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Virulence Profiling and Disease Association of Verocytotoxin-Producing *Escherichia coli* O157 and Non-O157 Isolates in Belgium

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Abstract

Whereas the association of verocytotoxin-producing *Escherichia coli* (VTEC) O157:H7 with the hemolytic uremic syndrome (HUS) is well established, the medical importance of many non-O157 serotypes remains unclear. Using polymerase chain reaction (PCR), we have investigated the distribution of the pathogenicity island O island 122 (OI-122) and other virulence genes in VTEC belonging to seropathotypes (SPT) A through D, and assessed their association with human disease. Two hundred sixty-five VTEC isolated from human stools comprising 52 O157 (of which 14 associated with HUS) and 213 non-O157 isolates (of which 19 associated with HUS) were studied. A complete OI-122 (COI-122) was detected in all O157, but in only 35 (16.4%) of non-O157 strains. A progressive decrease in the frequency of COI-122 was observed from SPT A through D, with a concomitant increase in the frequencies of incomplete and absent OI-122. We focused on the variable virulence profiles of the non-O157 serotypes and found that COI-122 was also more frequently present in isolates associated with HUS ($p = 0.001$). The individual genes *vtx2*, *eae*, *espP*, as well as the OI-122-associated genes *sen*, *nleB*, *nleE*, and the *efa* gene cluster were significantly more often present in non-O157 VTEC associated with HUS. Non-O157 isolates carrying the combined virulence profile *vtx2-nleE-efa* showed the strongest association with HUS ($p < 0.0001$). Molecular risk assessment by determination of virulence profiles of individual isolates may be useful in the identification of highly virulent non-O157 strains. We showed that the detection of a specific gene combination could assist in identifying non-O157 VTEC isolates that pose a serious public health concern.

Introduction

Verocytotoxin (VT)–producing *Escherichia coli* (VTEC), also called Shiga toxin–producing *E. coli* (STEC), are foodborne pathogens associated with sporadic cases and outbreaks of bloody diarrhea that may be complicated by hemorrhagic colitis and hemolytic uremic syndrome (HUS), mainly in young children and the elderly (Karch *et al*., 2005). The production of one or more types of bacteriophage-encoded VT is the cardinal virulence trait involved in HUS development. Over 400 VTEC serotypes have been identified (www.usc.es/ecoli/SEROTIPOSHUM.htm), but only a subset has been frequently associated with severe disease and outbreaks. This suggests that additional virulence markers are necessary for disease. Karmali *et al*. (2003) have classified VTEC serotypes into five seropathotypes (SPT) (A through E) according to their association with serious disease and outbreaks. SPT A comprises O157:H7/H-, which are most frequently associated with outbreaks and HUS; SPT B strains are associated with outbreaks and HUS, but less frequently than SPT A; SPT C strains are associated with sporadic HUS but not outbreaks; SPT D strains are associated with uncomplicated diarrhea; and SPT E strains are not associated with human disease and appear to be limited to animals.

Sequencing of VTEC O157:H7 EDL933 revealed a 4.1-Mb genomic backbone, which is homologous to *E. coli* K-12, and 1.34 Mb of O157:H7-specific DNA organized in genomic "O islands" (Perna *et al*., 2001). One class of genomic islands, pathogenicity islands, represents a flexible pool of virulence-associated genes, which can be transferred horizontally in the bacterial population contributing to pathogen evolution (Hacker *et al*., 2000) and possibly in the emergence of new pathogenic VTEC serotypes. One such pathogenicity island is the locus of enterocyte effacement (LEE), which encodes a type III secretion system involved in the formation of attaching and effacing lesions. While other O islands encoding putative virulence factors were identified in EDL933, O island 122 (OI-122) has received increasing attention. OI-122 is a 23-kb pathogenicity island containing 26 open reading frames (ORF), of which six show significant homology to known

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Table 1. Seropathotype Classification of Included Verocytotoxin-Producing *Escherichia coli*

Seropathotype (no. of isolates)	*Included serotypes (no. of isolates)*[a]
A (52)	**O157:H7** (33); **O157:H-** (19)
B (90)	**O26:H11/H-** (46); O103:H2 (14); **O111:H8**/H- (15); **O145:H28/H-** (15)
C (34)	**O2:H6** (3); O5:H- (3); O8:H14 (1); O15:H- (2); O84:H- (2); O91:H21/H- (4); O98:H- (2); O104:H2 (1); O105:H18 (1); O113:H21 (4); **O118:**H16/**Hunt** (3); **O121:H-** (3); O128:H2 (1); O168:H- (2); **O172:H-** (1); **O178:Hunt** (1)
D (89)	O3:H- (1); O4:H16/H- (2); O6:H10 (1); O8:H9/H- (3); O15:H27 (1); O20:H4/H45 (2); O24:H10 (1); O38:H26 (2); O45:H- (1); O55:H12 (2); O63:H6 (8); O76:H19 (1); O78:H- (1); O79:H14 (2); O80:H- (2); O84:H28 (3); O87:Hunt (1); O91:H14 (1); O109:H5 (1); O113:H2/H4/H- (4); O115:H- (1); O117:K1:H7 (2); O118:H- (1); O127:H40 (1); O128:H- (4); O132:H34 (1); O136:H20 (1); O146:H21/H28/H- (15); O147:H- (1); O150:H- (1); O153:H- (1); O162:H28/H- (2); O166:H28 (1); O171:H29 (1); O174:H21 (1); O176:H- (1); O177:H11 (1); O181:H40 (1); OX182:H34 (1); O183:H18 (3); Ount:H14 (2); Orough:H4/H10/H19/H21/H- (6)

[a]Serotypes associated with hemolytic uremic syndrome (HUS) in this study are shown in bold.

virulence genes. ORF Z4321 is homologous to *pagC* of *Salmonella enterica* serovar Typhimurium; ORF Z4326 is homologous to *sen* of *Shigella flexneri*; ORFs Z4328 and Z4329 are homologous to non-LEE effector (nle) genes *nleB* and *nleE* of *Citrobacter rodentium*; and ORFs Z4332 and Z4333 are homologous to the EHEC Factor for Adherence gene cluster *efa1* and *efa2* found in VTEC O157:H7. Thirteen ORFs are associated with mobile genetic elements, and the function of the remaining seven genes is unknown. While nearly all O157:H7/H- strains carry a complete OI-122 (COI-122), a progressive decrease in the prevalence of OI-122 genes in non-O157 VTEC belonging to SPT B through E with a concomitant decreasing pathogenicity was observed (Karmali *et al*., 2003). Wickham *et al*. (2006) have described a modular arrangement of OI-122 genes based upon their association with each other across HUS-associated non-O157 VTEC strains: module 1 contains Z4318, *pagC*, and Z4322; module 2 contains Z4323, *sen*, *nleB*, and *nleE*; and module 3 contains the *efa* gene cluster. The presence of putative transposases in OI-122 has led to the hypothesis that its elements are acquired or lost in a modular manner. It was shown that, while *pagC*, Z4322, *sen*, *nleB*, *nleE*, and *efa1* individually were more prevalent in non-O157 VTEC associated with HUS, the simultaneous presence of all of these genes strengthened the association with serious disease. Thus, after acquiring OI-122 modules from VTEC O157:H7 via horizontal transfer, less pathogenic non-O157 strains could cross a virulence threshold, resulting in sufficient pathogenicity to cause HUS.

Since no specific data on this pathogenicity island are available in Belgium, we have investigated the prevalence and distribution of OI-122 elements and other virulence factors by PCR in human VTEC isolates belonging to SPT A through D and correlated these data with clinical manifestations.

Methods

Bacterial strains

Two hundred sixty-five O157 ($n=52$) and non-O157 ($n=213$) VTEC isolates from patients with HUS ($n=33$), bloody diarrhea ($n=32$), diarrhea ($n=145$), abdominal pain ($n=28$), and disease other than HUS or diarrhea ($n=27$) recovered in our laboratory from 1990 to 2010 were included. These isolates were selected from our cryocollection to reflect the different O:H serotypes isolated from humans in Belgium. The isolates were classified into four SPT, A through D, according to known associations with outbreaks and severe disease (Karmali *et al*., 2003; Wickham *et al*., 2006) (www.usc.es/ecoli/SEROTIPOSHUM.htm) and clinical data available in Belgium (Table 1). SPT E was not taken into account, since all VTEC originated from clinical cases.

Detection of virulence genes by PCR

All primers used in this study are listed in Suppl. Table S1 (Supplementary Material is available online at www.liebertonline.com/fpd). We searched for the established virulence markers VT1 and VT2 (*vtx1* and *vtx2*), intimin (*eae*), and enterohemolysin (*ehxA*) according to the method described by Paton *et al*. (1998). Additional PCRs targeting plasmid genes for STEC auto-agglutinating adhesin (*saa*), the A subunit of subtilase cytotoxin (*subA*), extracellular serine protease (*espP*), catalase-peroxidase (*katP*), and a type II transporter system (*etpD*) were performed (Brunder *et al*., 1999; Paton *et al*., 2002, 2004; Schmidt *et al*., 1999). All strains were screened for the presence of OI-122 genes *pagC*, *sen*, *nleB*, *nleE*, and the *efa* gene cluster using primers described by Karmali *et al*. (2003) and Wickham *et al*. (2006). Strains with positive PCR results for the six genes were defined as strains carrying a complete OI-122 (COI-122), an incomplete OI-122 was appointed to strains with a negative PCR result for at least one OI-122 ORF, and strains negative for all OI-122 PCRs were labeled as "OI-122 absent," VTEC O157:H7 EDL933 was used as a positive control in all PCRs, except for *saa* and *subA,* for which a clinical VTEC O113:H21 isolate (strain EH1516) was used. PCR-grade water was used as negative control.

Statistical analysis

Associations between the presence or absence of investigated genes were determined using univariate or multivariate analysis where appropriate (SPSS Statistics 19; IBM, Armonk, NY). Probability value of $p<0.05$ was considered significant.

Results

Strain characterization

Included serotypes sorted according to SPT are shown in Table 1. The 265 VTEC isolates belonged to 81 different O:H

Table 2. Prevalence Data of Tested Virulence Genes in Verocytotoxin-Producing *Escherichia coli* Associated and Not Associated with Hemolytic Uremic Syndrome (HUS)

Category (no. of isolates)	No. of isolates PCR positive for (%)[a]															
										O island 122						
	Verocytotoxins		LEE	Plasmid genes						Module 1		Module 2		Module 3		
	vtx1	vtx2	eae	ehxA	saa	subA	espP	katP	etpD	pagC	sen	nleB	nleE	efa1	efa2	
All (265)	160 (60.4)	150 (56.6)	187 (70.6)	202 (76.2)	13 (4.9)	8 (3.0)	137 (51.7)	99 (37.4)	76 (28.7)	109 (41.1)	163 (61.5)	163 (61.5)	159 (60.0)	153 (57.7)	153 (57.7)	
O157 (52)	18 (34.6)	52 (100)	52 (100)	51 (98.1)	0 (0.0)	0 (0.0)	48 (92.3)	48 (92.3)	51 (98.1)	52 (100)	52 (100)	52 (100)	52 (100)	52 (100)	52 (100)	
Non-O157 (213)	142 (66.7)	98 (46.0)	135 (63.4)	151 (70.9)	13 (6.1)	8 (3.8)	89 (41.8)	51 (23.9)	25 (11.7)	57 (26.8)	111 (52.1)	111 (52.1)	107 (50.2)	101 (47.4)	101 (47.4)	
Non-O157 associated with HUS (19)	5 (26.3)	17 (89.5)[b]	17 (89.5)[b]	18 (94.7)[b]	1 (5.3)	1 (5.3)	13 (68.0)[b]	7 (37.0)	3 (15.8)	8 (42.1)	17 (89.5)[b]	17 (89.5)[b]	17 (89.5)[b]	17 (89.5)[b]	17 (89.5)[b]	
Non-O157 not associated with HUS (194)	137 (70.6)[c]	81 (41.7)	118 (60.8)	133 (68.6)	12 (6.2)	7 (3.6)	76 (39.0)	44 (23.0)	22 (11.3)	49 (25.2)	94 (48.4)	94 (48.4)	90 (46.4)	84 (43.3)	84 (43.3)	

[a]By polymerase chain reaction (PCR).
[b]Significantly more prevalent in non-O157 isolates associated with HUS.
[c]Significantly more prevalent in non-O157 isolates not associated with HUS.

serotypes. Thirty-three O157:H7 and 19 O157:H-, including one sorbitol-fermenting O157:H-, were included in SPT A. SPT B comprised 90 isolates belonging to serotypes O26:H11/H- ($n=46$), O103:H2 ($n=14$), O111:H8/H- ($n=15$), and O145:H16/H28/H- ($n=15$). SPT C comprised 34 isolates belonging to 18 serotypes, and 89 isolates belonging to 44 serotypes were included in SPT D. The prevalence of individual virulence genes is shown in Table 2. Variation in the *vtx* genotypes and/or the presence of plasmid genes *ehxA*, *saa*, *subA*, *espP*, *katP*, and *etpD* were found in individual serotypes. A close relationship was observed between the serotype and the *eae* gene, with isolates of the same serotype being either all *eae*-positive or all *eae*-negative. *saa* (13/265; 4.9%) and *subA* (8/265; 3.0%) were only present in *eae*-negative VTEC. All *subA*-positive strains were also positive for *ehxA*, *saa*, and *espP*.

Modular distribution of OI-122

The distribution of individual OI-122 genes is shown in Table 2. All VTEC O157 isolates (52/52; 100%) contained a COI-122. On the other hand, only 35 (16.4%) out of 213 non-O157 isolates contained a COI-122, 89 (41.8%) had an incomplete OI-122, and OI-122 was absent in 89 (41.8%) isolates. A progressive decrease in the frequency of COI-122 from SPT A through D was observed, with a concomitant increase in the frequencies of incomplete and absent OI-122. In SPT B, 28 (31.1%) out of 90 isolates were positive for COI-122, 59 (65.5%) carried an incomplete OI-122, and three (3.3%) isolates were PCR-negative for OI-122–associated genes. In SPT C, five (5/34; 14.7%) isolates of serotypes O121:H- ($n=3$) and O5:H- ($n=2$) carried a COI-122. In 13 (13/34; 38.2%) isolates, OI-122 was incomplete and OI-122 was absent in 16 (16/34; 47.1%). Only two O80:H- isolates belonging to SPT D carried a COI-122, 17 (17/89; 19.1%) had an incomplete OI-122, and 70 (70/89; 78.6%) were negative in every OI-122 PCR. A COI-122 occurred significantly more frequently in SPT associated with outbreaks (A and B) (80/142; 56.3%) as compared to those not associated with outbreaks (C and D) (7/123; 5.7%) ($p<0.0001$). COI-122 was also significantly more prevalent in SPT associated with HUS (A, B, and C) (85/176; 48.3%) than in and SPT D (2/89; 2.2%), which is not associated with HUS ($p<0.0001$).

O island 122 as a marker for virulence in VTEC

We first considered the OI-122 content in association with disease for all studied isolates. The prevalence of a COI-122 was highest in VTEC recovered from HUS patients (22/33; 66.7%) and decreased progressively in patient groups with bloody diarrhea (15/32; 46.9%), diarrhea (43/145; 29.6%), disease other than HUS or diarrhea (5/27; 18.5%), and abdominal pain (2/28; 7.1%; Fig. 1). A COI-122 was significantly more frequent in isolates recovered from patients suffering from HUS or bloody diarrhea (37/65; 56.9%) as compared to isolated from patients with diarrhea, abdominal pain, or non-gastrointestinal disease (50/200; 25.0%) ($p<0.0001$).

Since nearly all O157 VTEC contained the complete array of virulence genes that were searched for, we focused on the variable virulence profiles of non-O157 serotypes. Of 19 non-O157 isolates recovered from HUS patients, eight (42.1%) carried a COI-122, nine (47.4%) carried an incomplete OI-122, and two were negative for OI-122. Among those not associated with HUS, a COI-122 was detected in 27 (27/194; 13.9%) isolates, 80 (41.2%) carried an incomplete OI-122, and OI-122 was absent in 87 (44.8%). The difference between the occurrence of COI-122 in both patient groups was highly significant ($p=0.001$). We investigated whether there were epidemiological differences in the association of OI-122 modules with HUS after non-O157 infection (Table 3). Isolates carrying modules 2 ($p=0.0006$) or 3 ($p=0.0001$) were significantly associated with HUS, yet module 1 was not ($p=0.11$). The combined presence of modules 2 and 3 strengthened the association with HUS ($p<0.0001$).

Significant associations of individual virulence markers were observed when comparing HUS-associated non-O157 strains to those not associated with HUS (Table 2). While *vtx1* was significantly more frequent in VTEC not associated with HUS (70.6%; $p=0.0002$), 17 out of 19 (89.5%) non-O157 VTEC associated with HUS were *vtx2* positive ($p=0.0001$). *eae* ($p=0.013$), *ehxA* ($p=0.016$), and *espP* ($p=0.014$) were significantly more prevalent in HUS than in non-HUS strains. Among the OI-122 genes, *sen*, *nleB* (both $p=0.001$), *nleE* ($p<0.0001$), and the *efa* gene cluster ($p<0.0001$) were significantly more frequent in non-O157 VTEC strains associated with HUS as compared to those not associated with HUS. No

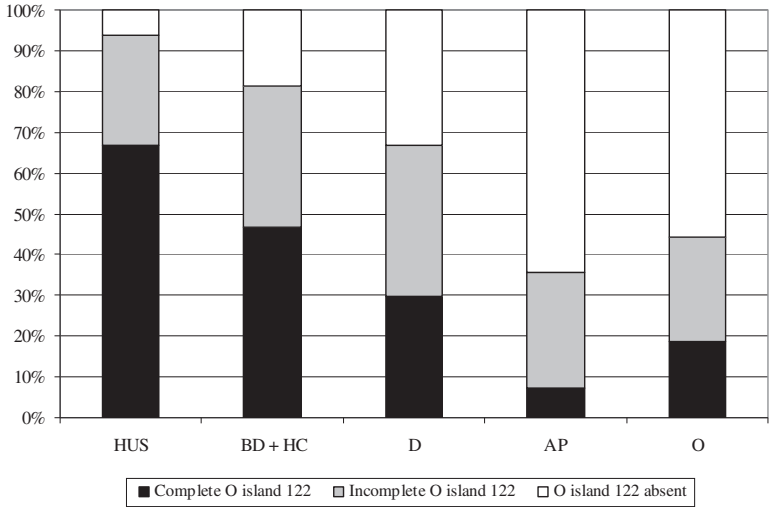

FIG. 1. Distribution of O island 122 in verocytotoxin-producing *Escherichia coli* in association with clinical manifestations. HUS, hemolytic uremic syndrome; BD, bloody diarrhea; HC, hemorrhagic colitis; D, diarrhea; AP, abdominal pain; O, disease other than HUS, diarrhea, or abdominal pain.

Table 3. Prevalence of O Island 122 Modules in Non-O157 Verocytotoxin-Producing *Escherichia coli* in Association with Hemolytic Uremic Syndrome (HUS)

Module	*Prevalence in HUS-associated isolates (%)*	*Prevalence in non-HUS-asociated isolates (%)*	p
1	8/19 (42.1)	49/194 (25.3)	0.11
2	17/19 (89.5)	94/194 (48.4)	0.0006
3	17/19 (89.5)	84/194 (43.3)	0.0001
2+3	17/19 (89.5)	82/194 (42.3)	<0.0001

significant differences were observed for *saa*, *subA*, *katP*, *etpD*, and *pagC*.

The combined presence of *vtx2-nleE-efa* in individual non-O157 isolates showed the strongest association with HUS ($p < 0.0001$), suggesting an additive effect of these virulence genes in the ability of VTEC to cause disease. For the collection of isolates in this study, the use of the *vtx2-nleE-efa* profile to detect non-O157 isolates causing HUS after infection had a sensitivity of 78.9%, a specificity of 94.8%, and positive and negative predictive values of 60.0% and 97.9%, respectively.

Discussion

We have investigated the distribution of pathogenicity island OI-122 and other virulence genes in O157 and non-O157 VTEC associated with human disease in Belgium. Because of the lack of distinct biochemical properties characteristic of non-O157 VTEC, many clinical laboratories only search for VTEC O157:H7, leaving most non-O157 infections undiagnosed. The absence of a widespread surveillance system of non-O157 VTEC opens a window of opportunity for the emergence of other serotypes with a pathogenic potential that is possibly comparable to that of O157:H7. In addition to careful evaluation of key genes associated with serious disease and outbreaks, there is a need for rapid diagnostic tools that allow clinical laboratories to quickly identify emerging pathogenic VTEC strains.

While the clinical importance of non-O157 VTEC is still underestimated, determining the virulence profile may help in the identification of potentially pathogenic strains. The overall prevalence of COI-122 (87/265; 32.8%) in our Belgian collection was lower as compared to previous data. Karmali *et al*. (2003) reported COI-122 in 40% of 70 strains of a North American collection. In that study, COI-122 was present in all strains of SPT A and in 60% of SPT B strains. Karama *et al.* (2009), on the other hand, showed that COI-122 was less frequent (31.8%) in a collection of O103:H2 VTEC strains, but that its prevalence was higher in North American human strains than in European human strains of the same serotype.

The present study confirms the strong correlation of COI-122 with VTEC strains belonging to SPT associated with outbreaks (A and B) and SPT associated with HUS (A, B, and C), as noted previously by Karmali *et al*. (2003) and Wickham *et al*. (2006). Moreover, all COI-122–positive strains in this study were also positive for *eae*, a marker for LEE. The association between OI-122 and LEE has been recently reported by Morabito *et al.* (2003). This provides more evidence for the assumption that differences in virulence between serotypes might be based on the presence or absence of specific pathogenicity islands rather than on the basis of individual genes. In this study, non-O157 VTEC associated with HUS were significantly more frequently positive for *vtx2*, *eae*, *ehxA*, *espP*, and the OI-122–associated genes *sen*, *nleB*, *nleE*, and the *efa* gene cluster. Hedican *et al.* (2009) argued that testing of samples to identify the presence of *vtx2* versus *vtx1* may not be reliable in predicting whether the infecting VTEC strain is capable of causing severe illness. Here, the combined presence of the genes *vtx2-nleE-efa* showed the strongest association with HUS. Identifying these virulence markers could aid in distinguishing non-O157 VTEC strains that pose a serious risk to public health from those strains that are associated with less severe disease. For the collection of isolates in this study, the use of the *vtx2-nleE-efa* profile to detect non-O157 isolates causing HUS after infection had a sensitivity of 78.9%, a specificity of 94.8%, and positive and negative predictive values of 60.0% and 97.9%, respectively. Obviously, this test is not completely appropriate for clinical practice. Testing for three genes limits its practical use, but this may be overcome by developing a multiplex PCR. In future experiments, subtyping of *vtx*, especially *vtx2*, may improve the diagnostic capabilities to detect highly pathogenic non-O157 strains.

The concept of detecting virulence markers to identify highly pathogenic VTEC is called "molecular risk assessment" and was recently introduced by Coombes *et al*. (2008). While *vtx2*-positive, LEE-positive, and OI-122–positive VTEC are associated with outbreaks and serious disease, this and previous studies have shown that strains lacking these virulence genes are also capable of causing important human illness (Karmali *et al*., 2003; Wickham *et al*., 2006). These observations suggest that additional genes, possibly organized in pathogenicity islands, may also enhance the virulence potential of VTEC strains. The precise contribution of all mobile genetic elements to VTEC virulence is currently unknown in most cases, and the complete virulence repertoire has not been determined for most non-O157 VTEC (Coombes *et al*., 2011). Recently, Ogura *et al*. (2009) compared the whole genome of three non-O157 strains to that of O157, but additional genome-wide studies are necessary to untangle the key players in non-O157 VTEC virulence.

The virulence potential of other *E. coli* pathotypes may be enhanced by the acquisition of *vtx* genes via mobile bacteriophages. This was recently evidenced by the German enteroaggregative, VT-producing *E. coli* O104:H4 strain, which had gained a *vtx2* gene as well as additional virulence genes and a CTX-M-15 extended β-lactamase through horizontal gene transfer (Bielaszewska *et al*., 2011; Rasko *et al*., 2011; Scheutz *et al*., 2011). To our knowledge, however, there are no data available on the presence of OI-122 in this O104:H4 outbreak strain.

In conclusion, virulence profiling of clinically relevant non-O157 VTEC may contribute to a better understanding of their virulence potential and could be used for the risk assessment of individual isolates. The World Health Organization has called the rapid identification of virulent non-O157 VTEC a public health priority (WHO, 1998). A continued surveillance of known and newly discovered putative virulence genes and pathogenicity islands seems therefore warranted. Virulence profiling by evaluation of genomic island content should be performed as quickly as possible to ensure prompt identification of highly pathogenic VTEC strains. To this aim, the

development of multiplexed real-time PCR or microarray techniques for the detection of selected virulence markers could be useful. This study provides more evidence that a subset of OI-122 genes could represent possible gene candidates.

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Disclosure Statement

No competing financial interests exist.

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