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Original article

Enterohaemolysin production and verotoxin genes in *Escherichia coli* strains isolated from neonatal calves in India

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Abstract

Objective: To screened all haemolytic strains of *Escherichia coli* (*E. coli*) for the presence of verotoxin genes and speculate the association between enterohaemolysin production and presence of verotoxin genes. **Methods**: A total 176 of *E. coli* strains of 64 serogroups isolated from neonatal calves were selected and screened for alpha-haemolysin and enterohaemolysin production on sheep blood agar and 5% washed sheep blood agar. Two types of haemolytic strains were further characterized by PCR for presence of Verotoxin gene (VT1 and VT2) and confirmed by verocell cytotoxicity assay. **Results**: Among 27 enterohaemolytic positive strains, 19 (70.37%) strains were found positive for presence of verotoxins (VTs) gene and verocell cytotoxicity assay. Whereas 34 alpha-haemolysin strains were found negative for VTs. Eight strains of *E. coli* were found VTs negative but Ehx positive. **Conclusion**: The close association between enterohaemolysin production and presence of verotoxin genes makes it useful epidemiological marker for rapid screening of Verotoxic *E. coli* but this phenotypic marker for screening of verotoxigenic *Escherichia coli* (VTEC) alone may raise a question in animal.

Keywords: Enterohaemolysin; Alpha haemolysin; Verotoxigenic *Escherichia coli*; Calves; India

INTRODUCTION

Haemolytic property of *Escherichia coli* (*E. coli*) is widely considered as an important virulence factor. Haemolysins form a family of lytic toxins which have been associated to pathogenesis of disease. Certain strains of haemolytic *E. coli* produce either alpha (α), beta (β) and enterohaemolysin (Ehx). Haemolytic strains of *E. coli* are frequently associated with extra-intestinal infections while diarrhoeagenic strains of *E. coli* tend not to produce haemolysin[1]. It has been thought that haemolysin production provides these bacteria with an essential source of iron during multiplication *in vivo*. It was revealed that strains of *E. coli* could produce both free (α) and cell- associated (β) haemolysins[2]. α-haemolysin has been demonstrated by observing zones of haemolysis around bacteria growing on blood agar plates. Both types of haemolysin are expressed in the exponential phase of growth, but only α-haemolysin requires calcium ions for lytic activity. It was found a close association between verotoxin (VT) production and a novel haemolysin called as enterohaemolysin[3]. This specific type of haemolysin is characterized by the production of small turbid zones of haemolysis after 18-24 h incubation on washed sheep blood agar. In contrast, *E. coli* α- haemolysin produces large clear zones of haemolysis apparent after 4 h incubation and occurring with both washed and unwashed erythrocytes[4]. *E. coli* isolates of sero-

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groups O157, O26, O111 commonly produce Ehx and it is therefore a useful epidemiological marker for potential verotoxin producing strains. It has been found three *E. coli*, Ehx positive strains belonging to O1:H-, O103:H- and O111:H19 serotypes were VT negative in human diarrhoea sample[5]. Whereas others[3] isolated belong to a VT positive porcine serotype which was α-haemolytic but Ehx negative. There is approximate 60 % isolates between Ehx and *E. coli* α-haemolysin. It was described that there was good correlation between Ehx production and verotoxin producing strain. It was reported that 28 of 28 *E. coli* O157, 16 of 19 human non-O157 and 13 of 26 animals verotoxigenic *Escherichia coli* (VTEC) were CVD419 probe positive[6,7].

The aim of the present study was to investigate the haemolysins production of *E. coli* with special emphasis on enterohaemolysin production and presence of verotoxin producing genes in *E. coli* isolated from one hundred ten fecal samples from neonatal claves (age 1-90 days). All the haemolytic strains were screened for the presence of VT producing genes.

MATERIALS AND METHODS

Collection of samples

A total of 110 faecal samples were collected from different farms situated in three different agroclimatic domain of India namely Western Himalayan region, Upper Gangetic Plains and Central Plateau and Hillus Region. Western Himalayan region is situated at the temperate region of the country. Upper Gangetic Plains Region and Central Plateau and Hillus Region belong to tropical region of northern and central part of India.

Out of 110 samples, 82 samples were from calves suffering from diarrhoea and rests of the samples were collected from apparently healthy animals. The age of the calves ranged between 1-90 days. All the calves exhibited symptoms of diarrhoea (slimy and bloody faeces, emaciation, and rough hair coat, weakening) were within the age group of 1-30 days. Whereas, apparently healthy calves were in between 31-90 days.

Microbiological procedures and serotyping of strains

In this study, strains of *E. coli* were isolated following standard methodology[8,9] and sent for serotyping to National *Escherichia coli* and *Salmonella* Center, Kasauli (Himachal Pradesh), India.

Detection of haemolysin (α-haemolysin and enterohaemolysin)

Strains of *E. coli* were tested for haemolysin production on sheep blood agar (SBA) plates and washed sheep blood agar supplemented with 1 % $CaCl_2$ (WBSA-Ca)[3]. Streaked plates were incubated at 37℃ and examined at 4 hrs interval upto 24 hrs. Strains of *E. coli*, which produced α-haemolysins, gave the typical haemolytic reaction after four hours on WSBA-Ca and frequently on the SBA. After 24 hours, clear haemolysis could be seen around all the colonies on both types of plate. Strains producing enterohaemolysin gave no reaction after four hours and only produced haemolysis on the WSBA-Ca plates after 24 hours. Non-haemolytic strains gave no reactions on either plate.

Molecular characterizitation of strains

PCR was performed[10,11] to evaluate the presence of virulence determinants using published primers for VT1 (forward, 5′-ATT TTT CTT TCT GTA TGT CTT-3′; reverse, 5′-CAC CCG GTA CAA GCA GGA TT-3′) and VT2 (forward, 5′-CTT CGG TAT CCT ATT CCC GG-3′; reverse, GGA TGC ATC TCT GGT CAT TG-3′). This primers produce amplicon size of 150 bp and 478 bp were taken positive for VT1 and VT2 genes respectively. PCR reaction was performed in 50 μL volumes containing Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (2 mM), gelatin (100 g/mL), glycerol (5% v/v), dATP, dCTP, dGTP and dTTP (200 M each), Taq polymerase (0.5U/23 L) and 20 Pmol of primers and 10 L of bacterial lysates. PCR reactions were for 40 cycles 94 ℃ for 45 s, 50 ℃ for 45 s, 72 ℃ for 90 s and a final extension step of 10 min at 72 ℃.

Cytotoxicity assay on verocell line for detection of verotoxin

Cytotoxicity of verotoxigenic *E. coli* strains (VTEC) in verocell was done according to OIE manual (OIE, 2004). The verocell lines procured from Center for Animal Disease Research and Diagnosis (CADRAD), IVRI, Izatnagar were propagated by trypsinization using trypsin-versene solution and maintained in GMEM (Sigma) supplemented with 10% newborn calf serum. *E. coli* isolates with positive enterohaemolysin were employed for cytotoxicity

assay using vero cell line system. Each of the *E. coli isolates was inoculated in* 5 mL of tryptic soy broth (TSB) and incubated at 37℃ for 24 hours. The 2 mL of broth culture was then reinoculated into 50 mL conical flask containing 20 mL TSB and incubated at 37℃ for 18-20 hours in a low speed shaking water bath. The bacterial cells were pelleted by centrifugation at 9 800 g for 30 min. at 4℃ and the supernatant was filtered through 0.22 m pore size membrane filter. The sterility of the cell free culture supernatant was checked by streaking onto tryptic soy agar, which was incubated at 37℃ for 24 hours. The sterile culture supernatants were then stored at −20℃ till used. Culture supernatant of *E. coli* O157:H7 [procured from National Escherichia and Salmonella Center, Kasauli (India)] was used as positive-control and non-toxic *E. coli* O4 as negative-control. The tissue culture plates were incubated at 37℃ under 5 % CO_2 atmosphere and examined daily (six hours) under the inverted microscope up to three days for characteristic morphological cytopathethic changes. Supernatant of *E. coli* strain causing CPE in more than 50 % vero cells were regarded as positive for verotoxin.

Table 1 Verotoxin (V1 and V2) production of *E. coli* strains from different serogroups isolated from claves in India.

SL. No.	*E. coli* Serotype	Origin	VT1	VT2	VT1 + VT2	Verocell cytotoxicity assay
1	O15	CPHR	-	+	-	+
2.	O26	UGPR	+	-	-	+
3.	O26	UGPR	+	-	-	+
4.	O26	UGPR	+	-	-	+
5.	O26	UGPR	-	+	-	+
6.	O26	UGPR	-	+	-	+
7.	O26	UGPR	-	-	+	+
8.	O26	UGPR	-	-	+	+
9.	O62	CPHR	+	-	-	+
10.	O62	CPHR	-	-	-	-
11.	O83	CPHR	-	+	-	+
12.	O87	WHR	-	-	-	-
13.	O87	WHR	-	-	-	-
14.	O91	WHR	+	-	-	+
15.	O92	WHR	-	-	-	-
16.	O103	WHR	+	-	-	+
17.	O111	UGPR	+	-	-	+
18.	O111	UGPR	+	-	-	+
19.	O111	UGPR	+	-	-	+
20.	O111	UGPR	+	-	-	+
21.	O111	UGPR	-	+	-	+
22.	O111	UGPR	-	+	-	+
23.	O111	UGPR	-	-	+	+
24.	O111	UGPR	-	-	-	-
25.	O111	CPHR	-	-	-	-
26.	O138	CPHR	-	-	-	-
27.	O168	CPHR	-	-	-	-
Total	27		10	6	3	19

Western Himalayan Region (WHR), Upper Gangetic Plains Region (UGPR) and Central Plateau and Hillus Region (CPHR)

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RESULTS

A total one hundred seventy six strains of 64 serogroups were selected and screened on SBA and 5% WSBA-Ca. On SBA, 34 (19.32 %) *E. coli* strains belonging to 18 serogroups O4, O25, O39, O73, O100, O115, O120, O149, O168, O172, O119 (one positive strains); O9, O20, O87 (two positive strains); O111 (three positive strains); O26, O173 (four positive strains) and O15 (six positive strains) produced typical clear haemolytic zone (Figure 1). All these 34 strains started revealed haemolysis after four hours incubation on WSBA-Ca following complete haemolytic zone after 24 hrs. On WSBA-Ca, 27 (15.34 %) strains belonging to O62, O83, O92, O103, O138, O168 (one positive strain); O87, O91 (two positive strain); O26 (seven positive strains) and O111 (nine positive strains) serogroups exhibited no haemolytic reaction after four hours but produced small turbid zone (Figure 2) after 18-24 hrs incubation and were taken as enterohaemolytic positive *E. coli*. Nineteen strains (70.37 %) out of 27 Ehx positive *E. coli* found VT positive by means of PCR and also in verocell toxicity assay (Figure 3). None of the α-haemolysin positive *E. coli* found positive for verotoxin. Whereas eight strains belonging to O92, O138, O168 (one positive strain); O87 (two positive strains) and O111 (three positive strains) serotypes were found VT negative but Ehx positive and culture fluid and cell lysate of these were not found to cause visible changes to cultured vero cell (Figure 4).

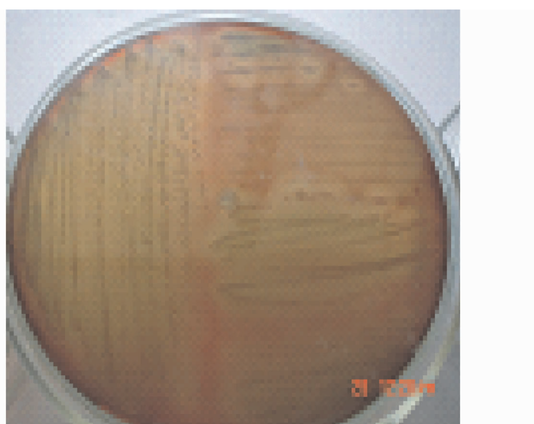

Figure 1 *E. coli* strain (O26) showing clear zone of haemolysis after 24 h on sheep blood agar.

Figure 2 *E. coli strain* (O111) showing turbid zone of haemolysis after 24 h on washed sheep blood agar with $CaCl_2$.

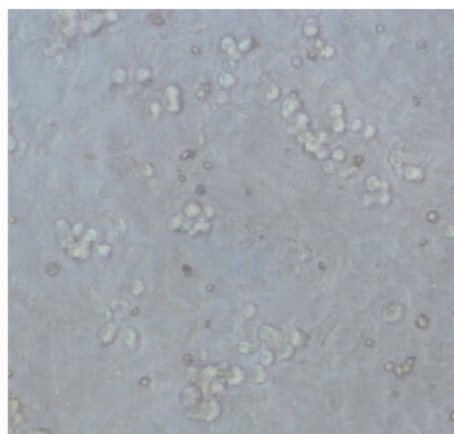

Figure 3 Clumping of vero cell due to presence of verotoxin in 18 h.

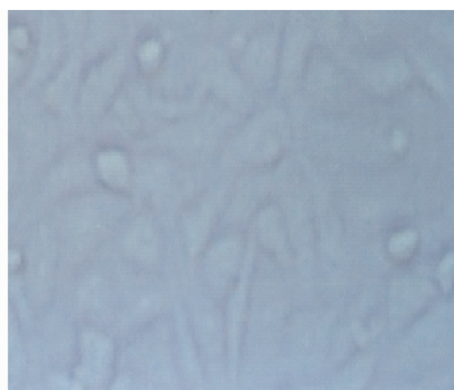

Figure 4 No effect on vero cell layer due to presence of enterohaemolysin.

DISCUSSION

The hemolytic activity might play an important role in the virulence of *E. coli*[12]. Haemolytic activity has been shown to be toxic to a wide variety of mammalian cells including sheep erythrocytes[3,8]. Ehx had no effect on human erythrocytes[13]. Production of Ehx *in vitro* can be markedly enhanced by growth

under conditions of low oxygen tension and anaerobic conditions[13]; similar conditions are found in the large bowel of man and animal, and suggest that the toxin may be produced maximally in these locations. The α-hemolysin is liberated extracellularly during active growth of bacteria[14]. Lysis of erythrocytes liberates the iron from haem of the erythrocytes and liberated iron from haem part of erythrocytes promotes the growth of *E. coli* in the host[11,15]. The role of enterohaemolysins remains unclear. Lysis of erythrocytes *in vivo* would release the haem and globin component that enhances the growth of *E. coli* O157 verotoxigenic strains. VTEC strains isolated from cattle depicted enterohaemolysin production probably are a suitable marker for the detection of such *E. coli*. In our study association between verotoxins production with enterohaemolysin production is agreement with similar studies in several countries[3,16-18]. However the previous data analysis regarding association between verotoxin production with enterohaemolysin production in human and animal, it seems that human VTEC isolates are more closely related to Ehx rather than reservoir animal. This finding may suggest that enterohaemolysin may play a significant role in *E. coli* pathogenesis in human comparatively to animal infections. Though close relationship between enterohaemolysin production and presence of verotoxin producing genes were reported by many workers but this phenotypic marker for screening of VTEC alone may raise a question in animal.

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