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Prevalence, molecular characterization and antimicrobial resistance of *E. coli* isolates from diarrhoeic lambs in Andhra Pradesh

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Abstract
A study was carried out on isolation, molecular characterization and antimicrobial resistance of *E. coli* isolated from faecal samples of diarrhoeic lambs with the aim to detect antimicrobial resistance in *E. coli* associated diarrhoeic lambs of this geographic region. A total of 212 faecal samples from diarrhoeic lambs of 1-7, 8-30, 31-60 and 61-90 days age groups were collected from Vizianagaram, West Godavari, Krishna, districts of Andhra Pradesh of which 170 *E. coli* were isolated with a prevalence rate of 80.18%. After cultural and biochemical confirmation of *E. coli* the isolates were further confirmed by molecular characterization. The antimicrobial resistance of isolates was tested by disk diffusion method. Among the three districts, higher prevalence of *E. coli* in diarrhoeic lambs was recorded in West Godavari district (86.41%), while lower (72.83%) prevalence was found in Krishna district. Irrespective of the districts, higher prevalence of *E. coli* was observed in 1-7 day-old diarrhoeic lambs (84%), while lower prevalence was detected in 61-90 day-old age group (72.72%). This study detected highest antibiotic resistance in *E. coli* isolates against colistin (98.82%) and sulphamethizole (89.41%), while enrofloxacin (5.88%), gentamicin (5.33%) and chloramphenicol (1.17%) were effective. This study provided baseline data on prevalence of *E. coli* associated diarrhoea and antimicrobial resistance against commonly used antimicrobials in sheep.

Keywords: Prevalence, molecular characterization, antimicrobial resistance, *E. coli*, lambs

1. Introduction
Diarrhoea in young lambs is a syndrome of great aetiological complexity that causes economic losses directly through mortality and indirectly from poor growth. The diarrhoea may be due to bacteria, virus, parasites and other aetiological agents while *Escherichia coli* (*E. coli*) is getting recognized as a leading cause (Islam *et al*., 2015) [9]. *E. coli* is a Gram-negative, rod-shaped, flagellated, non-sporulating and facultative anaerobic bacterium of the family *Enterobacteriaceae*, best known as commensal that grows in human and animal gut lumen (Buxton and Fraser 1977) [4]. According to the virulence properties of the organism and clinical symptoms in the host, diarrhoeagenic *E. coli* were recognized as six major pathotypes. These are Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Shiga-like toxin producing *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* and Diffusely Adherent *E. coli* (DAEC) (Nataro and Kaper, 1998) [15].
Antimicrobials are commonly used for therapy as well as for prophylactic and growth promotion purposes in this geographic area. However, indiscriminate use of antimicrobials since several decades, the development of drug resistance and the presence of multi drug resistant pathogenic bacteria increasing and were responsible for therapeutic failures in treating lamb diarrhoeia cases (Kumar *et al*., 2012) [10].
Therefore, the present study was undertaken on isolation, molecular characterization and antimicrobial resistance of *E. coli* isolated from faecal samples of diarrhoeic lambs with the aim to detect antimicrobial resistance in *E. coli* from diarrhoeic lambs of this geographic region.

2. Material and Methods

2.1 Sample collection

A total of 212 faecal samples from diarrhoeic lambs of 1 to 90 days age group were collected at random from organized sheep farms and individual flocks of Vizianagaram, West Godavari, and Krishna Districts of Andhra Pradesh (AP) during the period from October 2017 to June 2018. Age and sex of diarrhoeic lambs were recorded during sampling. The faecal samples were collected from rectum using sterile cotton swabs. After collection, the swabs were immediately transported to the Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram in ice-cooled containers for *E. coli* isolation.

2.2 Isolation and identification of *E. coli*

The rectal swabs were streaked on MacConkey (Hi Media) agar plates and incubated overnight at 37 ºC for 24h. The plates with pink colonies were selected and inoculated on Eosin methylene blue (EMB) agar plate and re-incubated at 37 ºC for 24h. Typical greenish metallic sheen colonies on EMB were tested for Gram's staining and motility. The organisms which were Gram negative were further tested for motility. The organisms which were Gram negative and motile were subsequently grown on nutrient agar (NA) slants in duplicate and stored at 4 ^{0}C for further biochemical tests (Hitchins *et al.,* 1992) [8] and (Cruikshank *et al.,* 1975) [6] and molecular characterization.

2.3 Biochemical tests

All the 170 *E. coli* isolates were subjected to biochemical tests such as IMViC tests, triple Sugar Iron agar test, oxidase test, catalase test, urease and nitrate tests. All the isolates were positive for indole and methyl red tests showing red coloured ring in indole test and red colour in methyl red test and were negative for voges-proskauer and citrate utilization tests. Triple sugar iron agar test showed yellow slant and butt, inferring acidic butt; acidic slant with gas production and no H_2S production. Oxidase disc showed colourless reaction, catalase test showed gas bubble production, urease test does not show any colour production and nitrate test produces red colour confirming *E. coli*. All the biochemical tests confirmed the presence of *E. coli.*

2.4 Molecular Characterization of *E. coli*

The biochemical results were confirmed by PCR amplification using *E. coli* 16s rRNA specific primers quoted by Sun Dong-bo *et al.* (2011) [7] (E16S-F: ATCAACCGAGATTCCCCCAGT E16S-R: TCACTATCGGTCAGTCAGGAG) with 231bp amplified product.

2.5 PCR conditions for detection *E.coli 16SrRNA*

PCR reactions were carried out in an Eppendorf thermal cycler. The amplification conditions were 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 1 min, 50 °C for 50 s, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. DNA amplified by PCR was subjected to 2% agarose gel electrophoresis as described by Sambrook and Russel (2001) [18].

Table 1: Optimized PCR mixture for *E. coli* specific PCR

***E. coli* specific uniplex PCR**		
Sl. No	**Components**	**Volume/ reaction**
1	Master mix	12.5µL
2	Forward primer (10 pmol/µL)	0.62µL
3	Reverse primer (10 pmol/µL)	0.62µL
4	Nuclease free water	10µL
5	Template DNA (50 ng/µL)	1.26µL
	Total	25µL

2.6 Detection of antimicrobial resistance

Antibiogram of *E. coli* isolates from lamb diarrhoea cases in the present study was carried out by Kirby Bauer disc diffusion method (Bauer *et al.,* 1966) [3] against 17 different and most commonly used antibiotics in veterinary practice. Susceptibility patterns of *E. coli* isolates were studied as per the zone diameter interpretative break points for *Enterobacteriaceae* as given in CLSI guidelines (CLSI M100-S24 document, 2018) [5].

Initially, *E. coli* isolates were sub-cultured in nutrient broth and incubated for 24 h at 37 °C. The turbidity was adjusted to 0.5 McFarland (equivalent to an approximate cell density of 1.5 x 108 CFU/ml, having absorbance of 0.132 at wavelength of 600 nm) units. About 200 µL of each inoculum was seeded on the Mueller Hinton (MH) agar using sterile cotton-tipped swab. Plates were allowed to dry and antibiotic discs were placed aseptically with sterile fine forceps. The plates were incubated at 37 °C for 48 h under favourable conditions and the diameter of the inhibition zones was measured to determine antibiotic susceptibility/resistance patterns for each isolate.

3. Results and Discussion

Screening of all the faecal samples (212) for *E. coli* by initial cultural and biochemical methods and furthur confirmation of *E. coli* by specific PCR yielding a 231 bp product of E16S rRNA gene revealed that 170 out of 212 samples were positive for *E. coli*, giving an overall prevalence rate of 80.18%. Similar to the present results, higher prevalence of *E. coli* in diarrhoeic buffalo calves (80.53%) reported by Srivani *et al.* (2017) [19] in AP, India and in diarrhoeic lambs (84%) by Aklilu *et al.* (2013) [2] in Ethiopia. However, compared to the prevalence rate observed in this study, lower prevalence rate of *E. coli* was reported in diarrhoeic lambs by Nasr *et al.* (2014) [14] in Egypt (34.20%) and Wani *et al.* (2008) [21] in Kashmir valley, India (22.18%).

Among various districts, higher prevalence of *E. coli* in diarrhoeic lambs was observed in West Godavari district (86.41%), while lower (72.83%) prevalence was found in Krishna district. The differences in the prevalence rates of *E. coli* may not be proper to compare between various geographic areas since feeding and management practices may vary from one area to another.

The present study detected higher prevalence of *E. coli* associated diarrhoea in lambs of younger age compared to older lambs. Higher prevalence (84%) was detected in faecal samples of one to seven day old lambs, followed by 80.80, 80.32 and 72.72% observed in 8-30, 31-60 and 61-90 day old lambs, respectively. The present results are corroborated with the findings of Srivani *et al*. (2017) [19] and Abdulgayeid *et al*. (2015) [1] who reported higher a prevalence of *E. coli* in 1-7 day old calves compared to older calves.

Higher prevalence of *E. coli* in young lambs may be due to poor immune status due to delay in first colostrum and ill developed rumen compared to older lambs, and also due to poor hygienic conditions in the farms. Matte *et al*. (1982) [12] also found that 61% of colostral immunoglobulin containing 80g/ml of IgG absorbed in six hours and decreases sharply, thereafter. This indicates that the first six hours are the period in which maximum absorption of colostral immunoglobulins takes place.

The present study observed that *E. coli* isolates from diarrhoeic lambs were resistant to at least one of the antimicrobials tested. The per cent of antimicrobial resistance observed in *E. coli* isolates from diarrhoeic lambs in the descending order was 98.82, 89.41, 73.52, 70.58, 67.64, 67.05, 58.23, 48.82, 34.11, 24.7 21.76, 20, 18.83, 10.58, 5.88, 5.3 and 1.17 to colistin, sulphamethazole, ceftazidime, cefotaxime, aztreonam, ceftriaxone, tetracycline, ampicillin, streptomycin, co-trimoxazole,ceftazidime/clavulanicacid, cefotaxime/clavualanic acid, amoxicillin-clavulanicacid, nitrofurantoin, enrofloxacin, gentamicin, chloramphenicol, respectively.

Higher antibiotic resistance was observed in *E. coli* isolates against colistin (98.82%) and sulphamethizole (89.41%), while enrofloxacin (5.88%), gentamicin (5.3%) and chloramphenicol (1.17%) were effective. Higher colistin resistance observed in this study might be due to sub-therapeutic, low doses of polymixins are being fed to lambs as growth promoters. The emergence of colistin resistance among clinical isolates of *Enterobacteriaceae* has been reported in recent years due to increase its use for carbapenem resistant isolates (Livermore and woodford, 2006 [11] and Walsh, 2010) [20]. The association of colistin resistance by plasmid that can be transferred between different Gram negative bacilli leads to arise of other danger in the era of antibiotic resistance and making the study of the presence of colistin resistance an important issue to limit its spread (WHO 2016) [22]. The present results are in accordance with the findings of Nguyen *et al*. (2016) [13] who revealed increased resistance in commensal *E. coli* due to extensive use of colistin in livestock and poultry industry.

The occurrence of colistin resistance based on the plasmid-encoded *mcr-1* gene in *Enterobacteriaceae* has been described in different European countries since it was first reported in November 2015 at European Centre for Disease Prevention and Control (ECDC). *E. coli* isolates in this geographical area were effectively inhibited by chloramphenicol which might be due to limited usage of this antibiotic in lambs. Similar to the present results Paul *et al*. (2010) [17] also observed higher sensitivity for *E. coli* isolates to chloramphenicol in Bangladesh.

The present study also detected multidrug resistance in 89 of 170 *E. coli* isolates (52.35%). Parallel to the present findings, Pereira *et al*. (2011) [16] reported 81% of the *E. coli* isolates obtained from faecal samples of calves were multidrug resistant. Increased prevalence of multidrug resistance in *E. coli* isolates from diarrhoeic lambs is an indication of indiscriminate use of antimicrobials in lambs. Therefore, awareness may be required for judicious use of antimicrobials in lamb production. Further, this study provided baseline data on prevalence of *E. coli* associated diarrhoea and antimicrobial resistance against commonly used antimicrobials in sheep.

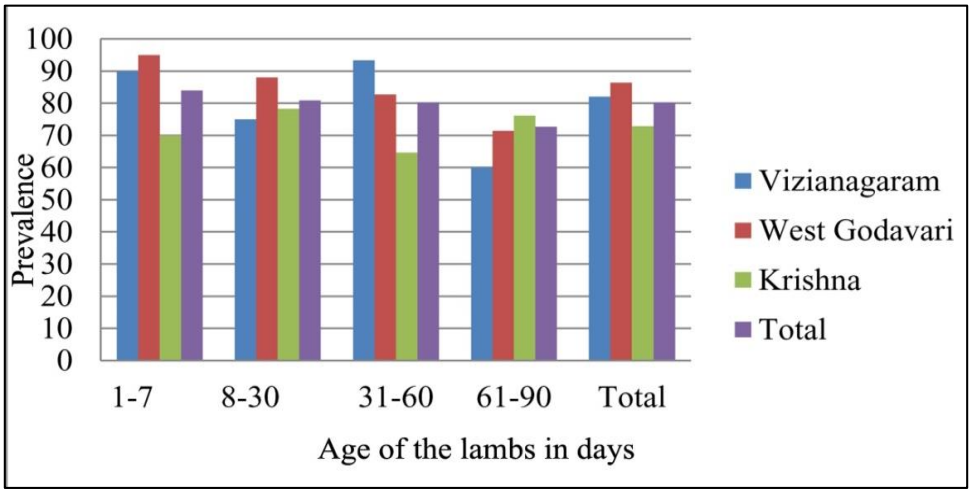

Fig 1: Prevalence of *E. coli* associated diarrhoea in lambs

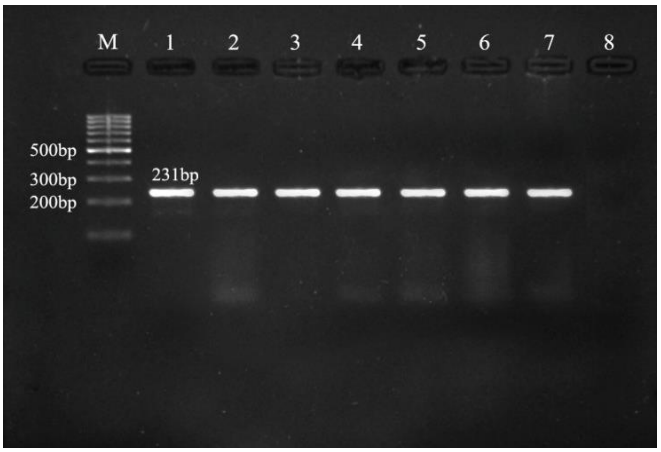

Fig 2: Gel Photograph of E 16 S rRNA species specific PCR for *E. coli*

Lane M : DNA Ladder (100 bp)
Lane 1 : *E. coli* 16 S rRNA positive control
Lane 2 to 7 : *E. coli* carrying 16 S rRNA gene
Lane 8: Negative control

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