

Phenotypic and molecular characterization of *Escherichia coli* isolated from food producing small ruminants and their environment in Awadh region of Uttar Pradesh

V YADAV^{1✉}, R P DIWAKAR², D P SHRIVASTVA³, S V SINGH⁴, D NIYOGI⁵ and RISHIKANT⁶

Acharya Narendra Deva University of Agriculture and Technology, Kumarganj Ayodhya 224 229 Uttar Pradesh India

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ABSTRACT

Antimicrobial resistance (AMR) and emergence of resistance to β -lactam antibiotics especially in *Enterobacteriaceae* is a major global concern. The present study, total 200 samples were collected from Ayodhya and Barabanki districts of Awadh region of U.P. Out of 200 samples 46.5% were confirmed as *E. coli* isolates by PCR analysis using species specific *uspA* gene with highest 60.0% from goat faecal samples followed by 56.0%, 40.0%, 36.0% and 28.0% from sheep faecal, environmental floor swab, chevon and water samples, respectively. About 74.19% and 72.04% isolates were confirmed as ESBL producers by DDST and ESBL E-strip tests, respectively. Genotypic analysis revealed the overall prevalence of *bla*_{CTX-M-1} (72.91%) followed by *bla*_{TEM} and *bla*_{SHV} with 43.75% and 29.17%, respectively. Antibigram of all phenotypically confirmed *E. coli* isolates was done against 20 antibiotics of 12 different classes, which revealed 76.47% to 100% resistance against ampicillin, and 3rd and 4th generation cephalosporins, while 100% sensitive to aminoglycosides, polymyxins B and chloramphenicol. Findings of this study suggested routine AMR monitoring of animal origin food, regulated use of antibiotics in food animals, awareness among stockholders and improved hygiene practices even small herds of sheep and goat and meat shops. Thus, a comprehensive one health strategy is need of hour to control AMR and secure food safety and public health.

Keywords: AMR, *E. coli*, ESBLs, Public health, Small ruminants

Animal reservoirs play an important role as carrier of antimicrobial resistance (AMR) genes of *Escherichia coli* (*E. coli*) and have drawn considerable global attention (Monstein *et al.* 2007). Food animals and their environment are considered as a potential source of AMR and have greater role in the spread of AMR has been debated for a long (Marshall and Levy, 2011). The β -lactams are among most popular antibiotics for the treatment of bacterial infections and multidrug resistant (MDR) bacteria producing Extended-spectrum β -lactamases (ESBLs) are also prevalent worldwide. The occurrence of ESBL producing *E. coli* is not only limited to nosocomial outbreak but also reported from food animals, their products and environment. National action plan on AMR (2017-2021) in India has reported lack of effective surveillance of

AMR pathogens of animal origin and limited information on MDR ESBLs and virulent strains in healthy livestock. ESBL producing *E. coli* infections is not only restricted to animal health and productivity but is also important from human health point of view, being zoonotic in nature. The ESBLs genes (CTX-M, TEM and SHV) expressed by *E. coli* are transmitted across the animal-human-environment and food chain (Nayakvadi *et al.* 2023).

Till date no in-depth study has been done on genotypic analysis of ESBL producing *Enterobacteriaceae* from small ruminants in Awadh region of Uttar Pradesh. So, the present study was designed to determine the ESBL producing *E. coli* isolates by targeting *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes in healthy goat, sheep, chevon and their environment, to provide a database to help the field veterinarians, pharmaceuticals and policy makers in appropriate selection of antibiotics for therapeutic use and also helpful in controlling the dissemination of microbial resistance in community setting.

MATERIALS AND METHODS

Study area: The study was carried out in Department of Veterinary Microbiology, C. V. Sc. and A. H. Kumarganj, Ayodhya. The samples were collected from Ayodhya and Barabanki districts of Awadh region of Uttar Pradesh, India between August 2024 and March 2025.

Present address: ¹Associate Professor, Department of Veterinary Microbiology, ²Assistant Professor, Department of Veterinary Microbiology, ³Assistant Professor, Department of Veterinary Pathology, ⁴Professor and Head, Department of Veterinary Pharmacology and Toxicology, ⁵Professor and Head, Department of Veterinary Pathology, ⁶Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, 224229, Ayodhya, (U.P.). ✉Corresponding author email: vibhavet2005@gmail.com

Sample collection: Total 200 samples from apparently healthy sheep (faecal sample 50), goat (faecal samples 50), raw meat (chevon samples 50) and environment (floor swab and water samples 25 each) were randomly collected from Ayodhya and Barabanki districts. Faecal and environmental floor samples were collected using sterilized swab technique while for environmental water samples, approximately 5mL were taken in sterilized collection test tube samples and Chevon samples, approximately 25 gram, were collected in sterile screw capped sample bottles. All collected samples were immediately transported to bacteriology laboratory in an icebox to ensure preservation of microbial viability during transportation.

Isolation and Identification: The samples except chevon were enriched with 2ml Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 hrs. The chevon samples were triturated and suspended in 225 mL of the 1% sterile peptone water and homogenized manually followed by overnight incubation at 37°C (Kharate *et al.* 2025). A loopful of inoculum was directly streaked on MacConkey lactose agar (MLA) plates added with 2mg/L cefotaxime, and incubated at 37°C for 24 hrs. Plates showing pink colonies (lactose fermenting) were picked up and streaked on Eosine Methylene Blue (EMB) agar (Hi-Media) plates. Colonies showing greenish metallic sheen (Fig.1) on EMB were tentatively considered as *E. coli*. A single pure colony was picked up and transferred to nutrient agar slant. Further identification of the isolates was done by various biochemical tests viz. IMViC pattern, catalase test (Fig. 2), nitrate reduction, urease test, triple sugar iron agar and sugar fermentation reaction as per the method of Edward and Ewing (1972) and PCR analysis.

Extraction of genomic DNA: The DNA templates were prepared by using snap-chill method as described by Franco *et al.* (2008).

Molecular identification of *E. coli*: All presumptively positive *E. coli* isolates were confirmed by PCR amplification using species specific *uspA* gene as per method described by Foti *et al.* (2011) (Table-1, Fig.3). The PCR reaction was carried out as a total 25 µl volume constituted by 12.5 µl (2X GeNei Red Dye) Master Mix, 8.5 µl nuclease free water, 1µl mixture of forward and reverse primers (0.5 µl each primer) and 3.0 µl of template DNA. Amplification was performed using thermal cycler (Bio-Rad, USA). The cycling conditions of PCR are mentioned in table-1. Simplex PCR was performed using 100bp ladder. Amplified products (5µl) were mixed with 3µl of bromophenol blue dye (6X) and electrophoresis was done in 2.0% the gel at 80 mA for 45 minutes and gel was visualized using the UV illuminator (GeNei Bangalore, India)

India)

Screening of ESBL producing isolates: Confirmed isolates were subjected to screening of ESBLs, using 3rd, 4th generation cephalosporins, monobactam and carbapenems group of antibiotics. The results were interpreted as per CLSI guidelines (2019). Isolates showing resistance to one or more of these antibiotics were screened for ESBL production.

Double disc synergy test (DDST) for ESBL confirmation: The screened isolates for ESBL production were further confirmed by DDST using ESBL kit 1 and Kit 3 (Hi-media) (Fig.4). The commercially available discs were placed at 25mm apart on Muller Hinton Agar (MHA) (Hi-media) plates inoculated with 1.5×10^8 organism/ml and incubated at 37°C for 24 hrs. The results were interpreted as per CLSI guidelines (2019) (Fig.4).

Minimum inhibitory concentration (MIC) ESBL E-test: For further confirmation ESBL, E-strip test was done by placing strip on MHA plates inoculated with 1.5×10^8 organism/ml and incubated at 37°C for 24 hrs. The results were interpreted as per CLSI guidelines (2019) (Fig.5).

Extraction of plasmid DNA: A single pure colony of all ESBL positive isolates were inoculated into 10 ml of Luria-Bertani (LB) broth medium (HiMedia, India) and incubated at 37°C for 18 hrs in shaking incubator. The bacterial suspension was centrifuged at 10,000 rpm for 3 min and pellet was collected. After that, plasmid DNA was isolated using GeneJet plasmid Miniprep kit (Thermo Scientific) as per the instruction of the manufacturers.

Detection of *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes: Genotypic confirmation of ESBL genes was done in a total reaction volume of 25 µl for *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes as per method described by Dallenne *et al.* (2010) and Bhattacharjee *et al.* (2007) respectively. Amplicon size and primer sequence of targeted genes alongwith conditions of thermal cycling for different primer pairs are mentioned in table-2. Simplex PCR was performed for all three mentioned genes using 1Kb ladder. Amplified products (5µl) were mixed with 3µl of bromophenol blue dye (6X) and electrophoresis was done in 0.8% gel at 60-70 mA for 45 min and gel was visualized using the UV illuminator (GeNei Bangalore, India).

Antimicrobial resistance study of all phenotypically confirmed ESBL producing isolates: Antibigram of all phenotypically confirmed *E. coli* isolates was done against 20 antibiotics (Hi-Media) of 12 different classes (Table 3). It was done by disc diffusion method on MHA plates seeded with 1.5×10^8 organisms/mL and incubated at 37°C for 24 hrs. and isolates were classified as susceptible and resistant as per interpretation guidelines of CLSI (2019).

Table 1. Oligonucleotide primer sequences used for amplification of *uspA* genes and PCR cycling conditions used

Targeted gene	Primer sequence (5'-3')	Amplicon size (bp)	PCR conditions and cycles	References
uspA	F- 5'CCGATACGCTGCCAATCAGT3' R-5'ACGCAGACCGTAGGCCAGAT3'	884	1 cycle of 2 minutes at 98°C, 35 cycles of 30 seconds at 98°C, 1 minutes at 56°C, 45 seconds at 72 °C, 1 cycle of 5 minutes at 72°C	Foti <i>et al.</i> 2011

Table 2. Details of primers used for molecular characterization of ESBL genes

Targeted genes	Primer sequence	Primers pair	Amplicon size (bp)	References
<i>bla</i> _{CTX-M}	F-5' TTAGGAARTGTGCCGCTGYA3' R- 5'CGATATCGTTGGTGGTRCCAT3'	1 cycle of 10 min.at 94°C, 30 cycles of 40 cycles of 40 sec.at 94 °C, 40 sec.at 60 °C, 1min.at 72 °C, 1 cycle of 7min.at 72 °C	688	Dallenne <i>et al.</i> 2010
<i>bla</i> _{TEM}	F-5'CATTTCGTCGTCGCCCTTATTC3' R-5'CGTTCATCCATAGTTGCCTGAC3'	1 cycle of 10 min. at 94°C, 30 cycles of 40 sec. at 94 °C, 40 sec.at 61 °C, 1min.at 72 °C, 1 cycle of 7min. at 72 °C	800	Dallenne <i>et al.</i> 2010
<i>bla</i> _{SHV}	F-5'AGGATTGACTGCCTTTTTG3' R-5' ATTTGCTGATTCGCTCG3'	1 cycle of 10 min. at 94°C, 40 cycles of 40 sec.at 94 °C, 40 sec.at 60 °C, 1min. at 72 °C, 1 cycle of 7min.at 72 °C	393	Bhattacharjee <i>et al.</i> 2007

Table 3. AMR pattern of phenotypically ESBL producing isolates of various sources

Group	Antibiotics	Conc. (µg/disc)	<i>E. coli</i> (n=67)
			Resistance
Aminoglycosides	Gentamicin (Gen)	10	0.0%
	Amikacine (Ak)	30	0.0%
Carbapenems	Imepenem (IMP)	10	8.96%
	Meropenem (MRP)	10	5.97%
3 rd and 4 th generation Cephalosporins	Cefotaxime (CTX)	10	100%
	Cefpodoxime(CPD)	10	82.0%
	Ceftazidime (CAZ)	30	76.47%
	Ceftriazone (CTR)	30	100%
Monobactams	Aztreonam (AT)	30	29.85%
2 nd generation Cephalosporins	Cefoxitin (CX)	30	14.92%
Penicillin	Ampicillin (AMP)	25	100%
Polypeptides	Polymyxin-B (PB)	300 unit	0.0%
Sulphonamides	Co-trimoxazole (COT)	25	35.82%
	Trimethoprim (TR)	30	22.38%
	Enrofloxacin (EX)	10	22.38%
Quinolones	Ofloxacin (OF)	2	32.83%
	Nalidixic acid (NA)	30	41.79%
Tetracycline	Tetracycline (TE)	30	22.38%
Amoxyclav	Amoxicillin/Clavulanic (AMC)	(20/10)	13.43%
Chloramphenicol	Chloramphenicol (C)	30	0.0%

Organisms showing resistance against one antibiotic of at least three classes of antibiotics were considered multidrug resistant (MDR).

RESULTS AND DISCUSSION

A total of 107 *E. coli* isolates were retrieved from total 200 samples of sheep and goat faecal samples, chevon and environmental samples consisting 50 each with over all prevalence of 53.5% on the basis of their morphology, cultural characteristics on selective media and biochemical tests (Table-4, Fig.1 and 2). Further PCR analysis using *uspA* gene, revealed overall prevalence of 46.5% isolates with highest 60.0% from goat faecal samples followed by 56.0%, 40.0%, 36.0% and 28.0% from sheep faecal,

environmental floor swab, chevon and water samples, respectively (Table 4, Fig. 3) which was in agreement with earlier studies (Kharate *et al.* 2025; Nayakvadi *et al.* 2023 and Kumar *et al.* 2022).

Total 93 confirmed *E. coli* isolated were subjected to screening, DDST and ESBL-E test for study of phenotypically ESBL producing isolates. On preliminary screening, out of 77/93 (82.79 %) isolates presumed as ESBL producer, 69/93 (74.19%) isolates and 67/93 (72.04%) isolates were confirmed as ESBL by DDST and ESBL-E strip test, respectively (Table 4 and 5, Fig. 4 and 5). Total 67/200 (33.5%) were ESBL positive isolates comprising 22/50 (44.0%), 20/50 (40.0%), 12/50 (24.0%), 8/25 (32.0%) and 5/25 (20.0%) from apparently

Table 4. Isolation rate and distribution of ESBL producing isolates, among various sources

Samples (Source/Origin)	Presumptive positive isolates (Biochemical tests)	Confirmed isolates (PCR analysis)	Phenotypically ESBL positive isolates
Sheep	Apparently normal faeces (n=50)	31 (62.0%)	28 (56.0%)
Goat	Apparently normal faeces (n=50)	33 (66.0%)	30 (60.0%)
	Raw meat (Chevon) (n=50)	21 (42.0%)	18 (36.0%)
Environment	Floor swab (n=25)	12 (48.0%)	10 (40.0%)
	Water (n=25)	10 (40.0%)	07 (28.0%)
Total	N=200	107 (53.5%)	93 (46.5%)

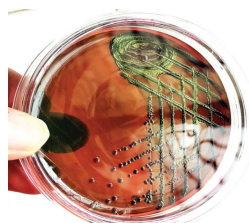
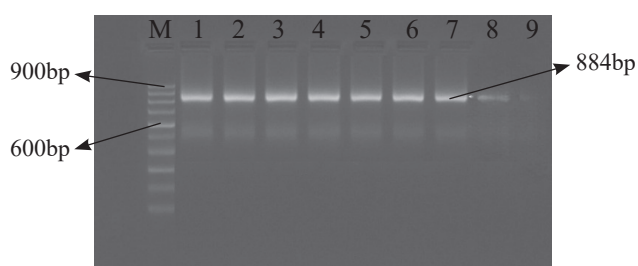
Fig. 1. Metallic sheen of *E. coli* on EMB agar

Fig. 2. Catalase positive test

Fig. 3. PCR amplification of *uspA* gene (884bp)

M: 1kb ladder, Lane 1, 2, 3, 4, 5, 6 & 7 (884bp), Lane 8 negative, Lane 9 negative control.

normal faecal samples of sheep and goat, chevon and environmental floor swab and water, respectively (Table 4). Phenotypically, ESBL positive isolate prevalence in sheep faecal samples was similar to the findings of Ranjan *et al.* (2024) while higher percentage in goat faecal samples, chevon and environmental samples was reported by several workers in India and abroad (Kharate *et al.* 2025, Ranjan *et al.* 2024, Bhoomika *et al.* 2016, Kamboj *et al.* 2024, Mahmud *et al.* 2020). There was little difference in the sensitivity among different phenotypic confirmatory test and findings are in conformity to Kharate *et al.* (2025).

Genotypic analysis of phenotypically confirmed ESBL positive isolates was done by targeting *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes using PCR and revealed the presence of ESBL genes in 51.61% (48/93) isolates (Fig.6, 7 and 8, Table 5). The present study showed an overall prevalence of *bla*_{CTX-M} (72.91%) followed by *bla*_{TEM} and *bla*_{SHV} with 43.75% and 29.17%, respectively (Table-6). The present finding revealed the predominance of *bla*_{CTX-M} gene in the area. Likewise, various co-workers across the world have also reported *bla*_{CTX-M} gene as the most prevalent one in different sample sources (Mahmud *et al.* 2020; Nayakvadi

Table 5. Confirmation of ESBL producing isolates by using screening, phenotypic and PCR analysis

Tests	Confirmed <i>E. coli</i> isolates (93)
Screening test	77/93 (82.79%)
Double disc synergy test (DDST)	69/93 (74.19%)
ESBL-E test (Phenotypically confirmed)	67/93 (72.04%)
PCR analysis (Genotypically confirmed)	48/93 (51.61%)

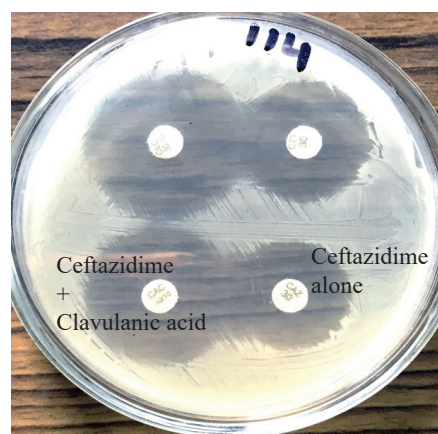


Fig. 4. DDST test

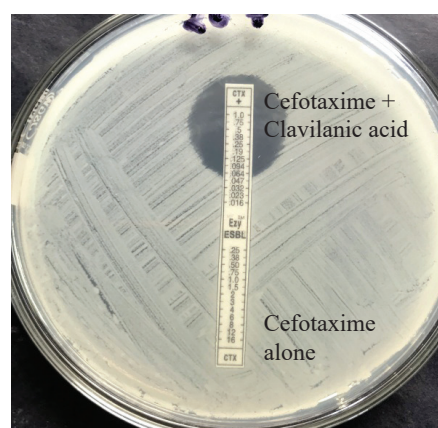


Fig. 5. ESBL E-strip test

et al. 2023, Ranjan *et al.* 2024, Kamboj *et al.* 2024). In this study, 18/48 (39.58%) isolates harboured two genes in different combination while 01/48 (2.08%) isolate was found to have combination of all three genes (Table 6).

Table 6. Distribution of ESBL genes among phenotypically ESBL positive isolates

No of genes	<i>bla</i> -genes	Genotypically positive ESBL isolates (48)	
		Number	Percent
Single gene	<i>bla</i> - _{CTX-M}	35	72.91%
	<i>bla</i> - _{TEM}	21	43.75%
	<i>bla</i> - _{SHV}	14	29.17%
Multiple genes	<i>bla</i> - _{CTX-M} and <i>bla</i> - _{TEM}	09	18.75%
	<i>bla</i> - _{CTX-M} and <i>bla</i> - _{SHV}	05	10.42%
	<i>bla</i> - _{SHV} and <i>bla</i> - _{TEM}	04	8.33%
	<i>bla</i> - _{CTX-M} , <i>bla</i> - _{TEM} and <i>bla</i> - _{SHV}	01	2.08%

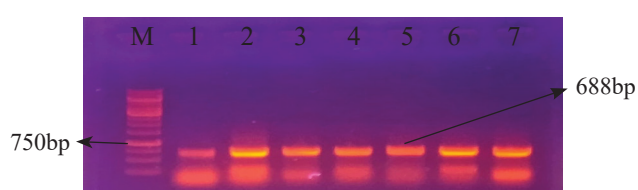


Fig. 6. PCR amplification of *bla*-_{CTX-M-1} gene (688bp)
M: 1kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7 positive for *bla*-_{CTX-M-1} (688bp)

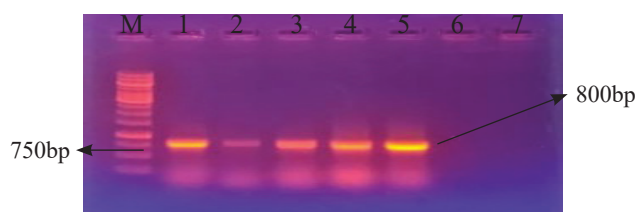


Fig. 7. PCR amplification of *bla*-_{TEM} gene (800bp)
M: 1kb ladder, Lane 1, 2, 3, 4 and 5 positive for *bla*-_{TEM} gene (688bp), Lane 6 and 7 negative control

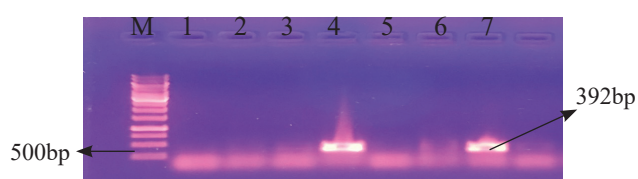


Fig. 8. PCR amplification of *bla*-_{SHV} gene (392bp)
M: 1kb ladder, Lane 4 and 7 positive for *bla*-_{SHV} gene (392bp), Lane 8 negative control

Co-expression of genes has been also observed in different source of sample by many workers (Yadav *et al.* 2022; Ranjan *et al.* 2024, Kamboj *et al.* 2024). Presence of two or more genes in combination suggested plasmid-mediated multi-gene carriage and potential for horizontal gene transfer while carriage of all three genes suggested the risk of highly resistant *E. coli* strains accessing the food chain

In present study, phenotypically confirmed ESBL producing isolates of various sources showed 100% resistance against ampicillin followed by 76.47% to 100% against 3rd and 4th generation cephalosporins, and 22.38

% to 41.79% against quinolones, sulphonamides and tetracycline alongwith 100% sensitivity to aminoglycosides, polymyxins B and chloramphenicol. There are abundant evidences that corroborate with the finding in India and abroad (Bhoomika *et al.* 2016, Mahmud *et al.* 2020, Kumar *et al.* 2022, Ranjan *et al.* 2024, kamboj *et al.* 2024). In this study, 72.5% isolates were found to be MDR i. e. resistant to at least one antibiotic of three or more classes of antimicrobial, which highlighted the potential threat by limiting the therapeutic options.

This study highlighted the presence of ESBL-producing *E. coli* and 75.0% to 100% isolates showed resistance to ampicillin, 3rd and 4th generation cephalosporins and most of them were found to be MDR, which is an alarming situation in this area. However, isolates found to be 100% sensitive to aminoglycosides, polymyxins B and chloramphenicol groups of antibiotics which may be alternative therapeutic option to this area. The detection of *bla*-_{CTX-M}, *bla*-_{TEM} and *bla*-_{SHV} genes alone and their co-existence is of utmost public health concern. These result explicated a cohesive and synchronized effort with immediate attention across the animal-human-environment interface. Findings suggested routine AMR monitoring of animal origin food, regulated use of antibiotics in food animals, awareness among stockholders and improved hygiene practices even small herds of sheep and goat and meat shops. Thus, a comprehensive one health strategy is the need of hour to control AMR and secure food safety and public health.

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