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Detection and molecular characterization of sorbitol fermenting non-O157 Escherichia coli from goats

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ABSTRACT

Shiga toxigenic *Escherichia coli* (STEC) O157 and several other serogroups of non-O157 STEC strains present as commensalism bacteria in small ruminants that possess a high potential of attaining pathogenic virulent genetic elements. The pathogenicity of non-O157 strains is emerging progressively in animals as well as in humans especially in the rural areas as they get transmitted through unsanitary practices of living, consumption of uncooked meat and milk, human-livestock close proximity as well as within livestock pathogenic bacterial transmission. The present study was carried out to determine the prevalence of non-O157 *E.coli* isolates and characterize based on clinical history, antibiotic sensitivity testing, multiplex PCR (mPCR) detection of virulence genes, genotype identification using pulse field gel electrophoresis (PFGE) and polyacrylamide gel electrophoretic separation of antigenic proteins to determine their prevalence, virulence and genetic comparison between host and environment for epidemiological significance. A total of 300 *E.coli* isolates were recovered from rectal swabs of goats and their surrounding environment over a period of one year (2016–2017) by selective isolation. Among which 50 isolates were confirmed to be from the non-O157 *E.coli* family. The mPCR analysis of these 50 isolates revealed the presence of two or more virulent genes, viz., *hylA* (90%), *fliC* (74%), *eaeA* (56%), *stx1* (48%) and *stx2* (22%).Four isolates exhibited multidrug-resistance to amoxiclav, doxycycline, ciprofloxacin and ceftriazone. The PFGE fingerprint profile showed six major clusters at 100% similarity from the 50 isolates. The major antigenic proteins identified from the isolates were *stx1*A, *stx2*B and *fliC*. This study has significant implications for understanding the molecular diversity of emerging pathotypes of non-O157 in young goats in terms of virulence and epidemiological aspects.

1. Introduction

Ruminants have been implicated as the principal reservoirs of pathogenic *Escherichia coli* which are a major source of foodborne outbreaks affecting human health (Ferens and Hovde, 2011; Swift et al., 2017). As long as these bacteria do not attain genetic elements and virulence factors they remain as benign commensalism bacteria within the gut (Osman et al., 2013). Several strains of pathogenic *E.coli* cause diarrhea in goats, sheep and cattle affecting mostly young ones leading to heavy mortality and morbidity (Etcheverría and Padola, 2013). Younger goats have a higher susceptibility rate (65%) of developing an infection to pathogenic *E.coli* as compared to adults (37.1%) (Islam et al., 2016). Numerous pathogenic *E.coli* expressing various combinations of virulence factors like shiga toxins and enteropathogenic toxins were recovered from clinical and non-clinical cases in calves, goats and sheep (Wani et al., 2006; Osman et al., 2013). Non-O157 *E.coli*serogroups are emerging to cause serious illness in humans and ruminants (Hussein, 2007). Therefore, the similarities in phylogenies of O157 *E.coli* and non-O157 *E.coli* suggest an ongoing micro evolutionary process in which the shiga toxin gene (*stx*) is transferred between the two groups causing an array of diverse strains (Eichhorn et al., 2015).

The west coast of south India is hot and humid with high rainfall and goat farming in this region is in the form of small holdings. The majority of goat farms are stall feeding reared on raised platforms. The incidence of diarrhoeagenic pathogens particularly *E.coli* is persistently prevalent during all the seasons more frequently in young goats due to poor housing and management conditions in this region. The most prominent genes in *E.coli* that correspond to the virulence factor of the bacteria are *stx1*, *stx2*, *eaeA*, *hylA* and *fliC* (Pan et al., 2002; Bai et al., 2010; Osman et al., 2012). The current outlook on virulence studies

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Table 1
PCR conditions of target gene along with their primer details.

Target Gene	Nucleotide Sequence (5′-3′)	Amplicon Size (bp)	PCR conditions			Reference
			Denaturation	Annealing Temp	Extension	
Stx1	TGTCGCATAGTGGAACCTCA TGCGCACTGAGAAGAAGAGA	655	95 °C 3 min	56° C 30sec	72 °C 2 min	(Paton and Paton, 1998)
Stx2	CCATGACAACGGACAGCAGTT TGTCGCCAGTTATCTGACATTC	477	95 °C 3 min	56° C 30sec	72 °C 2 min	(Paton and Paton, 1998)
EaeA	GACCCGGCACAAGCATAAGC GACCCGGCACAAGCATAAGC	384	95 °C 3 min	56° C 30sec	72 °C 2 min	(Paton and Paton, 1998)
hlyA	GCATCATCAAGCGTACGTTCC GACCCGGCACAAGCATAAGC	534	95 °C 3 min	56° C 30sec	72 °C 2 min	(Paton and Paton, 1998)
FLIC	AGCTGCAACGGTAAGTGATTT GGCAGCAAGCGGGTTGGTC	949	95 °C 3 min	55° C 30sec	72 °C 2 min	(Bai, et al. 2010)
Rfb	GGCAGCAAGCGGGTTGGTC TTAGAATTGAGACCATCCAATAAG	296	95 °C 3 min	55° C 30sec	72 °C 2 min	(Bai, et al. 2010)

indicates that *E.coli* of any serotype can acquire the toxin gene (*stx1*, *stx2*) but the ability of an organism to produce these toxins alone is insufficient for that organism to cause disease. Therefore, the *E.coli* strain would have to possess multifactorial virulence genes to induce pathogenicity (Law, 2000; Zschock et al., 2000). Additionally, in a study conducted by Jacob et al. (2013), non- O157 serotype of *E.coli* isolates from goats indicating the presence of two or more virulence genes (14.5%; 43/296) which confirms the bacterial pathogenic factor. In India, there is not enough information on the isolation and characterization of non-O157*E.coli* strains from livestock animals reported. Few studies have indicated the prevalence of shiga toxin-producing *Escherichia coli*(STEC) from non-diarrheic calves, lambs, chicken and feral pigeons in Kolkata andenteropathogenic E. coli (EPEC) strains from healthy goats in Jammu and Kashmir. (Wani et al., 2004, 2006)

In the present study, non-O157 *E.coli* strains were characterized from goat rectal swabs based on clinical history, antibiotic sensitivity testing and multiplex PCR (mPCR) detection of virulence genes. Furthermore, genotype identification was carried out using PFGE and PAGE separation of antigenic proteins to determine a comparison between host and environment for epidemiological significance.

2. Materials and methods

Sample collection: A total of 300 (250 rectal swabs and 50 environmental) samples were collected from multiple farms all over the region, where goats were reared under a free-range as well as stall feeding system in the west coastal state of India—Goa, over a period of one year (2016–2017). The coastal climate is hot and humid, which allows very few breeds of goats that are suitable to be reared. The rectal swabs were obtained from diarrhoeic and non-diarrhoeic goats cases along with their corresponding environmental surroundings. The majority of samples were collected from adults and young goats which were kept together with adults (80%) as younger goats are more susceptible to infections and thus were more liable to have pathogenic bacteria.

Selective isolation and screening: The rectal swabs were enriched with brain heart infusion (BHI) broth (HiMedia, Mumbai, India) at 37° C for 24 h and streaked on eosin-methylene blue agar (HiMedia, Mumbai, India). After incubation, colonies with cultural characteristics similar to *E.coli* were streaked onto selective *E.coli* O157 MUG (4-methylumbelliferyl-beta-D-glucuronide) identification agar plates (HiMedia, Mumbai, India) which were screened for O157 and non-O157 *E.coli* strains.All the multiple strains from a single sample were considered and subjected to phenol base broth biochemical assay and to antibiotic sensitivity assays.

Biochemical and antibiotic-sensitivity assays: The pure cultures were screened for sorbitol fermenting ability to distinguish between *E.coli* O157: H7 and non-O157: H7 *E.coli* using the phenol red sorbitol test. The selected isolates were then subjected to antibiotic agents by disk diffusion test on Muller-Hilton agar and data were classified as susceptible or resistant according to the specifications of Clinical and Laboratory Standards Institute, 2012. The antibiotic compounds used were amoxiclav (AMC), doxycycline (DO), ciprofloxacin (CIP) and ceftriaxone (CTR) as these are the most commonly used antibiotics for respiratory and enteric bacterial infections in India (Rasheed et al., 2014; Alves et al., 2017).

Genomic DNA isolation and multiplex PCR assay for virulence genes: One mLof overnight grown BHI broth culture was centrifuged at 7500 rpm for 5 min and the pellet was suspended in 180 µl of buffer (QIAamp, DNA mini kit). Proteinase K (QIAGEN, Germany) was added and the suspension was incubated at 56 °C for 2–3 h for complete lysis of bacterial cell wall. The lysate was washed, centrifuged and the supernatant containing DNA was quantified using a Nanodrop®N-1000 spectrophotometer (ThermoScientific, USA) for using as a template in PCR. All PCR reactions were performed using the gradient thermocycler (Eppendorf pro, Germany). Published primer sequences (Paton and Paton, 1998; Bai et al., 2010) were used (Table 1) and thermocycling conditions were standardized by gradient PCR. The following optimal conditions for the mPCR assay was established: reaction volume of 25 µl which consisted of 2X Gotaq green master mix (400 µM of each dNTPs, GoTaq® DNA polymerase, 2X Green GoTaq® Reaction Buffer (pH 8.5) and 3 mM MgCl2) (Promega, USA), 1.0 µM each of forward and reverse primers, 100 ng of the template DNA and nuclease-free water to make up remaining volume. The reaction was performed at 95 °C for 3 min of initial denaturation, 95 °C for 30 s of denaturation, annealing temperature for reaction set (*stx1*, *stx2, eaeAand hylA*) was at 56 °C and for reaction set (*fliC* and *rfbr*) was at 55° C for 2 mins, extension at 72 °C for 2 min and 72 °C for 10 min of final extension. The amplified DNA along with the positive strain control ATCC 25922 and a negative control (PCR mixture without template) was separated using gel electrophoresis at 1.2% agarose, stained with ethidium bromide and visualized using GelDocAplhaimager fluorescent imaging system (AlphInnotech, USA).

Pulse Field Gel Electrophoresis: Fifty isolates of non-O157 *E.coli* colonies from the overnight cultures were suspended in cell suspension buffer (100 mM Tris; 100 mM EDTA, pH 8) and cell concentration was adjusted to an optical density (OD) of 0.8–1 at 610 nm. Chromosomal bacterial DNA was prepared in 1% agarose gel plugs by mixing cell suspension buffer with an equal amount of low melting point agarose (Bio-Rad, USA) and Proteinase K (20 mg/mL) which was lysed in lysis buffer for four hours at 56 °C in the water bath. The plugs were washed three times with Milli-Q water, which was kept at 56 °C prior to the