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Phenotypic and Molecular Characterization of *Escherichia coli* Isolated from Food Animals for Antimicrobial Resistance

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ABSTRACT

Background: The use of antimicrobials in food animals significantly contributes to the development of antimicrobial resistance in nature. This study aimed to determine the prevalence of ESBL-producing *E. coli* isolated from cattle, buffalo, sheep and goat fecal samples.

Methods: In this study out of 2025 healthy animals from 10 different farms, fecal samples were randomly collected by rectal swabs from 200 animals (50 each from cattle, buffalo, sheep and goat). *E. coli* was isolated and further studied for AMR and ESBL production by phenotypic assays and PCR targeting ESBL genes.

Result: The overall prevalence of *E. coli* was 81.50% with the highest isolation rate from cattle and goat samples. Out of 163 isolates, 45.39% were ESBL-producers. Genes encoding beta-lactam resistance *viz.* bla_{SHV}, bla_{TEM}, bla_{CTX-M} *and* bla_{OXA} were detected in 44.59% ESBL-positive isolates. The bla_{SHV} was present in only cattle isolates, while bla_{CTX-M} was present in *E. coli* isolated from all animals. The prevalence of beta-lactam genes was found as bla_{SHV} (2.70%), bla_{TEM} (12.16%), bla_{CTX-M} (22.97%) and bla_{OXA} (6.75%). A high degree of resistance to multiple antimicrobials was observed. Maximum susceptibility of *E. coli* was observed for trimethoprim, imipenem and chloramphenicol. This study demonstrated that food animals could be the source of ESBL-producing multidrug-resistant *E. coli.* The present findings are significant in the context of AMR monitoring in India.

Key words: Antibiotic resistance**,** Beta-lactam genes, *E. coli*, ESBL producing, Food animals.

INTRODUCTION

Although the development of antimicrobial resistance (AMR) in the bacterial community is considered an ancient and natural phenomenon, its emergence and widespread distribution have escalated to a great extent in recent the past (Laxminarayan *et al.,* 2013). Livestock and pets are considered as a potential source of AMR spread in nature. The role of livestock, poultry and aquaculture in the spread of AMR has been debated for a long (Marshall and Levy, 2011). There is a correlation between the use of antibiotics and the emergence of resistance to pathogens (Allen *et al.,* 2010). Beta-lactam antibiotics are the most widely used agents for the treatment of bacterial infection, therefore, resistance against Extended Spectrum Beta-Lactamase (ESBL) has been recorded globally. Among ESBL-producing *Enterobacteriaceae, Klebsiella* species and *Escherichia coli (E. coli)* are the most commonly resistant bacteria causing community-acquired infections (Pormohammad *et al.,* 2019). Research on AMR in the livestock origin bacterial species with special reference to the critical antimicrobials is at a primitive stage. This study was undertaken on the isolation and characterization of *E. coli* from fecal samples of cattle, buffalo, sheep and goats in terms of AMR, ESBL production and prevalence of ESBL encoding genes.

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MATERIALS AND METHODS

Sampling

In this study, farms having at least 50 animals of a single species were identified for sampling. Accordingly, 10 farms located at distinct places in the Satara and Pune Districts of Maharashtra state were selected for sampling. From each farm, rectal swabs of apparently healthy animals were randomly collected by the veterinarian. The proportion of samples collected from each farm varies from 5-10%, which

depends on the number of animals at the farm. A total of 200 rectal swabs from livestock species (50 each from cattle, buffalo, sheep and goat) were collected. The convenient sampling plan was used to determine the sample size. *E. coli* was isolated as pure culture by enrichment of fecal swabs in *Enterobacteriaceae Enrichment Broth* (EEB) for 24 hr at 37°C followed by selective plating on Eosin Methylene Blue (EMB) agar and incubation at 37°C for 24 hr. Isolates were confirmed using biochemical tests *viz.* catalase, oxidase, indole, methyl red, Voges-Proskauer and citrate utilization tests.

Antimicrobial resistance study

The antimicrobial susceptibility pattern was studied by modified Kirby–Bauer disk diffusion and the results were interpreted according to the *Clinical and Laboratory Standards Institute* guidelines (CLSI, 2017). Antimicrobial discs were procured from HiMedia Laboratories, Mumbai. Antimicrobial discs used in this study were: ampicillin (10 μg^{-1}), cefazolin (30 μg^{-1}), cefixime (5 μg^{-1}), chloramphenicol (30 μg^{-1}), ciprofloxacin (5 μg^{-1}), gentamicin (10 μg^{-1}), imipenem (10 μg^{-1}), kanamycin (30 μg^{-1}), tetracycline (30 μg^{-1}) and trimethoprim (5 μg^{-1}).

ESBL screening

ESBL production was confirmed by the disc diffusion synergy test (DDST) as per the recommendations of CLSI. Antimicrobial discs used for initial screening were cefpodoxime (10 μg^{-1}), ceftazidime (30 μg^{-1}), aztreonam (30 μg^{-1}), cefotaxime (30 μg^{-1}), ceftriaxone (30 μg^{-1}). ESBL confirmation was done by using cephalosporin discs *viz.* cefotaxime (30 μg^{-1}), cefotaxime/clavulanic acid (10 μg^{-1}), ceftazidime (30 μg^{-1}) and ceftazidime/clavulanic acid (10 μg^{-1}). A difference of ≥5 mm in the zone of inhibition between cephalosporin alone and cephalosporin plus clavulanic acid indicates ESBL production (CLSI, 2017).

Detection of genes encoding beta-lactamases

ESBL-positive *E. coli* were further screened by multiplex polymerase chain reaction (PCR) designed previously targeting four beta-lactamase genes *viz. blaSHV, blaTEM, blaCTX-M and blaOXA* (Fang *et al.,* 2008). For PCR targeting beta-lactam genes, bacterial DNA was extracted by the heat lysis method. Multiplex PCR was performed in 25 µl volume containing 12.5 µl 2x PCR master mix,1 µl each forward and reverse primers (10 pmol/µl), 2 µl DNA template and 2.5 µl nuclease-free water to make final the volume. The cycling conditions were set as initial denaturation (95°C/ 15 min) followed by 30 cycles of denaturation (94°C/30 sec), annealing (62°C/90 sec) and extension (72°C/60 sec). The final extension was set at 72°C for 10 min and held at 4°C. Five µl of amplified product was further separated by electrophoresis in 1.5% agarose gel dissolved in 0.5x TBE stained by ethidium bromide (0.5 mg/mL).

RESULTS AND DISCUSSION

The prevalence of ESBL-positive *E. coli* strains and ESBL-encoding genes found in this study is depicted in Table 1. Maximum ESBL-producing *E. coli* strains were isolated from goats (58.69%), followed by sheep (45.94%), buffalo (42.10%) and cattle (33.33%). Altogether, 74 *E. coli* strains (45.39%) were ESBL producers. Bacteria belonging to the family *Enterobacteriaceae* are widely studied around the world for antimicrobial resistance. Among livestock species, cattle-origin *E. coli* has been extensively investigated for ESBL types at a global scale (Schmid *et al.,* 2013; Pehlivanoglu *et al.,* 2016; Aworh *et al.,* 2022). However, studies on ESBL types of *E. coli* isolated from other food animals *viz.* buffalo, sheep and goats are limited (Geser *et al.,* 2012). In India, ESBL production was studied by a few investigators in *E. coli* isolated from cattle feces (Rawat *et al.,* 2018) and raw foods of animal origin (Bhoomika *et al.,* 2016). All 74 isolates were further studied for the presence of ESBL-encoding genes by multiplex PCR. ESBL encoding genes were detected in 33 (44.59%) ESBL-positive *E. coli* (Fig 1, 2). Prevalence of bla_{CTX-M} (22.97%) was highest among the ESBL genes, followed by bla_{TEM} (12.16%), bla_{OXA} (6.75%) and bla_{SHV} (2.70%). ESBL gene bla_{SHV} was detected only in cattle-origin *E. coli.* Similarly, bla_{OXA} and bla_{TEM} genes were absent in the goat and cattle isolates, respectively. The bla_{CTX-M} was ubiquitous and present in *E. coli* isolates from cattle, buffalo, sheep and goats. Beta-lactam gene-specific prevalence in different food animal species was also determined among cattle (bla_{SHV} -22.22%; bla_{CTX-M} - 55.55%; bla_{OXA}- 22.22%); buffalo (bla_{TEM} -42.85%; bla_{CTX-M} -42.85%; bla_{OXA}-14.28%); sheep (bla_{TEM} -28.57%; bla_{CTX-M} -42.85%; bla_{OXA} 28.57%); and goat (bla_{TEM} -40%; bla_{CTX-M} -60%).

The antimicrobial resistance and susceptibility pattern of 163 isolates recorded during the study is displayed in Table 2. *Escherichia coli* expressed a high degree of resistance to cefazolin (96.31%) and tetracycline (61.96%),

Table 1: ESBL positivity and ESBL encoding genes in *Escherichia coli.*

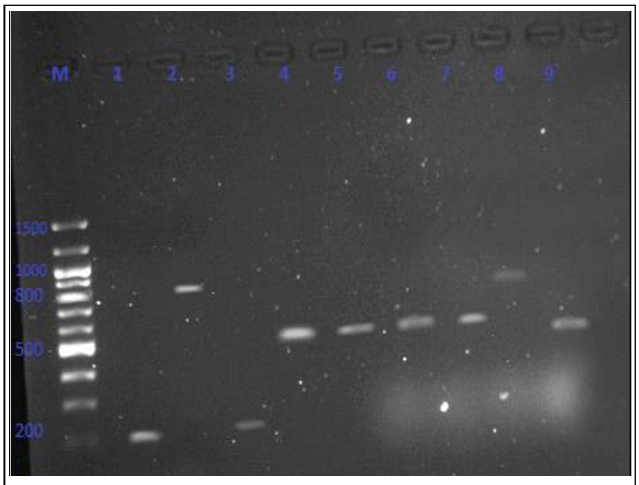
Animal species	Total *E. coli* (Number)	ESBL positive (Number)	Percentage (%)	ESBL genes present (Number)	Percentage (%)	ESBL genes (Number and %)			
						blaSHV	*blaTEM*	*blaCTX-M*	*blaOXA*
Cattle	42	14	33.33	9	64.28	2 (22.22%)	0	5 (55.55%)	2 (22.22%)
Buffalo	38	16	42.10	7	43.75	0	3 (42.85%)	3 (42.85%)	1 (14.28%)
Sheep	37	17	45.94	7	41.17	0	2 (28.57%)	3 (42.85%)	2 (28.57%)
Goat	46	27	58.69	10	37.03	0	4 (40%)	6 (60%)	0
Total	163	74	45.39	33	44.59	2 (2.70%)	9 (12.16%)	17 (22.97%)	5 (6.75%)

followed by kanamycin (52.76%), ampicillin (46.62%), cefixime and ciprofloxacin (42.94%, each). *E. coli* isolates were found highly sensitive to trimethoprim (90.18%), chloramphenicol (81.59%), imipenem (74.84%) and gentamicin (58.28%). *Escherichia coli* isolated from food animals irrespective of species were multi-drug resistant. Almost all the resistant strains exhibited resistance against a minimum of three antimicrobials tested. Greater percentages of isolates were resistant to kanamycin, ampicillin, ciprofloxacin, cefixime, tetracycline and gentamicin irrespective of the livestock species of its origin. In the present study, the majority of the ESBL-positive isolates were resistant to ampicillin, cefazolin, cefixime and tetracycline. Animal-origin ESBL-producing *E. coli* seems to be resistant to multiple antimicrobials (Islam *et al.,* 2016; Seni *et al.,* 2016). The present finding is also in agreement with previous researchers from India on the AMR pattern of *E. coli* isolated from food animals (Mahanti *et al.,* 2013).

Extensive studies were not conducted earlier in India on ESBL characterization of *E. coli* isolated from buffalo, sheep and goats. *E. coli* is an inhabitant of the gastrointestinal tract of all vertebrates and thus its presence in the fecal matter is obvious, however, detection of ESBL-positive strains is alarming. Present findings are in agreement with a study from Bavaria, Germany wherein, the prevalence of ESBL-producing *E. coli* in dairy cows and beef cattle was recorded to the tune of 32.8% (Schmid *et al*., 2013). Comparatively less prevalence of ESBL-positive *E. coli* from fecal samples of healthy cattle (13.7%) and sheep (8.6%) was recorded in Switzerland (Geser *et al.,* 2012). ESBL-carrying *E. coli* were isolated from 12.5% rectal samples of beef cattle from Japan which is far lesser than our findings (Hiroi *et al.,* 2012). Comparatively high prevalence of bla_{CTX-M} and less prevalence of bla_{SHV} were recorded as compared to the findings from India (Kar *et al.,* 2015). A study from China showed that food animals are the major reservoirs of multidrug-resistant *E. coli* (Ho *et al.,* 2011). The global spread and high prevalence of bla_{CTX-M} type ESBL in *E. coli* is a matter of Veterinary Public Health concern. The genetic basis for the global dissemination of bla_{CTX-M} is poorly understood. Molecular studies have shown a strong association of the bla_{CTX-M} gene with conjugative plasmid and successful bacterial clones (Tamang *et al.,* 2013).

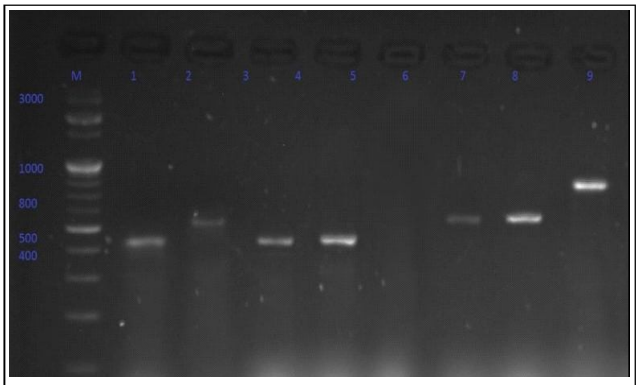
In support of the findings on the buffalo-origin *E. coli,* a study from Iran revealed the prevalence of bla_{CTX-M} (44.8%), bla_{TEM} (35.2%) and absence of bla_{SHV} in the *E. coli* isolated from fecal samples of water buffalo (Hoseini *et al.,* 2014). Buffalo-origin *E. coli* of our study also showed an almost similar pattern and bla_{SHV} was not detected. Diversity in the distribution of ESBL genes was detected in the present study, bla_{CTX-M} and bla_{TEM} were the predominant genes. Reports on ESBL characterization of *E. coli* from fecal samples of sheep and goats are scarce. In our study, more than 45% of sheep and goat isolates were ESBL- positive *E. coli.* Similarly, beta-lactam genes *viz.* bla_{TEM}, blaCTX-M and bla_{OXA} were detected in sheep isolates. Goat isolates were only positive for bla_{TEM} and bla_{CTX-M}. The incidence of ESBL-producing *E. coli* in small ruminants has been poorly studied in most parts of the world (Mandujano *et al.,* 2023). Findings from Turkey revealed bla_{CTX-M} and bla_{SHV} -positive *E. coli* in the fecal samples of sheep (Pehlivanoglu *et al.,* 2016).

Geographical variation persists in the ESBL types and their variants, therefore, the present study is significant in the context of determining the prevalence of ESBL-producing *E. coli* in livestock species. Due to its ubiquitous nature and ecological niche, *E. coli* is used as a sentinel species for studying emerging antimicrobial resistance trends. Some of the studies have also indicated that animals might be responsible for the transfer of ESBL-producing bacteria and the ESBL-encoding genes to humans *via* contact or animal-origin foods (Ewers *et al.,* 2012). These properties make *E. coli* a potential strain in the transmission of AMR at the animal-human interface. The study of animal-to-human



Lane M: 100 bp DNA ladder, Lane 1 and 3: bla_{SHV} (237 bp), Lane 2 and 8: bla_{OXA} (813 bp), Lane 4,5,6,7 and 9: bla_{CTX-M} (593 bp).

Fig 1: Detection of beta lactam genes in *E. coli* isolated from cattle.



Lane M: 100 bp DNA ladder, Lane 1, 3 and 4: bla_{TEM} (445 bp), Lane 2,7 and 8: bla_{CTX-M} (593 bp), Lane 9: bla_{OXA} (813 bp).

Fig 2: Detection of beta lactam genes in *E. coli* isolated from buffalo.

Table 2: Antimicrobial sensitivity and resistance pattern of *Escherichia coli.*

Antimicrobials used	Cattle origin (n=42)		Buffalo origin (n=38)		Sheep origin (n=37)		Goat origin (n=46)	
	Resistant No and %	Sensitive No and %	Resistant No and %	Sensitive No and %	Resistant No and %	Sensitive No and %	Resistant No. and %	Sensitive No and %
Ampicillin	19 (45.23%)	7 (16.66%)	23 (60.52%)	3 (7.89%)	11 (29.72%)	10 (27.02%)	23 (50%)	7 (15.21%)
Cefazolin	38 (90.47%)	2 (4.76%)	38 (100%)	0	36 (97.29%)	1 (2.70%)	45 (97.82%)	0
Cefixime	12 (28.57%)	1 (2.38%)	20 (52.63%)	5 (13.15%)	14 (37.83%)	16 (43.24%)	24 (52.17%)	10 (21.73%)
Gentamicin	10 (23.80%)	23 (54.76%)	3 (7.89%)	22 (57.89%)	9 (24.32%)	19 (51.35%)	4 (8.69%)	31 (67.39%)
Kanamycin	27 (64.28%)	6 (14.21%)	19 (50%)	1 (2.63%)	22 (59.54%)	4(10.81%)	18 (39.13%)	12 (26.08%)
Tetracycline	21 (50%)	9 (21.42%)	32 (84.21%)	3 (7.89%)	20 (54.05%)	12 (32.43%)	28 (60.86%)	3 (6.52%)
Ciprofloxacin	12 (28.57%)	11 (26.19%)	22 (57.89%)	12 (31.57%)	10 (27.02%)	16 (43.24%)	26 (56.50%)	9 (19.56%)
Imipenem	0	31 (73.80%)	1 (2.63%)	13 (34.21%)	0	33 (89.18%)	0	45 (97.82%)
Chloramphenicol	1 (2.38%)	37 (88.09%)	0	35 (92.10%)	1 (2.70%)	28 (75.67%)	1 (2.17%)	33 (71.73%)
Trimethoprim	0	38 (90.47%)	0	36 (94.73%)	0	37 (100%)	2 (4.34%)	36 (78.26%)

transmission of antibiotic resistance thus requires a greater understanding of genetic interaction and spread that occur in the larger arena of commensal and environmental bacteria. For better management of the emergence and dispersal of ESBL type of resistance, a complete analysis of abundance, diversity and dissemination of resistance genes in pathogenic and commensal *E. coli* strains has been suggested (Bajaj *et al.,* 2016).

CONCLUSION

Our study observed that cattle, buffalo, sheep and goats harbor ESBL-producing *E. coli.* The present study reported that ESBL-positive *E. coli* were multidrug-resistant strains and bla_{CTX-M} *and* bla_{TEM} are predominant beta-lactam genes present in them. Significant links between the use of antibiotics in food-producing animals and the development of resistance in indicator bacteria need to be elucidated.

Conflict of interest

All the authors declare that they have no conflict of interest.

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