RESEARCH ARTICLE

Phenotypic and Genotypic Characterization of ESBL Producing *E. coli* Isolates from the Ruminant Species of Namakkal Region, India

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Abstract

Antibiotic resistance in animals is a significant and highly challenging threat among animals. Extended spectrum β-lactamase (ESBL) producing *E. coli* amongst all bacteria have been categorized by the WHO as the most critical AMR pathogen to human health and a major public health concern. In an effort to study the prevalence of ESBL producing coliforms among the ruminant species, phenotypic screening and confirmation by Kirby Bauer diffusion method followed by genotypic characterization of the isolates were carried out to ascertain the type of β-lactamase involved in conferring resistance. A total of 50 fecal swabs were collected from the cattle (n=26), buffalo (n=11), goat (n=10) and sheep (n=3) species. A total of 72 (26/50) % and 68 (34/50) % of the coliform isolates from the ruminant species were found to be phenotypically resistant to most of the β-lactam group of antibiotics and third generation cephalosporins, respectively. The isolates showing phenotypic resistance to third generation cephalosporins were genotypically confirmed by multiplex PCR to determine the types of β-lactamase coding for the resistance. The multiplex PCR for TEM, SHV and OXA type ESBLs revealed the presence of 713 and 564 bp amplicon indicating the predominance of serine β-lactamases, *viz.,* SHV (Class A) and OXA (Class D) type ESBL in 85.7 % and 7.0 % of the coliform isolates of ruminants. This study confirms and emphasizes the prevalence of ESBL producing coliforms and the hidden threat underlying in healthy ruminant species.

Key words: Antimicrobial resistance, *E. coli*. Extended spectrum β-lactamase (ESBL), Genotypic, Multiplex PCR, Phenotypic.

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Introduction

The increasing rate of resistance to antimicrobial agents in the bacterial community of animals is often overlooked in the developing countries due to various constraints like availability over the counter, non-compliance in the use of antimicrobials, economic factors, lack of awareness etc. The important risk factor associated with the increase in antimicrobial resistance is the unnecessary and excessive use of antimicrobials that leads to the dissemination of resistant bacteria and resistance genes in animals and humans (Aguirre *et al.,* 2020: van den Bogaard and Stobberingh, 2000). These resistant populations have an impact on the public health by dissemination of antibiotic resistance genes through food chain. Extended spectrum β-lactamase (ESBL) producing *E. coli* have been categorized by the WHO as the most critical Antimicrobial resistant (AMR) pathogen to human health and a major public health concern. ESBLs now significantly threaten the continued effectiveness of cephalosporins in a number of clinical contexts (Chong *et al.,* 2018).

Broad-spectrum β-lactamase enzymes hydrolyze penicillins, cephalosporins (first-, second- and third-generation), and aztreonam, but not carbapenems. However, these enzymes are inhibited by clavulanate (Paterson and Bonomo, 2005). These enzymes are produced by the Gram negative opportunistic pathogens such as *Enterobacteriaceae* (e.g., *Escherichia coli*) and non-fermenting organisms (e.g., *Pseudomonas aeruginosa*) act by hydrolyzing the amide bond of the four-membered β-lactam ring. The β-lactamases are divided into four classes; the active-site serine β-lactamases (classes A, C and D) and the zinc-dependent or metallo-β-lactamases (MBLs; class B). The class A enzymes comprise the most widely distributed and intensively studied of all β-lactamases, and include PC1, TEM (Datta and Kontomichalou, 1965), SHV (Chaves *et al*., 2001), CTX-M (Bauernfeind *et al*., 1990), and KPC (*K. pneumoniae* carbapenemase) (Rapp and Urban, 2012).

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The ESBL production of the isolates are phenotypically determined by a difference of ≥5 mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate disc is taken to be phenotypic confirmation of ESBL production (CLSI, 2009). The genes encoding β-lactamases, extended-spectrum β-lactamases (ESBLs) could be identified employing a uniplex or multiplex PCR (Sivakumar *et al.,* 2021). The present study was aimed to record the prevalence of the ESBL producing *E. coli* isolates in healthy subjects of different ruminant species considering the potential for transmission either directly to the human populations involved in animal rearing or indirectly through the products like milk and meat obtained from those animals.

Materials and Methods

Samples Collection

A total of 50 fecal swabs were collected from the cattle (n=26), buffalo (n=11), goat (n=10) and sheep (n=3) species reared in the Namakkal district of Tamil Nadu, India during the period of August to December 2022. The samples were collected and transported to the laboratory on ice.

Isolation of *E. coli*

The fecal swabs were inoculated in to 5 mL of sterile nutrient broth and incubated overnight at 37°C. A loop of nutrient broth culture was streaked onto MacConkey agar and incubated overnight at 37°C and the pink colonies (lactose fermenting) were randomly selected from each sample and further inoculated onto the eosin methylene blue agar to confirm the colonies as *E. coli.* Simultaneously, the morphology and biochemical characteristics of the isolates were confirmed by Gram staining and Hi24 *Enterobacteriaceae* Identification Kit (Cat. No. KB016).

Antibiotic Sensitivity Test

The antibiotic sensitivity pattern of the *E. coli* isolates was determined by disc diffusion method following Kirby-Bauer method on Mueller-Hinton Agar (HiMedia India Pvt. Ltd against amoxicillin/clavulanate, cefotaxime, ceftazidime, oxytetracycline, gentamicin and enrofloxacin disc. The isolates which were not susceptible (either resistant or intermediate) to 3 or more antibiotics classes were considered as MDR (Magiorakos *et al.,* 2012).

The *E. coli* isolates exhibiting reduced susceptibility to ceftazidime (30 µg), cefotaxime (30 µg) were further phenotypically characterized for the ESBL production by combination disc method as described by CLSI (2014) using ceftazidime/clavulanic acid- CAC (30/10 µg), cefotaxime/ clavulanic acid CEC (30/10 µg). An increase in ≥5 mm growth inhibition zone for any antimicrobial associated with clavulanic acid in comparison with the inhibition zone of antibiotic tested alone are confirmed as ESBL producers (CLSI, 2016) and the results were interpreted.

Genotypic Characterization of ESBL Isolates by Multiplex PCR

The DNA from the ESBL isolates were extracted by suspending a colony in 100 µL of distilled water and heating at 95˚C for 10 min in a water bath followed by a brief centrifugation. The supernatant containing the DNA (2 µL) was used as template in the multiplex PCR targeting ESBL genes (bla_{OXA}, bla_{SHV}, and bla_{TEM}) in a 50 µL reaction mixture encompassing 25 µL of Taq DNA Polymerase Master Mix RED (Amplicon), 1 µL each forward and reverse primer (bla_{OXA}, bla_{SHV}, and bla_{TEM}), 2 µL template DNA from the isolates and 11 µL nuclease free water. The primers and the amplifying conditions mentioned by Dallenne *et al.* (2010) were followed in this study (Table 1).

PCR amplification of the target genes was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. The amplicons of the PCR amplification were analysed by agarose electrophoresis (1.5 %) at 100 V for one hour alongside 100 bp DNA ladder as a marker and visualized under UV illumination.

Results and Discussion

The nutrient broth culture inoculated with fecal swabs on the MacConkey and EMB agar produced pink, non-mucoid and green metallic sheen colonies, respectively, indicating the isolates as *E. coli.* The Gram staining of the isolates confirmed the presence of Gram negative rods. Further, the biochemical characteristics also endorsed the presence of *E. coli* in the inoculum. The antibiotic sensitivity pattern of the of the isolates against the mentioned antibiotics displayed 72 % (36/50) as multidrug resistant, 75% (38/50) were resistant to beta-lactam group of antibiotics and 68 % (34/50) were resistant to third generation cephalosporins in cattle (Fig. 1),

Table 1: Primers used in the multiplex PCR targeting ESBL genes

β lactamase targeted	Primer name	Primer Sequence 5'-3'	Amplicon Size (bp)
TEM variants including TEM-1 &TEM-2	MultiTSO-T_For	CATTTCCGTGTCGCCCTTATTC	800
	MultiTSO-T_Rev	CGTTCATCCATAGTTGCCTGAC	
SHV variants including SHV-1	MultiTSO-S_For	AGCCGCTTGAGCAAATTAAAC	713
	MultiTSO-S_Rev	ATCCCGCAGATAAATCACCAC	
OXA-1, OXA-4 & OXA-30	MultiTSO-O_For	GGCACCAGATTCAACTTTCAAG	564
	MultiTSO-O_Rev	GACCCCAAGTTTCCTGTAAGTG	

buffalo (Fig. 2) and goats (Fig. 3) denoting the heavy usage of these antibiotics in these species. Interestingly, the *E. coli* isolates of sheep (Fig. 4) did not illustrate resistance to any of the antibiotics tested in the study suggesting the limited use of these antibiotics in this species. The results of this study were in agreement with Abayneh *et al.* (2018), where it was reported that ESBL producing *E. coli* are resistant to other class of antibiotics like fluoroquinolones, aminoglycosides, trimethoprim, sulfonamide, chloramphenicol, and tetracycline.

The phenotypic characterization with combination disc cefotaxime/clavulanic acid - CEC (30/10 µg) indicated that 26.9 (7/26), 81.8 (9/11), 30.0 (3/10) and 0.0 (0/3) % isolates of cattle, buffalo, goat and sheep, respectively, were ESBL producers. Similarly, with ceftazidime/ clavulanic acid - CAC (30/10 µg) disc, 42.3 (11/26), 72.7 (8/11), 20.0 (2/10) and 0.0 (0/3) % isolates of cattle, buffalo, goat and sheep, respectively, were ESBL producers (Fig. 5).

Genotypic characterization by multiplex PCR of the 14 isolates identified phenotypically as ESBL producers revealed the presence of either of the ESBL genes bla_{OXA}, bla_{SHV}, and bla_{TEM} encoded by them. The amplification of 713 bp amplicon (Fig. 6) indicated the predominance of *SHV* type ESBL in 85.7 (24/28) % of the isolates and amplification of both 713 bp as well as 564 bp amplicons indicated the existence of *SHV* and *OXA* type ESBL in 7 (1/28) % of the coliform isolates. The remaining one isolate from goat identified phenotypically as ESBL producer did not show amplification of either of the ESBL genes indicating the absence of all the 3 types of ESBL genes (Fig. 6, Lane 4) signifying the ESBL resistance conferred by some other ESBL gene. Interestingly, none of the isolates possessed the bla_{TEM} type of ESBL gene.

Kanokudom *et al.* (2021) reported that high MDR profiles existed mostly in ESBL positive *E. coli,* which implies a crisis in antibiotic usage. As reported by Liu *et al.* (2016) and Mohsin *et al.* (2017), ESBL genes are located on plasmids, which are usually co-expressed with other plasmid-mediated drug resistance genes, such as quinolone, aminoglycosides, trimethoprim, and tetracyclines which could be the reason for the ESBL isolates of this study for being multidrug resistant.

The findings of this study discloses the moderate to low prevalence of ESBL producing *E. coli* among the ruminants species in this locality and also signifies the likelihood of dissemination of AMR to the human beings as reported by Augirre *et al.* (2020) and in turn to the environmental microbes through horizontal transmission. The hidden threat of AMR from the one health perspective needs to be deliberated critically to save the life saving drugs for the near future.

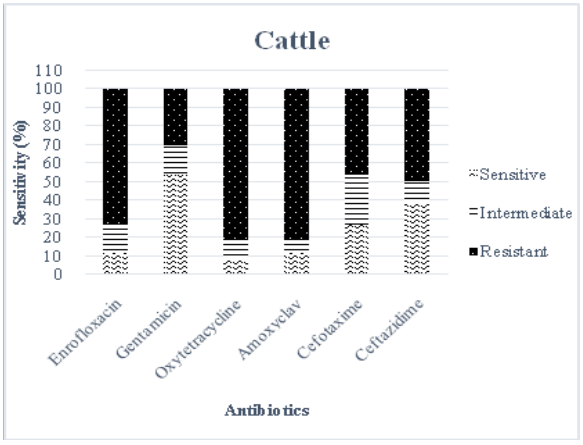

Fig. 1: AMR pattern in *E.coli* isolates ofCattle

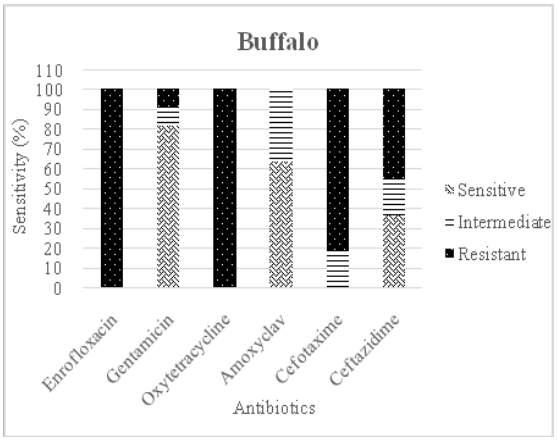

Fig. 2: AMR pattern in *E.coli* isolates of Buffalo

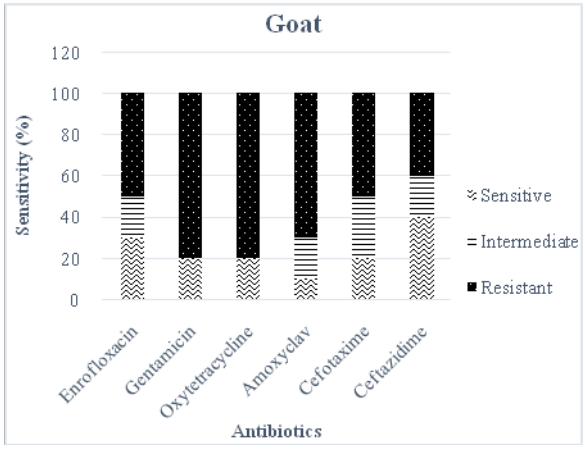

Fig. 3: AMR pattern in *E.coli* isolates of Goat

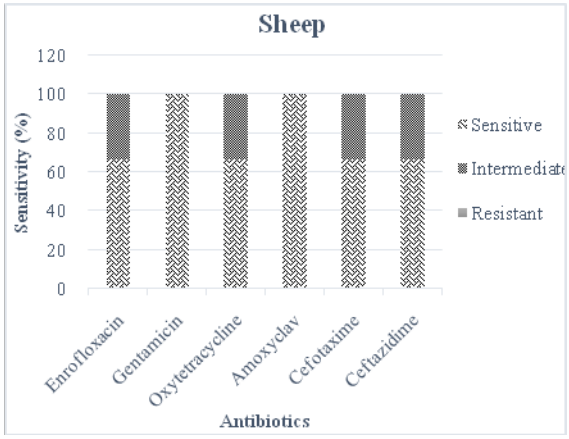

Fig. 4: AMR pattern in *E.coli* isolates of Sheep

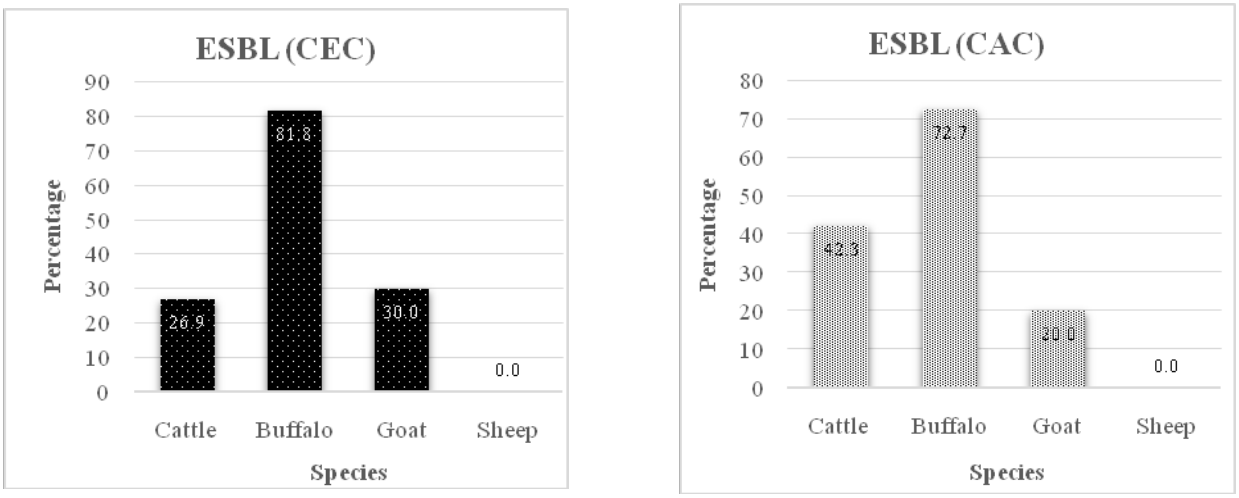

Fig. 5: Prevalence of ESBL producing *E. coli* among ruminant species by combination disc method

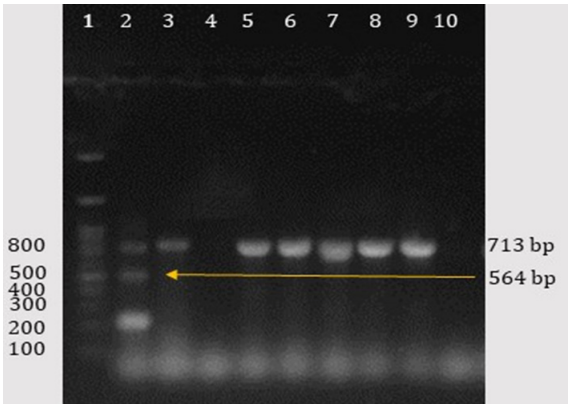

Fig.6: Genotypic characterization by multiplex PCR (TEM, SHV and OXA genes):
Lane 1: 100 bp DNA ladder; Lane 2: Isolate having both 564 bp amplicon indicating the OXA type and 713 bp amplicon SHV type ESBL genes; Lane 3, 5, 6, 7, 8, 9: Isolate having 713 bp amplicon SHV type ESBL gene alone; Lane 4: Absence of all the 3 types of ESBL genes; Lane 10: NTC

Conclusion

The results of the phenotypic characterization of ESBL producing *E. coli* by combination disc correlates very well by genotypic assessment too, and the presence of ESBL *E. coli* in the faecal samples of the ruminants could be a source of infection to other animals and humans. The genotypic characterization gives the insight on the type of ESBL gene involved in conferring resistance to beta-lactam antibiotics. Hence, multiplex PCR targeting more than one gene would be a rapid, reliable and cost effective approach to identify the gene responsible for resistance to beta-lactam antibiotics.

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References

Abayneh, M., Tesfaw, G., &Abdissa, A. (2018). Isolation of extended-spectrum β-lactamase- (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* from patients with community-onset urinary tract infections in Jimma University Specialized Hospital, Southwest Ethiopia. *Canadian Journal of Infectious Diseases and Medical Microbiol*ogy, *2018,* 8 pages. https://doi.org/10.1155/2018/4846159.

Aguirre, L., Vidal, A., Seminati, C., Tello, M., Redondo, N., Darwich, L., & Martín, M. (2020). Antimicrobial resistance profile and prevalence of extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamases and colistin resistance (mcr) genes in *Escherichia coli* from swine between 1999 and 2018. *Porcine Health Management*, *6*(1), 1-6.

Bauernfeind, A., Schweighart, S., & Grimm, H. (1990). A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection*, *18*(5), 294-298.

Chaves, J., Ladona, M.G., Segura, C., Coira, A., Reig, R., & Ampurdanés, C. (2001). SHV-1 β-lactamase is mainly a chromosomally

encoded species-specific enzyme in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, *45*(10), 2856-2861.

Chong, Y., Shimoda, S., & Shimono, N. (2018). Current epidemiology, genetic evolution and clinical impact of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. *Infection, Genetics and Evolution*, *61*, 185-188.

CLSI - Clinical and Laboratory Standards Institute. (2009). Performance Standards for Antimicrobial Susceptibility Testing: *19th Informational Supplement M100-S19. Wayne*, PA: CLSI.

CLSI - Clinical and Laboratory Standards Institute. (2014). Performance standards for antimicrobial susceptibility testing: 24th Informational supplement, *CLSI document M100-S24*. Wayne, PA: CLSI.

CLSI - Clinical and Laboratory Standards Institute. (2016). Performance Standards for Antimicrobial Susceptibility Testing (M100S). Wayne, PA: CLSI.

Dallenne, C., Da Costa, A., Decré, D., Favier, C., & Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. *Journal of Antimicrobial Chemotherapy*, *65*(3), 490-495.

Datta, N., & Kontomichalou, P. (1965). Penicillinase synthesis controlled by infectious R factors in *Enterobacteriaceae*. *Nature*, *208*, 239-41.

Kanokudom, S., Assawakongkarat, T., Akeda, Y., Ratthawongjirakul, P., Chuanchuen, R., & Chaichanawongsaroj, N. (2021). Rapid detection of extended spectrum β-lactamase producing *Escherichia coli* isolated from fresh pork meat and pig cecum samples using multiplex recombinase polymerase amplification and lateral flow strip analysis. *PLoS One*, *16*(3), e0248536.

Liu, X., Thungrat, K., & Boothe, D.M. (2016). Occurrence of OXA-48 carbapenemase and other β-lactamase genes in ESBL-producing multidrug resistant *Escherichia coli* from dogs and cats in the United States, 2009-2013. *Frontiers in Microbiology*, *7*, 1057.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Monnet, D.L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3), 268-281.

Mohsin, M., Raza, S., Schaufler, K., Roschanski, N., Sarwar, F., Semmler, T., ... & Guenther, S. (2017). High prevalence of CTX-M-15-Type ESBL-producing *E. coli* from migratory avian species in Pakistan. *Frontiers in Microbiology*, *8*, 2476.

Paterson, D.L., & Bonomo, R.A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews, 18*(4), 657-686.

Rapp, R.P., & Urban, C. (2012). *Klebsiella pneumoniae* carbapenemases in *Enterobacteriaceae*: history, evolution, and microbiology concerns. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *32*(5), 399-407.

Sivakumar, M., Abass, G., Vivekanandhan, R., Anukampa, Singh, D.K., Bhilegaonkar, K., & Dubal, Z. (2021). Extended-spectrum beta-lactamase (ESBL) producing and multidrug-resistant *Escherichia coli* in street foods: A public health concern. *Journal of Food Science and Technology*, *58*, 1247-1261.

Van den Bogaard, A.E., & Stobberingh, E.E. (2000). Epidemiology of resistance to antibiotics: Links between animals and humans. *International Journal of Antimicrobial Agents*, *14*(4), 327-335.