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Original Article

Virulence factors in *Escherichia coli* isolated from calves with diarrhea in Vietnam

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This study was conducted to determine the prevalence and characteristics of pathogenic *Escherichia* (*E.*) *coli* strains from diarrheic calves in Vietnam. A total of 345 *E. coli* isolates obtained from 322 diarrheic calves were subjected to PCR and multiplex PCR for detection of the *f5, f41, f17, eae, sta, lt, stx1,* and *stx2* genes. Of the 345 isolates, 108 (31.3%) carried at least one fimbrial gene. Of these 108 isolates, 50 carried genes for Shiga toxin and one possessed genes for both enterotoxin and Shiga toxin. The *eae* gene was found in 34 isolates (9.8%), 23 of which also carried *stx* genes. The Shiga toxin genes were detected in 177 isolates (51.3%) and the number of strains that carried *stx1, stx2* and *stx1/stx2* were 46, 73 and 58, respectively. Among 177 Shiga toxin-producing *E. coli* isolates, 89 carried the *ehxA* gene and 87 possessed the *saa* gene. Further characterization of the *stx* subtypes showed that among 104 *stx1*-positive isolates, 58 were the *stx1c* variant and 46 were the *stx1* variant. Of the 131 *stx2*-positive strains, 48 were *stx2*, 48 were *stx2c*, 11 were *stx2d*, 17 were *stx2g*, and seven were *stx2c/stx2g* subtypes. The serogroups most prevalent among the 345 isolates were O15, O20, O103 and O157.

Keywords: diarrheic calves, enterotoxigenic *E. coli*, Shiga toxin-producing *E. coli*, Stx variants, virulence genes

Introduction

Escherichia (*E.*) *coli* is an important cause of diarrhea in farm animals. According to virulence properties and the clinical symptoms of the host, pathogenic *E. coli* strains are designated as enterotoxigenic *E. coli* (ETEC), attaching and effacing *E. coli*, enteropathogenic *E. coli*, Shiga toxin-producing *E. coli* (STEC), and necrotoxigenic *E. coli* [11, 19]. ETEC can cause severe diarrhea in newborn calves via the production of heat-stable enterotoxin (STa). The most common observed fimbriae on ETEC from calves with diarrhea is K99 (F5) and F41; however, strains with F17 fimbriae have also been isolated [18]. STEC strains are a well recognized cause of colibacillosis in newborn calves. Even though both healthy and diarrheic calves harbor STEC in their intestine, natural outbreaks and experimental infections have documented the association of STEC with diarrhea and dysentery in young calves [9,30]. In humans, STEC can cause severe diseases, including haemorrhagic colitis and haemolytic uremic syndrome (HUS), via production of Shiga toxins. These toxins are subdivided into two groups, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Stx1 is a homologous group in which three variants (Stx1, Stx1c and Stx1d) have been described [8,39]. Stx2 is more heterogeneous and composed of several subtypes (Stx2, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g and activatable Stx2d) [15,19,28,32]. In addition to toxin production, STEC strains may possess other virulence factors such as intimin (encoded by the *eae* gene) [19], the plasmid-encoded enterohemolysin (encoded by the *ehxA* gene) [31] and the STEC autoagglutinating adhesion (Saa) [26].

In Vietnam, 40% of farm animals have diarrhea, which results in major economic losses [20]. However, no studies regarding the prevalence of pathogenic *E. coli* as a cause of diarrhea in calves have been published to date in Vietnam. This study was designed to investigate the prevalence and characteristics of pathogenic *E. coli* strains from diarrheic calves in Vietnam.

Materials and Methods

Sampling and isolation of *E. coli* strains

Between 2006 and 2008, samples from 322 diarrheic calves (< 3 months of age) were cultured for *E. coli*. The calves resided on 247 farms, 234 of which had one to four animals and 13 of which had > 50 animals. The farms were located in six different provinces in Central Vietnam. Of the 322 calves, 46 were < 7 days old and 276 were between 8- and 90-days old. None of the calves had been vaccinated

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and all showed symptoms of watery diarrhea at the time of sampling. Fecal samples were collected using sterile rectal swabs that were placed into tubes containing Stuart medium and immediately transported to the Laboratory in ice-cooled containers. The samples were then plated onto MacConkey's agar and incubated overnight at 37°C. From each sample, 4 to 5 lactose positive colonies were selected and confirmed to be *E. coli* by standard biochemical tests (Indole, methyl red, Voges-Proskauer and citrate utilization tests). One or two isolates per calf were selected for screening of virulence genes. *E. coli* isolates were stored in tryptic soy broth containing 20% glycerol at −70°C for further characterization.

Detection and genotypic characterization of virulent factors of *E. coli* strains

The primers used for PCR and multiplex PCR are shown in Table 1. Bacterial DNA was obtained by boiling the cells at 100°C for 15 min and then pelleting the cells by centrifugation. The supernatant was then used in the PCR reaction. All isolates were examined for the presence of the *stx1, stx2, sta, f5, f41* and *eae* genes by multiplex PCR as described by Franck *et al.* [12]. The genes for F17 fimbriae and enterotoxin (LT) were screened as described by Vu-Khac *et al.* [35]. The STEC strains were further tested for the presence of *ehxA* [31] and *saa* [25] genes by PCR. To distinguish the *Stx2*, *Stx2c*, and *Stx2d* genes, the restriction fragment length polymorphism-PCR method described by Piérard *et al.* [28] was used. The detection of *stx2g* was conducted as described by Leung *et al.* [15]. The DNA of isolates positive for *stx1* was amplified using the Stx1cF and Stx1cR primers, which are specific for the *stx1c* subtype [39].

Reference strains, O antiserum, and O serogroup determination

The *E. coli* strains used as positive controls were E329 (*f5*), E322 (*f17*), E320 (*f41*), E281 (*lt*), E256 (*sta, stb*), P80 (*eae*), E389 (*stx1*), E391 (*stx2*), BKH-4.1 (*saa/ehxA/stx1c/stx2c*), CT55 (*stx2g*), and BHK (*stx2d*). O antiserum and antisera for the H7 flagella were purchased from Denka-Seiken (Japan). O serogroup determination of the *E. coli* isolates was conducted using standard slide agglutination techniques according to the manufacturer's

Table 1. Primers used for PCR and multiplex PCR

Target gene	Primer	Sequences	Size of product (bp)	References
stx1	STx1-F	TTCGCTCTGCAATAGGTA	555	[12]
	STx1-R	TTCCCCAGTTCAATGTAAGAT		
stx2	STx2-F	GTGCCTGTTACTGGGTTTTTCTTC	118	[12]
	STx2-R	AGGGGTCGATATCTCTGTCC		
sta	STa-F	GCTAATGTTGGCAATTTTTATTTCTGTA	190	[12]
	STa-R	AGGATTACAACAAAGTTCACAGCAGTAA		
lt	LT-F	ATTTACGGCGTTACTATCCTC	281	[34]
	LT-R	TTTTGGTCTCGGTCAGATATG		
eae	EAE-F	ATATCCGTTTTAATGGCTATCT	425	[12]
	EAE-R	AATCTTCTGCGTACTGTGTTCA		
f5	F5-F	TATTATCTTAGGTGGTATGG	314	[12]
	F5-R	GGTATCCTTTAGCAGCAGTATTTC		
f41	F41-F	GCATCAGCGGCAGTATCT	380	[12]
	F41-R	GTCCCTAGCTCAGTATTATCACCT		
f17	F17-F	GGGCTGACAGAGGAGGTGGGGC	411	[34]
	F17-R	CCCGGCGACAACTTCATCACCGG		
saa	SAA-F	CGTGATGAACAGGCTATTGC	119	[24]
	SAA-R	ATGGACATGCCTGTGGCAAC		
ehxA	Hly-F	GGTGCAGCAGAAAAAGTTGTAG	1,551	[31]
	Hly-R	TCTCGCCTGATAGTGTTTGGTA		
stx1c	STx1c-F	TTTTCACATGTTACCTTTCCT	498	[37]
	STx1c-R	CATAGAAGGAAACTCATTAGG		
stx2, stx2c	VT2-e	AATACATTATGGGAAAGTAATA	348	[27]
stx2d	VT2-f	TAAACTGCACTTCAGCAAAT		
stx2g	STx2g-F	GTTATATTTCTGTGGATATC	573	[15]
	STx2g-R	GAATAACCGCTACAGTA		

instructions. The flagella antigen H7 was determined in strains belonging to serogroup O157.

Results

Prevalence of the genes of fimbriae, toxins and intimin in *E. coli* isolated from calves with diarrhea

A total of 345 *E. coli* isolates were analyzed by PCR and the results are summarized in Table 2. Overall, 108 of the 345 isolates (31.3%) carried genes of one of the fimbriae tested (F5, F17, and F41). Of the isolates that had fimbriae genes, 50 also carried genes for STx, one had both Stx and enterotoxin genes and 57 did not have any toxin genes. More than half of the isolates (177/345) carried genes for Stx and 23 also possessed the *eae* gene. Amongst isolates that carried the *stx* genes, 46 were *stx1*, 73 were *stx2* and 58 were *stx1/stx2*.

Characterization of virulence genes

The STEC strains were further tested for two additional virulence factors, enterohemolysin (EHEC-hly) and autoagglutinating adhesion (Saa). The *ehxA* and *saa* genes were detected in 51% and 49% of the STEC isolates, respectively. The majority of the strains (73/87) carrying the *saa* gene were also positive for *ehxA*. Further characterization of the 104 isolates carrying the *stx1* gene showed that 58 (55.7%) strains were positive for the Stx1c variant and 46 strains (44.2%) were classified as Stx1. Of the 131 strains carrying the genes for Stx2 variants, 48 (36.6%) were *stx2*, 48 (36.6%) were *stx2c*, 11 (8%) were *stx2d*, 17 (12.9%) were *stx2g*, and seven (5.3%) contained both *stx2c* and *stx2g*.

O serogroups

The isolates were tested against 33 different O serogroups commonly associated with pathogenic bovine *E. coli* and 89 strains (25.8%) belonged to one of these groups. The majority of strains (60) belonged to only four O serotypes, which included O15 (21 strains), O20 (9 strains), O103 (20 strains) and O157 (10 strains) (Table 3). Two of the ten O157 strains belonged to serotype O157:H7 and one carried genes for the Shiga toxin and intimin.

Discussion

This study is the first report of the prevalence of virulence factors in *E. coli* isolated from calves with diarrhea in Vietnam. It is well known that among the pathotypes of *E. coli* that cause diarrhea in calves, the ETEC strains are the most important agents, and strains expressing F5 and/or F41 and producing STa toxin are the most common [18]. F5 and/or F41 play a role in the colonization of bovine small intestine epithelial cells by ETEC and are primarily isolated from 1- to 7-day-old diarrheic calves [18]. In the present study, 53 of 345 isolates carried the *f5* (36 isolates) and *f41* (17 isolates) genes, and all of the F5-positive

Table 2. Virulence genes of 345 *Escherichia (E.) coli* strains isolated from calves with diarrhea

Pathotypes	No. of strains	Virulence genes									
		f5	*f41*	*f17*	*lt*	*stx1*	*stx2*	*stx1stx2*	*saa*	*ehxA*	*eae*
ETEC	2	1	1	0	2	0	0	1	0	0	0
nt-EC*	53	19	8	30	0	0	0	0	0	0	2
STEC	177	16	8	25	0	46	73	58	87	89	23
AEEC	9	0	0	0	0	0	0	0	0	0	9
Total	241	36	17	55	2	46	73	59	87	89	34

*nt-EC: *E. coli* strains did not have toxin genes. ETEC: enterotoxigenic *E. coli*, STCE: Shiga toxin-producing *E. coli*, AEEC: attaching and effacing *E. coli*.

Table 3. Distribution of virulence genes from O15, O20, O103 and O157 *E. coli* strains recovered from diarrheic calves

Serotypes	No. of strains	Virulence genes									
		f5	*f41*	*f17*	*lt*	*stx1*	*stx2*	*stx1stx2*	*saa*	*ehxA*	*eae*
O15	21	0	0	5	0	3	4	2	7	7	0
O20	9	1	1	2	0	1	3	1	1	0	0
O103	20	2	1	1	1	2	8	3	2	2	3
O157	10	2	2	2	0	1	2	2	2	1	1

strains isolated from diarrheic calves were less than 7-day old. The susceptibility of the calves to ETEC in this age period is in agreement with previous reports [21,38]. Interestingly, in this study, we found that none of the F5-positive strains encoded enterotoxin genes (STa) when analyzed by PCR and they were also negative when tested with baby mice (data not show). However, more than half of the strains contained genes for Shiga toxin. While the F5-positive strains do not have enterotoxin genes, they do contain genes for Shiga toxin, which would indicate the emergence of a new phenotype causing diarrhea in calves. The observation that a high number of *E. coli* strains carried genes for fimbriae but did not have toxin genes has been reported before [17]. The F17 fimbriae, which are formally known as FY or Att25, are prevalent in *E. coli* strains isolated from calves with diarrhea or septicemia. In the present study, we found that 55 (16%) of 345 *E. coli* isolates from diseased calves carried genes for F17 fimbriae. Our results are in agreement with those of previous studies [24,33,34], which showed that F17 fimbriae are commonly found among *E. coli* strains isolated from 12% to 19% of diarrheic calves. Surprisingly, 50 (46%) of the 108 strains carrying fimbrial genes also possessed genes for Shiga toxins. The high prevalence of the Shiga toxin genes in these strains is particularly striking and has not been previously reported.

STEC has been implicated as an etiological factor of calf diarrhea [9,23,30,37], and these animals form a principle reservoir of STEC that is pathogenic for humans [1,6]. In the present study, we found that a total of 177 isolates (51%) were positive for the *stx* genes. Similarly, high percentages (40% or more) of *stx* gene positive *E. coli* strains have been reported in Brazil [29] and India [2]. However, other authors [23,24] have reported a lower rate (less than 10%) of STEC in diarrheic calves. STEC strains belonging to serogroups O15, O20, O103, and O157 have previously been found to be associated with diarrhea and enteritis in calves in Belgium [16], Spain [22], and the United States [10]. The majority of the STEC strains isolated in this study did not belong to the serogroups (O8, O9, O26, O111, O113, O126, O145) that have previously been found to be associated with diarrhea and enteritis in calves in other countries [16]. Interestingly, we found that four of the eight strains belonged to serotype O157:H7 and carried genes for both Shiga toxin and fimbriae. All of these O157:H7 strains were isolated from eight different diarrheic calves and fermented in sorbitol sugar (data not shown). The isolation of two strains belonging to serotype O157:H7 is of concern to human health [4,5]. Several studies [16,23,37] have demonstrated that most STEC from diarrheic calves only produce Stx1, whereas Stx2-positive strains are the dominant types in healthy calves [16,23,37]. This is in contrast to the results of the present study, wherein the *stx2* gene (131 isolates) was observed as frequently as *stx1* (104 isolates). Among the 23 STEC strains containing the *eae* genes, 11 isolates had only the *stx1* gene, seven had the *stx2* gene, and five had both genes.

It has previously been reported that the *stx2c* toxin genes were predominant among the *stx2*-positive *E. coli* strains isolated from diarrheic calves [24]. This is consistent with the results of the present study in which 55/131 *stx2*-positive strains were found to possess genes encoding Stx2c. Several studies have demonstrated that toxin types and variants could be important in the pathogenicity of STEC strains [27,28]. In humans, infection with isolates producing Stx2 toxins resulted in HUS more frequently than was observed for Stx1-producing strains [1,6,19]. Conversely, Stx2d-producing strains showed a low cytotoxicity toward Vero cells and were less frequently associated with diarrhea and HUS [27]. In the present study, *stx2d* was detected in 11 of 131 *stx2*-positive strains. The novel variant of bovine Stx2, which was designated Stx2g [15], was detected in 24/131 isolates in which seven isolates also possessed the genetic determinants for the Stx2c toxin variant. Previous studies have shown the toxin type 1c (Stx1c) to be closely associated with sheep [7,14], but not with cattle [7,40]. However, in this study, we found the *stx1c* genes in 44.2% of 104 *stx1*-positive strains, which suggests that Stx1c-producing strains are not restricted to sheep.

Recently, Paton *et al.* [26] described a novel megaplasmid-encoded adhesin, denoted Saa, in a LEE-negative O113:H21 STEC strain responsible for an outbreak of HUS. These authors also showed that the *saa* gene was associated with the *ehxA* gene [25,26]. In the present study, we found the *saa* gene in 87 STEC strains, and the majority of these strains also carried the *ehxA* gene. Enterohemolysin is widely distributed among STEC strains isolated from calves [3,13,24], although the role of enterohemolysin in causing diarrhea in calves has not been demonstrated. It has been suggested that this virulence factor may compliment the effects of Shiga toxin [3,19], and that it can be used as a diagnostic marker because its presence is strongly correlated with Shiga toxin [3,36]. The percentage of enterohemolysin among bovine STEC strains has been reported to range from 46.7 to 70% [3,13,24,36]. Our results showed that 51% of STEC isolates possessed the *ehxA* gene.

In conclusion, the results of the present study indicate that a high prevalence of strains carrying fimbrial genes also contained Shiga toxin genes. This suggests that *stx* genes, which are encoded on bacteriophages, are continuing to expand in the *E. coli* population. The high number of STEC strains isolated from diarrheic calves implies that these animals are an important reservoir of STEC strains that are potentially pathogenic toward farm animals and humans.

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