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Epidemiology and Molecular Characterization of Shiga Toxigenic *Escherichia coli* from Diarrhoeic Lambs in Andhra Pradesh

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A B S T R A C T

A study was carried to investigate the epidemiology and molecular characterization of Shiga toxigenic *Escherichia coli* (STEC) from diarrhoeic lambs in Andhra Pradesh. 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. The *E. coli* isolates were confirmed by cultural, biochemical and molecular methods. The virulence genes of STEC were detected using multiplex PCR. The results of the study revealed that the prevalence rate of *E. coli* in diarrhoeic lambs was 80.18% of which 87.05 % were identified as STEC. Among the virulence genes of STEC studied, *eaeA&hlyA* genes were more prevalent (35.13%), followed by *stx1* (12.83%) and *stx2* (10.13%) genes. The combinations of *stx1&eaeA*, *stx2& eaeA* genes carrying isolates detected were 5.4% and 8.1%, respectively. Only four (2.70%) STEC isolates carried all the four (*stx1*, *stx2*, *eaeA* and *hlyA*) genes. This study indicated that diarrhoeic lambs are the the reservoirs of STEC in this geographic region.

Introduction

Diarrhoea is one of the important causes of mortality in neonatal lambs causing economic loss to the sheep producers in this geographic area. The most important enteropathogen known to be associated with lamb diarrhoeia is *Escherichia coli* (*E. coli*). Shiga-like toxin producing *E. coli* (STEC) infections have been described in a wide range of domestic and wild animal species, but the natural pathogenic role of STEC has been demonstrated only in weaning pigs, young calves and dogs (Caprioli *et al.*, 2005). Domestic ruminants, especially sheep and cattle, are well known reservoirs of STEC (Blanco *et al.*, 2003 and Caprioli *et al.*, 2005).

The pathogenicity of STEC is mediated mainly through Shiga toxins 1 (which is almost identical to *stx* produced by *Shigella dysenteriae*) and 2 encoded by *stx1* and *stx2*

genes, respectively (Paton and Paton 1998). Many STEC also produce intimin, an outer membrane surface adhesion encoded by the chromosomal *eaeA* gene (Blanco *et al.,* 2004). Intimin is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (Mora *et al.,* 2005). A factor that may also affect the virulence of STEC strains is the 60-mDa plasmid borne enterohaemolysin (*Ehly*) which is encoded by the *hlyA* gene (Schmidt *et al.,* 1995).

Limited studies are available on isolation and characterization STEC from sheep in India (Wani *et al.,* 2003 and Bhat *et al.,* 2007). Therefore, the present research was under taken on epidemiology and molecular characterization of STEC in Andhra Pradesh in order to provide baseline data for taking prophylactic measure in preventing lamb diarrhoeia.

Materials and Methods

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.

Isolation and identification of *E. coli*

The rectal swabs were streaked on MacConkey (Hi Media) agar plates and incubated overnight at 37^{o} C for 24h. The plates with pink colonies were selected and inoculated on Eosin methylene blue (EMB) agar plate and re-incubated at 37^{o} C 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^{o} C for further biochemical tests (Hitchins *et al.,* 1992) and (Cruikshank *et al.,*1975) and molecular characterization.

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) (E16S-F: ATCAACCGAGATTCCCCCAGT E16S-R: TCACTATCGGTCAGTCAGGAG) with 231bp amplified product.

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).

Multiplex PCR for detection of virulence genes

The primers used in the present study for the detection of virulence genes (*stx1, stx2, eaeA, hlyA*) of STEC were as described by Paton and Paton (1998).

Standardization of multiplex PCR protocol for detection of *stx1, stx2, eaeA & hlyA* virulence gene

Polymerase chain reaction for amplification of the *stx1, stx2, eaeA, hlyA* genes was set up in 25 µL reaction. Following initial trails with varying concentration of components, the reaction mixture was optimized as below.

PCR tube containing the reaction mixture was flash spun in a microcentrifuge to settle the reactants at the bottom. Polymerase chain reaction assay was performed in Eppendorf thermal cycler with heated lid. Samples were subjected to 35 cycles as per the procedure (Paton and Paton, 1998).

Results and Discussion

The faecal samples (212) were screened for *E. coli* by cultural and biochemical methods followed by confirmation of *E. coli* by molecular methods with PCR revealed that 170 out of 212 samples were positive for *E. coli*, giving an overall prevalence rate of 80.18%. Consistent with present findings, Aklilu *et al.,* (2013) reported 84% prevalence rate in diarrhoeic lambs and Srivani *et al.,* (2017) reported 80.53%) in diarrhoeic buffalo calves in India (AP) and Ethiopia, respectively. Contrary, lower prevalence rate of *E. coli* was reported in diarrhoeic lambs by Nasr *et al.,* (2014) in Egypt (34.20%) and Wani *et al.,* (2008) in Kashmir valley, India (22.18%).This study observed higher prevalence of *E. coli* 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 be due to differences in feeding and management practices among the districts studied.

Table.1 Details of the primers used for the detection of *stx1, stx2, eaeA* and *hlyA* genes

Primer	Target gene	Primer sequence	Amplicon size(bp)	Reference
***Stx1*F**	*stx1*	ATAAATCGCCATTCGTTGACTAC	180	**Paton and Paton. (1998)**
***Stx1*R**		AGAACGCCCACTGAGATCATC		
***Stx2* F**	*stx2*	GGCACTGTCTGAAACTGCTCC	255	
***Stx2* R**		TCGCCAGTTATCTGACATTCTG		
***eaeA*F**	*eaeA*	GACCCGGCACAAGCATAAGC	384	
***eaeA*R**		CCACCTGCAGCAACAAGAGG		
***hlyA*F**	*hlyA*	GCATCATCAAGCGTACGTTCC	534	
***hlyA*R**		**AATGAGCCAAGCTGGTTAAGCT**		

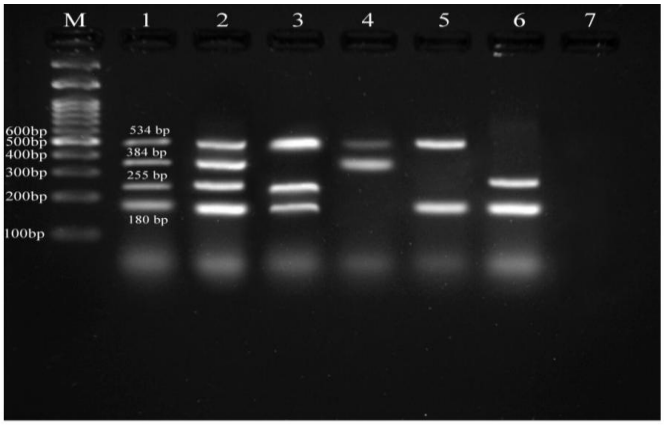
Table.2 Optimized PCR mixture for amplification of STEC virulence genes

Components	Quantity (µL)
Master mix	**12.5 µL**
Primer F - (20 pmol)	**0.12 µL X4**
Primer R- (20 pmol)	**0.12 µL X4**
Template	**5.00 µL**
NFW water	**6.54 µL**
Total	**25 µL**

Table.3 Distribution of virulence genes in *E. coli* isolates from diarrhoeic lambs

Virulence Gene	No.of *E. coli* isolates from diarrhoeic calves with the virulence gene (N=148)	%
Stx1	19	**12.83**
Stx2	15	**10.13**
hlyA	17	**11.48**
Stx1,eaeA	8	**5.40**
Stx1,hlyA	7	**4.72**
Stx2,eaeA	12	**8.10**
Stx2,hlyA	7	**4.72**
eaeA, hlyA	52	**35.13**
Stx1,stx2,hlyA	7	**4.72**
Stx1,stx2,eaeA,hlyA,	**4**	**2.70**

Fig.1 PCR assay targeting virulence genes of STEC



Lane M : DNA ladder of 100bp
Lane :1 slandered *E.coli* positive control carrying *Stx1, Stx2,eaeA* and *hlyA* genes
Lane 2 to 6 : Isolates carrying shigatoxin genes
Lane 7: Negative control

The prevalence of *E. coli* associated diarrhoea was high in younger lambs compared to older lambs. Higher prevalence (84%) was detected in one to seven day old lambs, followed by 80.80% in 8-30 days, 80.32% in 31 to 60 days, while 72.72 % was observed in 61-90 day old lambs. Parallel to this findings of Srivani *et al.*, (2017) and Abdulgayeid *et al.*, (2015) reported a higher prevalence of *E. coli* in 1-7 day old calves compared to older calves. Higher prevalence of *E. coli* associated diarrhoea in young lambs may be

due to ill developed rumen and poor immune status in those lambs that deprived of colostrum or delay in colostrum feeding compared to older lambs. Matte *et al.,* (1982) 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.

Out of 170 isolates from diarrhoeic lambs, 148 (87.05%) were identified as STEC. The prevalence of STEC detected in the present study was higher compared to the prevalence of STEC reported by Kiranmayi *et al.,* (2011) (54.80%) and lower compared to the results reported by Ferreira *et al.,* (2015) (78.3%) in sheep.

Multiple virulence genes are associated with the pathogenicity of STEC. Among the virulence genes of STEC studied, *eaeA&hlyA* were more prevalent (35.13%) in diarrhoeic lambs. Similar results of higher frequencies of *eaeA&hlyA* genes than *stx1*and *stx2* genes were reported by Wani *et al.,* (2003) in diarrhoeic calves in Kashmir valley, India. The present findings are also parallel to the results reported by Badouei *et al.,* (2010) who detected higher prevalence of *eaeA&hlyA* genes in healthy and diarrhoeic calves in Iran. Similar results of higher prevalence of *eaeA&hlyA* genes in STEC isolates from diarrhoeic calves were also reported in other studies (Blanco *et al.,* 2004 and Aidar *et al.,* (2007). Several investigators have observed strong association between the carriage of *eaeA* gene and the capacity of STEC to cause severe human disease (Blanco *et al.,* 2004 and Beutin *et al*., 2004).

This study also detected a high percentage (12.83%) *stx1*gene compared to *stx2*gene (10.13%) in diarrhoeic lambs. The present results are comparable with Srivani *et al.,* (2017) who reported highest *stx1*gene (16.04) compared to *stx2* gene (12.64%) in diarrhoeic calves. Contrary to the present findings, Wani *et al.,* (2003) reported more prevalence of *stx2* than *stx1*gene in diarrhoeic calves and lambs in Kashmir valley, India.

The combinations of *stx1&eaeA, stx2&eaeA* genes carrying isolates detected were 5.4% and 8.1%, respectively. Similar to our findings Rigobelo *et al.,* (2006) detected the association of *eae* gene with the *stx2* gene in STEC isolates from diarrhoeic calves in Brazil. However, Wani *et al.,* (2004) observed uniform association of *eae* gene with *stx1* and *stx2* in diarrhoeic calves in Kashmir valley, India.

In this study, only four (2.70%) STEC isolates carried all the four (*stx1, stx2, eaeA* and *hlyA*) genes. However, Maldonado *et al.,* (2005) reported that 84% of the food and clinical isolates from outbreaks carried all four virulence genes. This discrepancy may be due to the fact that the samples were not from outbreaks, implying more diversity amongst isolates and therefore, less prevalence of the target virulence genes combination. On the other hand, the isolates reported by Maldonado *et al.,* (2005) were of outbreak origin, were perhaps more homogenous in their genetic makeup, resulting in higher carriage rate of virulence genes. The presence of virulent strains of *Escherichia coli* in the environment may be a potential source of contamination of food and the water supply. Moreover, these strains may comprise a potential reservoir of virulence genes acquired from different sources (e.g., bacteriophages and plasmids). *E. coli* is a very dynamic organism with capacity for horizontal gene transfer to increase genetic diversity and under certain circumstances this can lead to the emergence of new pathogenic strains (Donnenberg and Whittam, 2001).

The present study concluded that higher prevalence of STEC in diarrhoeic lambs may indicate that diarrhoeic lambs are the potential reservoirs of STEC in this geographic region which may have public health significance.

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References

Abdulgayeid, M., Hazem Shahin., Seham Foad., and Madiha, S., Ibrahim. (2015). Molecular characterization of *Escherichia coli* isolated from buffalo calves in El-Behera Governorate. *Alexandria Journal of Veterinary Sciences,* 47 (1): 90-96.

Aidar, U L., Blanco, J., Blanco, M., Blanco, J. E., Leomil, L., Dahbi, G., Mora, A., Onuma, D L., Silveira, W. D., and Pestana de Castro, A, F. (2007). Serotypes, virulence genes, and intimin types of Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic*E. coli (*EPEC) isolated from calves in Sao Paulo, Brazil. *International Journal of Food Microbiology*, 115(3):297-306.

Aklilu, M., Sisay, T., Tefera, G., and Tekalign, B. (2013). Identification and biotyping of *Escherichia coli* from diarrheic lambs in and around Debre Birhan town, Ethiopia. *Journal of Environmental and Analytical Toxicology*, 3: 6.

Badouei, M, A., Salehi, T. Z., Khorasgani, M. R., Tadjbakhsh, H., Brujeni, G. N., and Nadalian, M. G. (2010). Virulence gene profiles and intimin subtypes of Shiga toxin-producing *Escherichia coli* isolated from healthy and diarrhoeic calves. *Veterinary Record,* 167(22):858-861.

Beutin, L., Geier, D., Steinruck, H., Zimmermann, S., and Scheutz, F. (1993). Prevalence and Some Properties of Verotoxin (Shiga-Like Toxin)-Producing *Escherichia coli* in Seven different species of healthy domestic animals. *Journal of Clinical Microbiology*, 31 (9): 2483-2488.

Bhat, M. A., Nishikawa, Y., and wani, S, A. (2007). Prevalence and virulence gene profiles of shiga toxin-producing *Escherichia coli* and enteropathogenic *Escherichia coli* from diarrhoeic and healthy lambs in India. *The Official Journal of the International Goat Association,* 75(1): 65-75.

Blanco, M., Blanco, J. E., Mora, A., Dahbi, G., Alonso, M. P., Gonzalez, E. A., Bernardez, M. I., Blanco, J. (2004). Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (eae-xi). *Journal of Clinical Microbiology*, 42:645-650.

Blanco, M., Blanco, J. E., Mora, A., Rey, J., Alonso, J. M., Hermoso, M., Hermoso, J., Alonso, M. P., Dahbi, G., Gonzalez, E. A., Bernardez, M. I., and Blanco, J. (2003). Serotypes, virulence genes, and intimin types of shiga toxin (Verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. Journal of Clinical Microbiology, 41(4): 1351–1356.

Caprioli, A., Morabito, S., Brugere, H. and Oswald, E. (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary Research.*, 36:289-311.

Cruickshank, R., Duguid, J. P., Marmion, B. P. and Swain, R. H. A. (1975). *Medical*

Microbiology, (12th edition)

Dong-bo, S U N., Rui, W. U., Xian-jing, H. E., Shuang Wang., Yun-cheng Lin., Xu, H. A. N., Yue- qiang Wang., Ting-ting, G. U. O., Guo-jun, W. U. and Ke-li, Yang. (2011) Development of a Multiplex PCR for Diagnosis of *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* from Cows with Endometritis. *Agricultural Sciences in China.,* 10(10): 1624-1629.

Donnenberg, M. S. and Whittam, T. S. (2001). Pathogenesis and evolution of virulence in enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Journal of Clinical Investigation.,* 39: 270-74.

Feria, C. E., Ferreira, J. D., Correira, J., Goncalves., and Canica. M. (2002). Patterns and mechanisms of resistance to beta-lactams and beta-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. Journal of *Antimicrobial Chemotherapy*, 49:77-85.

Hitchins, A. D., Hartman, P. A., Todd, E. C. D. (1992) Coliform *Escherichia coli* and its toxins. In: Vanderzant, C., Splittstoesser, D. (Eds), *Compendium of Methods for the Microbiological Examination of Foods*, pp. 327-329.

Kiranmayi, C. B., Krishnaiah, N., and Mallika, E. N. (2011) *Escherichia coli* O157:H7 - An emerging pathogen in foods of animal origin. *Veterinary World,* 3: 382- 389.

Maldanado, Y., Fiser, J. C., Nakatsu, C. H., and Bhunia, A. K. (2005). Cytotoxicity potential and genotypic characterization of *Escherichia coli* isolates from environmental and food sources. *Applied and Environmental Microbiology*, 71: 1890-98

Matte, J. J., Girard, C. L., Seoane, J. R., Brisson, G. J. (1982) Absorption of colostral immunoglobulin G in the newborn dairy calf. *Journal of Dairy Science* 65: 1765-1770

Mora, A. (2007) Phage types, virulence genes and PFGE profiles of shiga toxin-producing *E. coli* O157:H7 isolated from raw beef, soft cheese and vegetables in Lina (Peru). *International Journal of Food Microbiology,* 114: 204-210

Nasr, M., Bakeer, N. M., Hammouda, H. A., and Omar, A. A. (2014) Epidemiological, clinical and bacteriological studies on bacterial lamb enteritis at Behera Province, Egypt. *Alexandria Journal of Veterinary Sciences,* (43): 8-16.

Paton, A. W. and Paton, J. C. (1998) Detection and Characterization of Shiga Toxigenic *Escherichia coli* by Using Multiplex PCR Assays for stx 1, stx 2, eaeA, Enterohemorrhagic *E. coli* hlyA, rfb O111, and rfb O157. *Journal of Clinical Microbiology*, 36(2), 598-602.

Rigobelo, E.C, Gamez, H.J., Marin, J.M., Macedo, C., Ambrosin, J.A., and Ávila F.A, (2006). Virulence factors of *Escherichia coli* isolated from diarrheic calves. *Arquivo Veterinary Research and Animal Science*, 37(2): 1-7.

Sambrook, J., and Russell, D. W. (2001) Molecular cloning - a laboratory manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schmidt, H., Beutin, L., and Karch, H. (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infection and Immunity*, 63(3): 1055-1061.

Srivani, M., Reddy, Y. N., Subramanyam, K. V., Reddy, M. R., and Rao, T. S. (2017) Prevalence and antimicrobial resistance pattern of Shiga toxigenic Escherichia coli in diarrheic buffalo calves. *Veterinary world*, 10(7): 774.

Wani, S, A., Hussain, I, Fayaz, I., Mir, M. A., and Nishikawa, Y. (2008). Subtype

analysis of *stx*1, *stx*2 and *eae* genes in shiga toxin-producing *Escherichia coli* (STEC) and typical and atypical enteropathogenic *E. coli* (EPEC) from lambs in India. *The Veterinary Journal*, 182: 489-490.

Wani, S, A., Pandit, F, I., Samanta, I., Bhat, M. A., Buchh, A. S. (2004) Molecular epidemiology of of shiga toxin producing *Escherichia coli* (STEC) in India. *Current science,* 87(10): 1346-135

Wani, S. A., Bhat, M. A, Munshi, Z. H., Qureshi, S., and Buch, A. S. (2003) Isolation and *in- vitro* sensitivity pattern of pathogenic *Escherichia coli* from diarrhoeic lambs and calves. *Journal of Animal Sciences,* 73 (2): 168-170.

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