ESCHERICHIA COLI PATHOTYPES ASSOCIATED WITH DIARRHEA IN HUMAN AND DOMESTIC ANIMALS

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ABSTRACT

Ruminants are important reservoirs for zoonotic pathogenic *E. coli*. The objective of this study was to characterize pathogenic *E. coli* isolates from cattle and sheep linked to human illness with respect to their path types, serotypes and genotypes. *E. coli* O157:H7 isolated from cattle, sheep and human patients were compared for their genomic similarity by Pulsed-Field Gel-Electrophoresis (PFGE). PCR detection of virulence factors associated with different *E. coli* path types (VTEC, ETEC, EPEC, EAEC and EIEC) revealed that VTEC was the most prevalent path type (22/45; 48.9%), followed by EAEC (3/45; 6.7%), EPEC (1/45; 2.2%), a EPEC (3/45; 6.7%), ETEC (1/45; 2.2%) and EIEC (1/45; 2.2%). *E. coli* O157:H7 represented the most prevalent VTEC serotypes (11/22; 50%). Pulsed field gel electrophoresis typing revealed exact matches between *E. coli* O157:H7 isolates from the human patients, sheep and cattle in the same municipality. VTEC play an important cause of diarrhea in human, sheep and cattle. The molecular relatedness between PFGE profiles of *E. coli* O157:H7 isolates from human, sheep and cattle supported the hypothesis that ruminants especially cattle and sheep act as reservoirs of *E. coli* O157:H7 for human infection.

Keywords: DEC, Human, Ruminants, O157, PFGE

1. INTRODUCTION

Diarrheal diseases are a leading cause of morbidity and mortality in developing countries. *Escherichia coli* is recognized as an important cause of both sporadic cases and outbreaks of diarrhea all over the world. Nguyen *et al.* (2005). Diarrheagenic *Escherichia Coli* (DEC) strains can be classified into five major categories on the basis of the nature of their infection and pathogenic mechanisms: They are Enter Pathogenic *E. Coli* (EPEC), Enter Hemorrhagic *E. Coli* (EHEC), Enterotoxigenic *E. Coli* (ETEC), enter invasive *E. Coli* (EIEC) and Enter Aggregative *E. Coli* (EAEC) (Tamaki *et al.*, 2005). O157:H7 and related non-O157 VTEC strains are pathogens of public health concern worldwide. They may cause severe outbreaks of gastrointestinal illness with clinical symptoms ranging from diarrhea and hemorrhagic colitis to the life-threatening Hemolytic Uraemic Syndrome (HUS) (Lynn *et al.*, 2005). VTEC strains have been isolated from a variety of animals and cattle are considered to be the major reservoir for VTEC strains (Ferens and Hovde, 2011). However, recent evidence has indicated that small domestic ruminants, including sheep and goats, are also key reservoirs of VTEC (La Ragione *et al.*, 2009). In particular, sheep and their products have been documented as reservoirs for VTECs that belong to a diverse set of non-O157 Serogroup that harbor genes encoding key virulence factors that have been implicated in human disease (Djordjevic *et al.*, 2001). Transmission of VTEC to humans occurs through direct or indirect contact with animals or their environment, consumption of contaminated food or water and through person-to-person contact (Gillespie *et al.*, 2005).

In order to prevent and control DEC epidemics, a reliable procedure should be followed to identify and categorize DEC isolates. Since several virulence factors have been identified in DEC strains, modern molecular detection methods, including PCR have been developed (Teng *et al.*, 2004). They are performed at the genetic

level and directly detect genes for specific virulence factors, which themselves determine the pathogenicity. The presence of these genes is the evidence that renders the virulence and that can be used to categorize DEC strains (Yang *et al*., 2007).

This study aimed to document the association of pathogenic *Escherichia coli* isolates with diarrhea in human and animals, based on the PCR-based identification of their intrinsic virulence. And try to find the genetic relatedness among VTEC strains from human, cattle and sheep to identify the zoonotic possibility.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

Stool samples were collected from patients with diarrhea and rectal swabs were collected from cow and sheep with diarrhea. Typical *E. coli* isolates selected were selected from Eosin Methylene Blue (EMB) agar, which discourage the growth of gram positive bacteria and give a distinctive metallic green sheen, these isolates were then subjected to the IMViC test for the further selection of *E. coli* strains.

2.2. Reference Strains

E. coli strains used as positive controls were: 241 (*LT*), 422 (*aggR*), 418 (*bfp*) 226 (*ST*), S103 (*E-hly*), G200 (*ipaH*), 206 (*EAST1*), ED33 (*V1*, *V2* and *eae*) and *E. coli* A300 was used as a negative control. Reference strains were kindly provided by the Niigata Prefectural Institute of Public Health and Environmental Sciences, Department of Bacteriology, Niigata, Japan.

2.3. Somatic and Flagella Serotyping

Isolates were grown on nutrient agar plates at 37 8C overnight, then the cells were collected and suspended in 0.9% sterile normal saline (154 mEq/L sodium and 154 mEq/L chloride, pH adjusted to 7) and autoclaved at 121 8C for 15 min and then the cells were collected by centrifugation and resuspended in an appropriate volume of sterile normal saline. Detection of O-serogroup was performed using a commercially available O-serogrouping Kit (Denka Seiken, Tokyo, Japan) (Takahashi *et al*., 2008). The Flagellar phase inversion was carried out using the standard Craigie tube technique by passage through semi-solid agar containing the appropriate flagellar antis era (Davies and Wray, 1997).

- O-antis era used were,
- Polyvalent I: O1, O26, O86a, O111, O119, O127a, O128

- Polyvalent 2: O44, O55, O125, O126, O146, O166
- Polyvalent 3: O18, O114, O142, O151, O157, O158
- Polyvalent 4: O6, O27, O78, O148, O159, O168
- Polyvalent 5: O20, O25, O63, O153, O167
- Polyvalent 6: O8, O15, O115, O169
- Polyvalent 7: O28ac, O112ac, O124, O136, O144
- Polyvalent 8: O29, O143, O152, O164
- Polyvalent 9: O74, O91, O103, O121, O145, O161, O165
- Flagellar antis era (H-antis era) used were, H2, H4, H5
- H6, H7, H9, H10, H11, H12, H16, H18, H19, H20, H21, H27,H28, H34, H40, H41, H42, H45, H51

2.4. Template DNA Preparation

Single colonies obtained from nutrient agar were inoculated into Luria Bertani (LB) broth (Merck) and were grown at 37°C for 12 h. Cells were harvested from 2 mL of the cell suspension by centrifuging at 16 000×g for10 min and discarding the supernatant. Genomic DNA was then isolated by boiling method (Usein *et al*., 2009).

2.5. Detection of Virulence Factors

Detection of virulence factors was performed by PCR. Primer sequences and PCR conditions used for the study listed in **Table 1**, (Moyo *et al*., 2007; Ochoa *et al*., 2008; Vu-Khac *et al*., 2007; Mendez-Arancibia *et al*., 2008: Zweifel *et al*., 2006; Osek, 2003; Shabana *et al*., 2013). PCR performed in the Takara thermal cycler (Takara, Tokyo, Japan). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose (Wako, Japan) in Tris-Acetate-EDTA (TAE) buffer at 100 V. And a 100 or 500 bp DNA ladder (one-step ladder, Wako) was included in each agarose run, accordingly the amplified product.

2.6. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to CDC (the Pulse Net protocol of the (CDCP, 1998). The agarose-embedded bacterial genomic DNA was digested with restriction enzyme XbaI at 37 8C for 4 h. Electrophoresis was performed on 1% agarose gel with 0.5-Tris-borate-EDTA buffer. The electrophoretic conditions were optimized for the separation of the 24-to 600-kB XbaI-digested macro restriction fragments. The following PFGE parameters were applied: Voltage of 6 v/cm, initial switch time of 2.2 s, final switch time of 54.2 s and run time of 19 h. Electrophoresis was conducted by using a CHEF-DRII (Bio-Rad Laboratories, Tokyo, Japan). The gel was stained with ethidium bromide and imaged with the Gel Doc 2000 and Multi-Analyst program (Bio-Rad).

Table 1. PCR primers and conditions for amplification of virulence genes

Target gene	Primer designation	Primer sequence(5'-3')	PCR conditions *	PCR product (bp)	Reference
Vero toxin 1 (V1)	KS7 KS8	CCCGGATCCATGAAAAAAACATTATTAATAGC CCCGAATTCAGCTATTCTGAGTCAACG	95°C,30 s; 52°C,30 s; 72°C,30 s	285	Zweifel *et al.* (2006)
Vero toxin 2 (V2)	VT2e VT2f	AATACATTATGGGAAAGTAATA TAAACTGCACTTCAGCAAAT	95°C,30 s; 52°C,30 s; 72°C,30 s	348	Zweifel *et al.* (2006)
eae (all intimin genes)	SK1 SK2	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	95°C,30 s; 61°C,30 s; 72°C,30 s	863	Zweifel *et al.* (2006)
E-hly (EHEC-hemolysin)	HlyA1 HlyA4	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	95°C,30 s; 61°C,30 s; 72°C,30 s	1550	Zweifel *et al.* (2006)
(LT) Heat-labile Enterotoxin	elt1 elt2	ATTTACGGCGTTACTATCCTC TTTTGGTCTCGGTCAGATATG	95°C,30 s; 58°C,30 72°C,30 s	281	Vu-Khac *et al.* (2007)
(St)Heat-stable Enterotoxin	St5 St3	TTAATAGCACCCGGTACAAGCAGG CTTGACTCTTCAAAAGAGAAAATTAC	95°C,30 s; 52°C,30 s; 72°C,30 s	147	Mendez-Arancibia *et al.* (2008)
(EAST1) Eagg EaggEC heat-stable enterotoxin	AstA1-1 AstA1-2	CCATCAACACAGTATATC GGTCGCGAGTGACGGCTTTGT	95°C,30 s; 52°C,30 s; 72°C,30 s	111	Osek (2003)
(aggR) Fimbrial antigen-specific gene	aggR1 aggR2	CTAATTGTACAATCGATGTA AGAGTCCATCTCTTTGATAAG	95°C,30 s; 55°C,30 s 72°C,30 s;	457	Moyo *et al.* (2007)
(bfp) bundle-forming pilus	EP1 EP2	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	95°C,30 s; 52°C,30 s;	326	Ochoa *et al.* (2008)
(ipaH)Invasion plasmid antigen	EI-1 EI-2	GCTGGAAAAACTCAGTGCCT CCAGTCCGTAAATTCATTCT	95°C,30 s; 52°C,30 s; 72°C,30 s	424	Shabana *et al.* (2013)

Dendrograms were created with a Molecular Analyst (Bio-Rad) by using the Dice coefficient, Un weighted Pair Group Method with Arithmetic means (UPGMA) and a position tolerance of 1.3%.

3. RESULTS

3.1. Isolation and Confirmation of *E. Coli* Isolates from Diarrheic Cases

A total of 45 presumptive *E. coli* isolates were obtained from diarrheic human (n = 29), cow (n = 9) and sheep (n = 7). The presumptive *E. coli* isolates were confirmed as *E. coli* as they were UidA-positive by PCR, UidA-F, CCAAAAGCCAGACAGAGT, UidA-R GCACAGCACATCAA AGAG (Moyo *et al.*, 2007).

3.2. Serotyping

O antigen and H antigen typing using conventional method revealed a variety of sertypes, 22 *E. coli* isolates correlated with VTEC (O157:H7, O157:H11, O26:H11, O121: HNM, O165: HUT), there was only one ETEC isolate (O8:H12), three EAEC belonging to (OUT: H16, OUT: H28, OUT: HNM), EPEC represented by only one serotype (O115:HUT), three serotypes were aEPEC (OUT:H21, OUT:HNM, O128:H2), 1 EIEC belonging to serotype O74:H9. Fourteen serotypes were negative for the tested virulence genes belonging to serotypes (O166: HUT, O18: HUT, OUT: HUT, OUT: H27, O1: HUT, O153: HUT, O8: HUT, OUT: H21, OUT: H4 (**Table 2**).

3.3. Virulence Genes Detection and Categorization of DEC:

From the total of 45 isolates, 31 isolates were positive for at least one of the targeted virulence genes. The minimal criteria for the determination of DEC were as follows: The presence of *V1* and/or *V2* for VTEC, the presence of *LT* and /or *ST* for ETEC, the presence of *aggR* and or *astA* for EAEC, the presence of *bfpA* and *eae* for typical EPEC and the presence of *eae* only for atypical EPEC, the presence of *ipaH* for EIEC. Based on these criteria, the 31 isolates were classified as VTEC (22 isolates) expressed either V1 or V2 or both, ETEC (1 isolate) from calf, two isolates were EAEC positive for *aggR* and *astA* and one isolate EAEC (*astA*) was heterogenous among the putative DEC, typical EPEC represented by only one strain, 3 a EPEC isolates and 1 isolate EIEC, respectively (**Table 2**).

Table 2. Genotypic profile of diarrhegenic *Escherichia coli*

| DEC	Genotype	Serogroup (no. of isolates)	Host
VTEC	VT1, eaeA	O26:H11(5)	Human
		O157:H7(1)	sheep
	VT2, eaeA	O157:H7(1), O121:HNM (1), O165:HUT (1)	Human
		O157:H7(1)	sheep
	VT1,VT2, Ehly, eaeA	O157:H7(4)	Human
		O157:H7(2), O8:H4(2)	sheep
		O157:H11(2)), O157:H7(2)	cow
ETEC	LT, ST, astA	O8:H12(1)	cow
EAEC	aggR, astA	OUT:H16(1)	Human
	astA	OUT:H28(1), OUT:HNM(1)	Human
EPEC	eaeA, bfpA	O115:HUT(1)	Human
aEPEC	eaeA	OUT:H21(1), OUT:HNM(1), O128:H2(1)	Human
EIEC	ipaH, astA	O74:H9(1)	cow
Untypable		O166:HUT(2), O18:HUT(3), OUT:HUT(2),	Human
		O8:HUT(1), OUT:H21(2)	cow
		OUT:H4(1)	Sheep
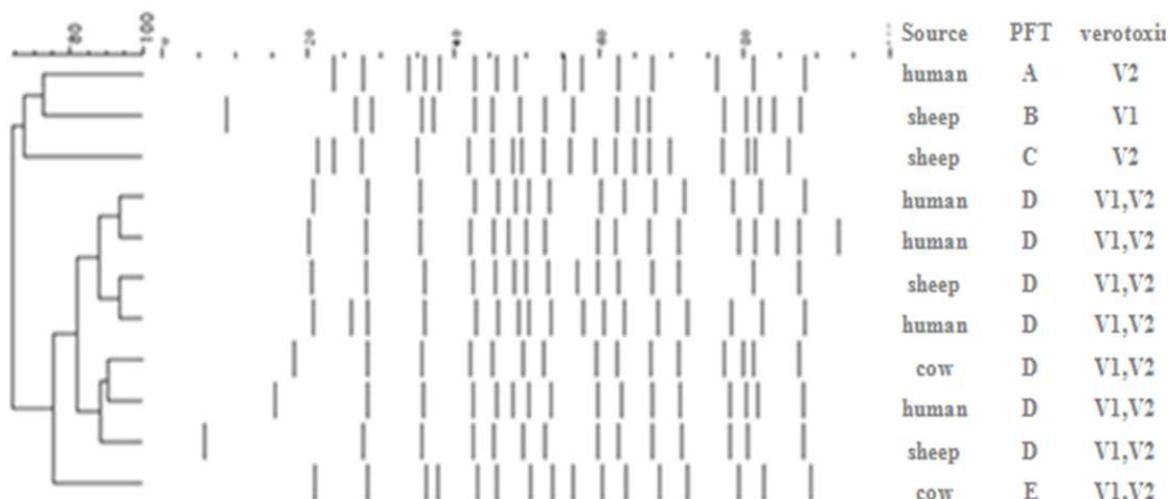
Fig. 1. Dendogram of PFGE patterns calculated with Dice coefficient for E. coli O157:H7 strains from different hosts. The Pulsed-field Gel Electrophoresis (PFGE) types (PFTs) were defined by 80% similarity. Isolates with PFGE patterns with similarity greater than 95% were considered to belong to the same PFT patterns

3.4. PFGE

A PFGE analysis was conducted to identify the genetic relatedness in the recovered O157 isolates from the diarrheic human (n = 6), calf (n = 3) and sheep (n = 2), **Fig. 1.** the strains had more than 80% similarity in the PFGE pattern, the results indicated five PFTs, A: (n = 1) and B: (n = 1), C: (n = 1), D (n = 7) and E (n = 1); briefly pulsotype A represented by single human strain expressing *V2*, pulsotype B represented by single calf strain expressing *V1* and pulsotype C represented by single human strain expressing *V2*. Strains with the pulsotype D were recovered from the three types of feces and all shared expression of both *V1* and *V2*, Pulsotype E also represented by single isolate from sheep expressing both *V1* and *V2*.

4. DISCUSSION

The reference diagnostic approach in the present study was The PCR-based detection of specific genes that render virulence to *E. coli*. In addition, the traditional method of Serotyping, which still occupies a central place in the history of *E. coli*. Specific *E. coli*

Serogroup can be associated reproducibly with certain clinical syndromes, the serotypes and Serogroup serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whittam *et al*., 1993).

Almost half of the isolates carried virulence genes common to Verotoxin-producing *Escherichia Coli* (VTEC), which appeared to be the most prevalent DEC category in this study (48.9%), VTEC distributed among human, calf and sheep isolates and assigned to serotypes O157:H7, O26:H11, O121: HNM, O165: HUT and O8:H4 harbored either *V1* or *V2* or both in addition to *Ehly* and *eaeA*. This is in concordance with other report highlighting the involvement of VTEC in human, calf and sheep illness (Blanco *et al*., 2003).

EPEC represented by four strains restricted to human isolates; three were atypical EPEC possessed only the virulence marker eae, indicative for the presence of the Locus of Enterocyte Effacement (LEE) pathogenicity island and single typical EPEC strain possessed an inducible bundle-forming pilus (bfpA) associated with the presence of the EPEC Adherence Factor (EAF) plasmid and Localized Adherence (LA) on HEp-2 cells in addition to LEE-encoded virulence factors. These findings agreed with previous report about the prevalence of EPEC and how atypical EPEC had more implication than typical EPEC (Afset *et al*., 2003). The majority of EPEC isolates belong to classic serotypes derived from 12 classical O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 andO158) (Scaletsky *et al*., 2010). This study reported O115: HUT as EPEC which agreed with (Saito *et al*., 2005).

ETEC was detected in only one strain from cow assigned for serotype O8:H12, the classical serotype of ETEC (Huasai *et al*., 2012). It possessed heat-labile, heat-stable enterotoxins and plasmid-borne *astA* gene encoding the EAST1. Although the lack of references, which monitors the presence of Enteroinvasive *Escherichia coli* (EIEC) in cow, the current study recorded one strain assigned for O74:H9 and possessed Invasion plasmid antigens (Ipa) proteins which encoded in the ipa operon.

Theoretical digestion of sequenced genomes of *E. coli* O157 strains should generate 41 fragments with XbaI. Some smaller bands run off the gel, while others are too similar in size and co migrate. As a consequence only half to three quarters of these bands can usually be distinguished by PFGE analysis. (Kudva *et al*., 2002) demonstrated that PFGE diversity in *E. coli* O157 is primarily attributed to insertions and deletions, not to point mutations. PFGE was used to genetically discriminate VTEC O157:H7 isolates from different hosts, similar patterns were found among calf, sheep and human strains in Lineage D, which accounted for 63.3% of the tested strains. The strains had expressed the same virulence genes, both *V1* and *V2*, in addition to *Ehly* and *eaeA*, while other lineages were distinctly different due to the variation in expression of virulence genes.

Enterohaemorrhagic *Escherichia Coli* (EHEC) O157:H7 is a zoonotic enteric pathogen of worldwide importance; ruminants cited as its primary reservoirs. Cattle considered to be the sole source of *E. coli* O157:H7 outbreaks in humans; the pathobiology of *E. coli* O157:H7 in small domestic ruminants does appear to differ significantly from that described in cattle and with cattle considered to be the primary reservoir (La Ragione *et al*., 2009).

Transmission of *E. coli* O157:H7 to humans is principally via contamination of food by animal feces (Jaros *et al*., 2013). Food products of animal origin have been confirmed as vehicles of disease transmission in case-control studies of VTEC outbreaks and sporadic VTEC infections; these included raw milk (Guh *et al*., 2010) and undercooked meat products (Chapman *et al*., 2001).

World Health Organization (WHO) listed *Escherichia coli* spp. as one of the most important Pathogenic bacteria which transmitted by food especially Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. Coli* (EPEC), Enterohaemorrhagic *E. Coli* (EHEC), enteroinvasive *E. coli* (EIEC), however the restricted number of the strains in the present study; EHEC, ETEC and EIEC strains were reported in cattle,. The findings refer to the importance of cattle and sheep as sources for zoonotic *Escherichia coli* strains.

5. CONCLUSION

High prevalence of VTEC among diarrheic human, sheep and cattle and highly similar genotypes of VTEC O157:H7 are found in both cattle and sheep and cause human illness suggest that pathogenic strains can circulate freely between them. This information will be of importance for future efforts to trace sources of infection and reduce the burden of disease caused by O157:H7.

6. ACKNOWLEDGEMENT

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