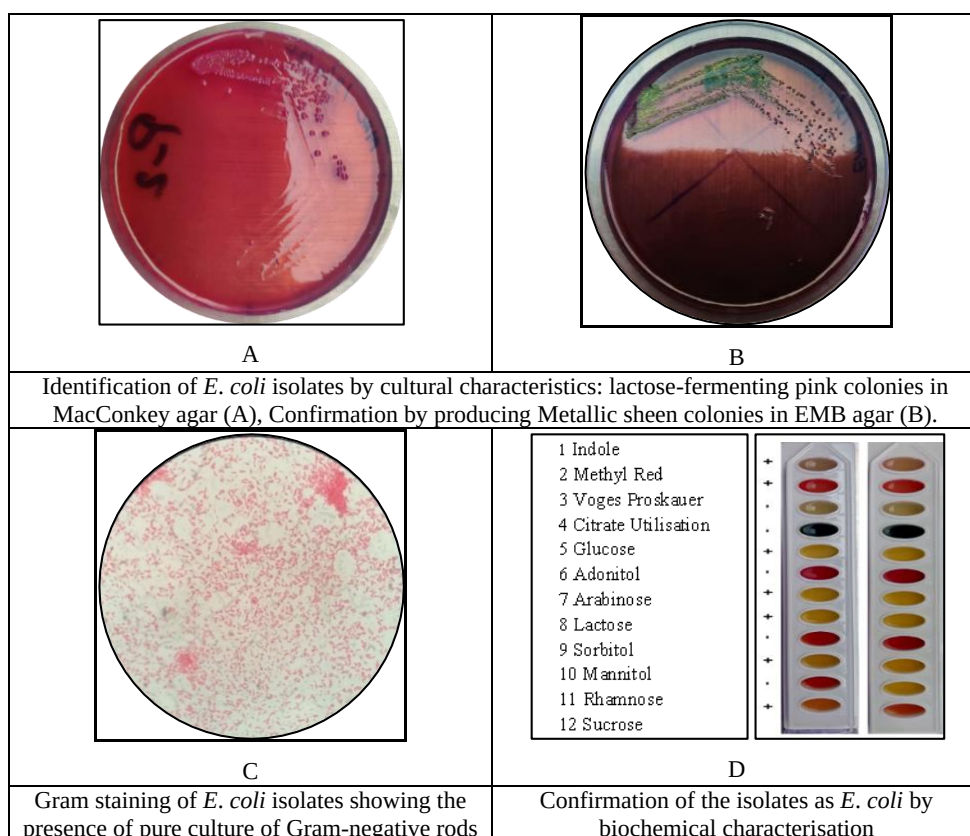


Fig 1: Isolation, Identification and Phenotypic Characterisation of *E. coli* isolates

The antibiotic sensitivity test to determine the susceptibility of the *E. coli* isolates in MHA agar using five antibiotics revealed the highest percentage of resistance against amikacin (72%) followed by cotrimoxazole (36%), cefotaxime (24%), ceftazidime (20%) and amoxicillin-clavulanic acid combination (8%) and about 24% and 16%

of isolates were found to be partially resistant to amikacin and ceftazidime respectively (Fig. 2). With regard to the resistance to third-generation cephalosporins, comparatively, a higher percentage [24% (6/25)] of isolates displayed resistance to cefotaxime against 20% (5/25) for ceftazidime. With respect to the ESBL production, among

the 25 *E. coli* isolates, 28% (7/25) isolates were found to be resistant to third generation cephalosporins of either cefotaxime or ceftazidime by combination disk method (Fig.3, 4). Further, 57.14% (4/7) of the resistant isolates were exhibiting resistance to both cefotaxime and ceftazidime, and the remaining 42.85% (3/7) isolates were found to be resistant to either cefotaxime or ceftazidime.

Interestingly, only 50% (3/6) of the cefotaxime-resistant isolates were found to be ESBL producers, whereas all the ceftazidime-resistant isolates were identified as ESBL producer (Fig. 4) in the combination disc method. Notably, 12% (3/25) of isolates were discovered as multidrug-resistant which were non-susceptible to at least 3 antibiotics belonging to different categories.

Sample No.	CTX 30 (µg)	CAZ 30 (µg)	COT 25 (µg)	AMC 30 (µg)	AK 30 (µg)	MDR (Yes/No)
1.	S	S	R	S	I	-
2.	S	I	R	S	I	-
3.	S	S	R	S	I	-
4.	S	S	R	S	R	-
5.	R	I	S	S	R	-
6.	S	S	S	S	R	-
7.	S	S	S	S	R	-
8.	R	R	S	S	I	-
9.	R	R	S	S	R	R
10.	R	R	R	S	R	R
11.	S	S	R	S	R	-
12.	R	I	S	S	R	-
13.	S	S	S	S	I	-
14.	S	R	S	S	R	-
15.	S	S	S	S	R	-
16.	S	S	S	S	R	-
17.	S	S	R	S	R	-
18.	S	S	S	S	R	-
19.	S	S	S	S	I	-
20.	R	R	R	R	R	R
21.	S	S	S	S	I	-
22.	S	S	S	S	R	-
23.	S	I	S	R	R	-
24.	S	S	S	S	R	-
25.	S	S	R	S	R	-
Total (R)	6	5	9	2	18	3
% (R)	6/25 (24%)	5/25 (20%)	9/25 (36%)	2/25 (8%)	18/25 (72%)	3/25 (12%)

Legend: S Sensitive I Intermediate R Resistant

MDR (Yes): Resistant to ≥ 3 different classes of antibiotics

CTX: Cefotaxime (30 µg) CAZ: Ceftazidime (30 µg) COT: Co-trimoxazole (25 µg) AMC: Amoxycylav (30 µg) AK: Amikacin (30 µg)

Fig 2: Heat map showing the antimicrobial susceptibility (Sensitive, Intermediate, and Resistant) and multidrug resistance patterns of the *E. coli* isolates by disc diffusion method

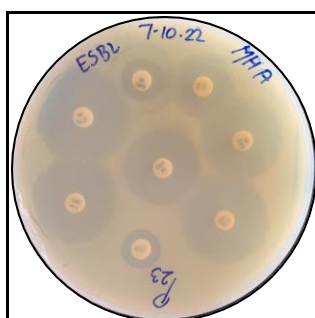


Fig 3: Phenotypic characterization of *E. coli* isolates to determine the ESBL production using combination discs (CTX-30, CEC-30/10, CAZ-30 & CAC 30/10)

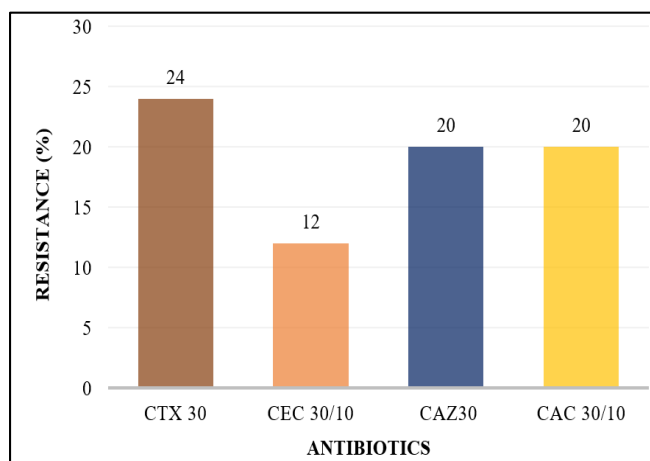


Fig 4: Antibiotic resistance of the *E. coli* isolates against third-generation cephalosporin and its combination with β -lactamase inhibitors by the combination disc diffusion method

The *E. coli* isolates phenotypically identified as ESBL producers were confirmed by subjecting them to genotypic characterization by multiplex PCR targeting *bla*_{OXA}, *bla*_{SHV}, and *bla*_{TEM} genes and uniplex PCR targeting *bla*_{CTX-M} gene. The multiplex PCR amplified a product just above 700bp (Fig 5), disclosing that the resistance to third-generation cephalosporins was conferred by the SHV type of ESBL in all seven isolates (100%). Curiously, none of the seven ESBL producing isolates encoded the *bla*_{CTX-M} genes in the uniplex PCR, confirmed by the absence of amplification.

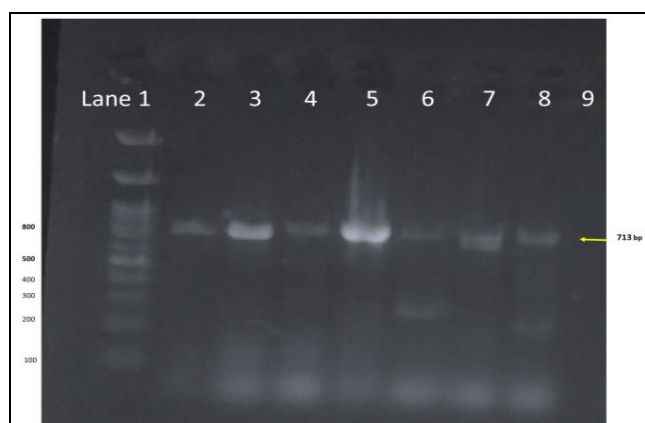


Fig 5: Multiplex PCR targeting TEM, SHV, and OXA types of ESBL variants. Lane1-100 bp DNA ladder, Lane 2-8 Phenotypically confirmed ESBL *E. coli* isolates amplified 713 bp SHV type of ESBL gene in all seven isolates, Lane 9-NTC control

Discussion

The present study provides insights on the prevalence of ESBL-producing *E. coli* in poultry farms across the

Namakkal region, which is one of the most densely populated poultry belts in India that supplies a major portion of the eggs to the state of Tamil Nadu and other neighbouring states. The results of the phenotypic observations correlated well with the molecular characterization underscoring the role of either of the gene encoding ESBL in conferring resistance to the isolates. The presence of ESBL-*E* among the layer birds could significantly impact the food products obtained from them as there is a high probability for the dissemination of the genes conferring resistance through either via plasmids, transposons, and integrons (Carattoli, 2013; Zheng *et al.*, 2025)^[20, 21] to other pathogens in humans as well as into the environment. This transfer of resistance to human beings happens through the animal-sourced food products, resulting in a major public health risk (Seiffert *et al.*, 2013)^[22]. The mode of transfer of ESBL resistance in low-and middle-income countries most often occurs between humans, animals and the environment rather than human-to-human transmission, which is quite common in high-income countries (Beaumont *et al.*, 2026)^[15] implying the role of poultry and the dissemination of resistance to manpower associated with poultry operations and eventually to the environment. Furthermore, the present study is in accordance with the observation made by Beaumont *et al.*, (2026)^[15] revealing the potential for resistance transfer from animals to humans.

The occurrence of ESBL producing bacterial isolates could be majorly attributed to the usage of antibiotics as growth promoters which is quite a common practice among commercial poultry farm owners. The main contributors to the development of antimicrobial resistance in livestock rearing are the use of antibiotics to promote growth and for prophylactic measures and therapeutic purposes. The reckless usage of massive amounts of antibiotics in livestock rearing, often outweighing the human usage could potentially render antibiotics useless in fighting against the pathogens in the near future (Van Boeckel *et al.*, 2017)^[16].

Overall, a cautious usage of antibiotics and routine monitoring of the prevalence of ESBL producing *E. coli* among food animals is a crucial step to prevent the emergence and dissemination of antimicrobial resistance and to provide a safe and healthy environment where the all these biotic coexist. Additionally, the availability of fitting and comprehensive data plays an essential role in policy-making regarding the reduction in the usage of antibiotics in livestock rearing. The presence of resistance in the livestock microbiome poses a significant threat to public health given the close contact between humans and animals and the dependency of human beings on livestock for various food commodities. Given these scenario, strategic studies on antimicrobial resistance at regular intervals covering the most possible interfaces could contribute towards the antimicrobial resistance stewardship in near future.

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