

OCCURRENCE OF BETA-LACTAM RESISTANT *ESCHERICHIA COLI* AMONG CLINICAL CASES OF LIVESTOCK IN ANDHRA PRADESH

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Abstract: A total of 83 clinical samples encompassing diarrhoeic faeces (30), mastitis milk (25) and endometritis uterine fluid samples (28) were collected from cattle, buffaloes and sheep presented to Veterinary polyclinics of Chittoor and Gudivada, Andhra Pradesh. Out of 83 samples, 52 (62.6%) were found positive for *E. coli* based on cultural and biochemical tests as well as by PCR targeting *E16S* gene. Serological typing of PCR confirmed *E. coli* (52) revealed O120 (9 isolates), O83 (6), O84 (5), O44 (4), O15 (4), O9 (3), O49 (2) serotypes and 19 isolates were found to be 'O' group untypable (UT). Extended spectrum beta-lactamase (ESBL) resistant phenotype was detected in nine (17.3%) isolates using combination disc method. Multiplex PCR assay for the detection of beta-lactamase (*bla*) genes revealed 13 (25%) isolates to possess any one of the three beta-lactamase genes. Incidence of *bla*_{SHV}, *bla*_{TEM} and *bla*_{OXA} genes in *E. coli* was found to be 15.3, 9.6 and 5.7%. The present study revealed the presence of beta-lactamase antimicrobial resistance in *E. coli*, which may pose problem in the treatment of infections in which it is implicated.

Keywords: Beta-lactamase, *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, *E.coli*, *E16S* gene, ESBL.

INTRODUCTION

Extended-spectrum beta-lactamase (ESBL) production in *E. coli* is of particular significance that confer resistance to beta-lactam antibiotics like penicillins, first, second, third generation cephalosporins, monobactams and are inhibited by beta-lactamase inhibitors (Bush and Jacoby, 2010). Detection of beta-lactamase resistance in advance helps in selection of suitable antibiotics to avoid treatment failures. Beta-lactamase (*bla*) genes located on the bacterial plasmids or on the chromosome mediate beta-lactamase production (Bush *et al.*, 1995).

Beta-lactamase genes *bla*_{TEM} (Temoniera beta-lactamase), *bla*_{SHV} (sulfhydryl variable beta-lactamase), *bla*_{OXA} (oxacillinase beta-lactamase) encodes broad spectrum beta-lactamases that confer resistance to first and second generation cephalosporins (Bush and Jacoby, 2010).

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Point mutations in TEM, SHV and OXA beta-lactamases extended their substrate profile to third generation cephalosporins over the years, leading to evolution of TEM, SHV, and OXA type ESBLs (Bush and Jacoby, 2010). Over the past few decades, production of beta-lactamases among gram-negative bacteria has increased drastically (Dallenne *et al.*, 2010). The present study aimed at the phenotypic and molecular detection of beta-lactamase resistance in *E. coli* isolated from clinical cases of cattle, buffaloes and sheep in Andhra Pradesh, India.

MATERIALS AND METHODS

Sample collection: A total of 83 clinical samples encompassing diarrhoeic faeces from cattle (12), buffaloes (11) and sheep (7); mastitis milk from cattle (14) and buffaloes (11); endometritis uterine fluids from of cattle (16) and buffaloes (12) were collected from livestock presented to Veterinary polyclinics of Chittoor and Gudivada, Andhra Pradesh.

Isolation and identification of *E. coli*: Clinical samples were inoculated into nutrient broth and incubated aerobically at 37°C for 24 h. Loopful of enriched broth culture was streaked onto MacConkey agar and eosin methylene blue (EMB) agar plates, incubated at 37°C for 24 h. Lactose fermenting pink colonies and metallic sheen colonies were subjected to standard biochemical tests for the characterization of *E. coli* (Sneath and Holt, 2001). Biochemically characterized *E. coli* isolates were subjected to PCR using primer pair (F, 5'- ATC AAC CGA GAT TCC CCC AGT-3' and R, 5'- TCA CTA TCG GTC AGT CAG GAG-3') targeting *E16S* gene (Sun *et al.*, 2011). Reaction mixture was optimized in 25 µl volume containing 1.5 µl of DNA template; 2.5 µl of *Taq* buffer [10x]; 0.5 µl of dNTP mix [10mM]; 1.5 µl of MgCl₂ [25mM]; 1 µl each of forward and reverse primer [10 pmol/µl]; 1 µl of *Taq* DNA polymerase [1 U/µl] and 16.0 µl of nuclease free water for the amplification of *E16S* gene (231 bp). The DNA of *E. coli* (ATCC 25922) was used as positive control and amplification was performed in Kyratec thermal cycler (Australia) under the following standardized cycling conditions: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec, final elongation at 72 °C for 10 min.

Serotyping of *E. coli* strains: All the PCR confirmed *E. coli* isolates were serotyped on the basis of their 'O' antigen at National *Salmonella* and *Escherichia* Centre (NSEC), Kasauli (Himachal Pradesh, India).

Phenotypic screening and confirmation of ESBL resistance: Clinical and Laboratory Standards Institute (CLSI) recommends two-step procedure for ESBL detection, which

includes an initial 'screening test' followed by 'confirmatory test'. Isolates were screened for resistance against four indicator antibiotics i.e. cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg) and aztreonam (AT, 30 µg) by disc diffusion method on Mueller Hinton agar (Bauer *et al.*, 1966). Resistance to at least one of the indicator antibiotics was considered as 'positive' screening test (Drieux *et al.*, 2008 and CLSI, 2014). Positive isolates in the screening test were subjected to 'confirmatory test' using combination disc method where three pairs of discs (i.e., with and without clavulanic acid) i.e. ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg) were placed. ESBL production was confirmed when the inhibition zone diameter around combination discs was ≥ 5 mm when compared to discs containing respective cephalosporin alone (Drieux *et al.*, 2008 and CLSI, 2014).

Detection of beta-lactamase (*bla*) genes: Multiplex PCR assay was standardized for the detection of beta-lactamase genes *bla*_{TEM} (F: CAT TTC CGT GTC GCC CTT ATT C and R: CGT TCA TCC ATA GTT GCC TGA C), *bla*_{SHV} (F: AGC CGC TTG AGC AAA TTA AAC and R: ATC CCG CAG ATA AAT CAC CAC) and *bla*_{OXA} (F: GGC ACC AGA TTC AAC TTT CAA G and R: GAC CCC AAG TTT CCT GTA AGT G) with amplicon sizes of 800, 713 and 564 bp, respectively (Dallenne *et al.*, 2010). Known positive DNA was used as positive control. Reaction mixture was optimized in 25 µl volume containing 2 µl of DNA template; 3 µl of *Taq* buffer [10x]; 1 µl of dNTP mix [10mM]; 1.5 µl of MgCl₂ [25mM]; three forward primers [10 pmol/µl] - each 0.5 µl; three reverse primers [10 pmol/µl] - each 0.5 µl; 1 µl of *Taq* DNA polymerase (1 U/µl) and 13.5 µl of nuclease free water. PCR assay was standardized in Kyratec thermal cycler (Australia) under the following standardized cycling conditions - initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, elongation at 72°C for 1 min, final elongation at 72 °C for 7 min.

RESULTS AND DISCUSSION

Out of 83 clinical samples analyzed, *E. coli* was isolated from 52 (62.6%) samples. Among diarrhoeic faecal samples, *E. coli* was predominantly isolated from cattle (10/12, 83.3%) followed by buffaloes (9/11, 81.8%) and sheep (5/7, 71.4%). *E. coli* was isolated from 64.2 (9/14) and 54.5% (6/11) of mastitis milk samples collected from cattle and buffaloes, respectively and 43.7 (7/16) and 50.0% (6/12) of uterine fluid samples collected from endometritis cases of cattle and buffaloes, respectively. All the biochemically characterized

E. coli isolates were further confirmed using PCR targeting *E16S* gene. Gel photograph of *E16S* gene amplicons was shown in Figure 1.(A).

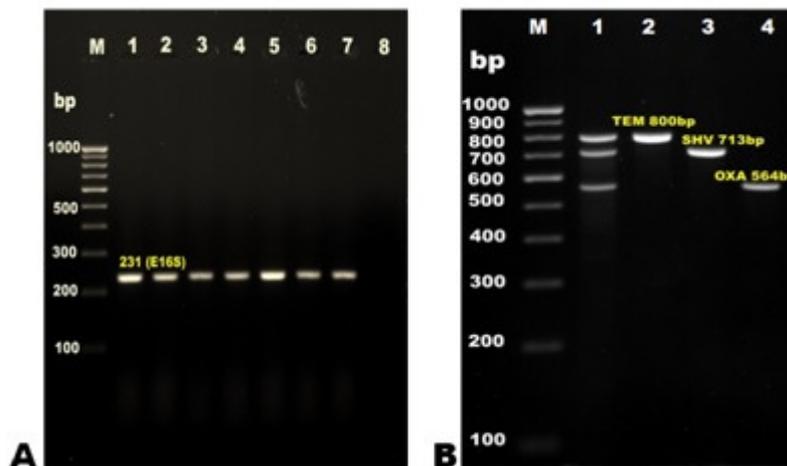


Fig. 1: (A). Gel photograph of amplicons of *E16S* gene (231 bp) PCR for the confirmation of *E. coli* isolates. Lane M: Molecular weight marker (100 bp), Lane 1: DNA standard of *E. coli* (ATCC 25922), Lane 2 and 7: *E. coli* isolates, Lane 8: Negative control. (B). Gel photograph of amplicons of *bla*_{TEM}, *bla*_{SHV} and *bla*_{OXA} genes. Lane M: Molecular weight marker (100 bp), L1: DNA standard positive for *bla*_{TEM}, *bla*_{SHV} and *bla*_{OXA} genes, L2: *bla*_{TEM} gene (800 bp) of *E. coli* isolate, L3: *bla*_{SHV} gene (713 bp) of *E. coli* isolate, L4: *bla*_{OXA} gene (564 bp) of *E. coli* isolate.

Wani *et al.* (2003) observed isolation of *E. coli* from 63.6 and 59.4% of diarrhoeic cattle and sheep, respectively. Rangel and Marin (2009) reported 34.4% isolation of *E. coli* from bovine mastitic milk. In a study by Ingale *et al.* (2016) from Uttar Pradesh, 24.2% incidence of *E. coli* in endometritis uterine fluid samples of buffaloes was observed. Increased incidence of *E. coli* in endometritis cases might be due to unhygienic artificial insemination practices resulting in uterine contamination with dung (Ingale *et al.*, 2016).

Serological typing of PCR confirmed *E. coli* (52) revealed O120 (9 isolates), O83 (6), O84 (5), O44 (4), O15 (4), O9 (3), O49 (2) serotypes and 19 isolates were found to be 'O' group untypable (UT). Among *E. coli* isolated from diarrhoeic cattle, O120 serotype was the frequent one detected (4 of 10) followed by O84 (3), O83 (2) and O49 (1). Among *E. coli* isolated from diarrhoeic buffaloes, O120 serotype was the frequent one detected (5 of 9) followed by O84 (2), O44 (1) and O49 (1). Of the *E. coli* isolated from diarrhoeic sheep, O83 (4) and O44 (1) serotypes were detected. Of the *E. coli* isolated from mastitic cattle, O44 (2) was the frequent serotype detected whereas seven isolates were found to be O-group untypable (UT). Of the *E. coli* isolated from mastitic buffaloes, O9 (2) was the frequent serotype detected whereas four isolates were found to be O-group untypable (UT). Of the *E.*

coli isolated from endometritic cattle, O15 (4) was the frequent serotype detected whereas three isolates were found to be O-group untypable (UT). Of the *E. coli* isolated from endometritic buffaloes, UT (5) and O9 (1) serotypes were detected.

Mohammad *et al.* (1986) reported isolation of O3, O5, O6, O7, O20, O21, O49, O75, O76, O80, O84, O86, O88, O103, O116, O117, O118, O137, O153 and O159 serotypes of *E. coli* from diarrhoeic cattle and buffaloes. Bandyopadhyay *et al.* (2011) reported O-serogroups O2, O8, O25, O30, O60, O75, O86, O113, O120, O127, O146, O159, O170 and O172 among *E. coli* from diarrhoeic sheep in Arunachal Pradesh, India. Majority of *E. coli* (30.5%) isolated from acute coliform mastitis of cattle were reported to be untypable (UT) in studies conducted by Wenz *et al.* (2006) from Colorado. Wenz *et al.* (2006) isolated O2, O3, O8, O15, O16, O18, O19, O20, O21, O25, O36, O37, O48, O53, O65, O78, O82, O86, O113, O129, O135, O140, O141, O146, O158 and O169 serotypes of *E. coli* from acute coliform mastitis cases of cattle in Colorado.

Thirteen out of 52 (25.0%) *E. coli* isolates screened were found to be resistant to one or more of the indicator antibiotics, with an overall incidence of 25.0% beta-lactamase resistance, which includes seven (7/24, 29.1%) isolates from diarrhoeic faeces, five (5/15, 33.3%) from mastitis milk and one (1/13, 7.6%) from uterine fluid samples. Overall incidence of resistance to ceftazidime, ceftriaxone, aztreonam and cefotaxime was found to be 11.5 (6/52), 11.5 (6/52), 7.6 (4/52) and 5.7% (3/52), respectively. Out of 13 *E. coli* isolates that were found positive in screening test, ESBL production was confirmed in nine isolates by combination disc method, which includes five (5/24, 20.8%) isolates from diarrhoeic faeces, three (3/15, 20.0%) from mastitis milk and one (1/13, 7.6%) from uterine fluid samples. In the remaining four isolates, enhancement of inhibition zone by ≥ 5 mm in the presence of clavulanic acid was not detected. This might be due to concurrent production of other non-ESBL beta-lactamases that were resistant to beta-lactamase inhibitors, masking the synergy in the confirmatory test (Drieux *et al.*, 2008). The overall incidence of ESBL phenotype in *E. coli* was found to be 17.3% (9/52).

Rangel and Marin (2009) reported resistance to ceftriaxone (17.7%) among *E. coli* strains isolated from mastitis milk. In a study on antimicrobial sensitivity of *E. coli* strains isolated from endometritis cases of buffaloes in Uttar Pradesh, 6% of isolates were reported to be resistant to cefotaxime/clavulanic acid combination (Ingale *et al.*, 2016). Kar *et al.* (2015) observed 3.1% incidence of ESBL phenotype among *E. coli* isolated from mastitis milk, with

100% resistance to cefotaxime, ceftriaxone, ceftazidime and aztreonam. Haenni *et al.* (2014) observed 29.4% incidence of ESBL phenotype among *E. coli* isolated from cattle.

Multiplex PCR assay for the detection of beta-lactamase genes revealed an overall incidence of 25.0% (13/52) beta-lactamase genes. Among the 52 *E. coli* isolates, one or more beta-lactamase genes were detected in a total of 13 (25.0%) isolates (i.e. 3 isolates were positive for *bla*_{TEM}; 6 for *bla*_{SHV}; 1 for *bla*_{OXA}; 1 for *bla*_{TEM} and *bla*_{SHV}; 1 for *bla*_{TEM} and *bla*_{OXA}; 1 for *bla*_{SHV} and *bla*_{OXA}). Beta-lactamase genes detected were as follows – *bla*_{SHV} gene in 8 (15.3%) isolates followed by *bla*_{TEM} gene in 5 (9.6%) and *bla*_{OXA} gene in 3 (5.7%) isolates. Gel photograph of *bla*_{TEM}, *bla*_{SHV} and *bla*_{OXA} amplicons was shown in Figure 1(B). Beta-lactamase genes were detected in all the 13 *E. coli* isolates with beta-lactam ‘resistant’ phenotype. Beta-lactamase genes were not at all detected among the 39 *E. coli* isolates with beta-lactam ‘sensitive’ phenotype. Kar *et al.* (2015) reported the detection of *bla*_{TEM} and *bla*_{SHV} genes in *E. coli* isolates with ESBL phenotype from cattle mastitis milk. Valat *et al.* (2012) observed 3.4% incidence of *bla*_{TEM} gene among *E. coli* strains in cattle.

Conclusion

The present study revealed the occurrence of beta-lactam resistant *E. coli* among various clinical cases of livestock in Andhra Pradesh, with an overall incidence of 25% beta-lactam resistance and 17.3% incidence of ESBL phenotype, which may pose problem in the therapeutics of *E. coli* associated diarrhea, endometritis and mastitis. The present study also signifies the need for a comprehensive antimicrobial surveillance programme to determine the prevalence and distribution of beta-lactamase resistant types among various pathogenic bacteria of livestock in India.

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