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.

Keywords
Epidemiology, Shiga toxigenic E. coli, Diarrhoeic lambs

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 diarrhoea 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 chromosom al 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 undertaken on epidemiology and molecular characterization of STEC in Andhra Pradesh in order to provide baseline data for taking prophylactic measure in preventing lamb diarrhoea.

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°C for 24h. The plates with pink colonies were selected and inoculated on Eosin methylene blue (EMB) agar plate and re-incubated at 37°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°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: TCACTATCGGTAGTCAGGAG) 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 |
|--------|-------------|----------------|-------------------|-----------|
| Stx1F  | stx1        | ATAAATCGCCCATTCGTTGACTAC | 180            | Paton and Paton. (1998) |
| Stx1R  |             | AGAACGCCACTGAGATCATC       |                 |           |
| Stx2F  | stx2        | GGCACTGTCTGAAACTGCTCC      | 255             |           |
| Stx2R  |             | TCGCAGTTATCTGACATTCTG      |                 |           |
| eaeAF  | eaeA        | GACCCGGCAACAAGCATAAGC      | 384             |           |
| eaeAR  |             | CCACCTGCAAGCAACAAGAGG      |                 |           |
| hlyAF  | hlyA        | GCATCATCAAAGCTACGGTTC     | 534             |           |
| hlyAR  |             | AATGAGCCAAAGCTGGTAAAGCT    |                 |           |

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           | 1                                                                              | 12.83 |
|                | 9                                                                              |     |
| Stx2           | 1                                                                              | 10.13 |
|                | 5                                                                              |     |
| hlyA           | 1                                                                              | 11.48 |
|                | 7                                                                              |     |
| Stx1,eaeA      | 8                                                                              | 5.40  |
| Stx1,hlyA      | 7                                                                              | 4.72  |
| Stx2,eaeA      | 1                                                                              | 8.10  |
|                | 2                                                                              |     |
| Stx2,hlyA      | 7                                                                              | 4.72  |
| eaeA, hlyA     | 5                                                                              | 35.13 |
|                | 2                                                                              |     |
| 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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