Production of Verotoxin and Distribution of O Islands 122 and 43/48 among Verotoxin-Producing *Escherichia coli* O103:H2 Isolates from Cattle and Humans[▽]

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This study investigated variations in the occurrence of markers of O islands 122 and 43/48 and in verotoxin 1 production in 91 verotoxin-producing *Escherichia coli* (VTEC) O103:H2 strains of bovine and human origins. None of the genes that were investigated appear to be virulence indicators for human O103:H2 VTEC.

Cattle are the main reservoir of verotoxin (VT)-producing *Escherichia coli* (VTEC) that have been incriminated in food-borne outbreaks of human diseases characterized by diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome (17). The majority of human VTEC illnesses have been attributed to VTEC O157:H7, but there is increasing recognition of the importance of non-O157 VTEC serogroups (3, 7, 9, 11). A non-O157 serotype that is receiving particular attention as an important emerging food-borne pathogen is O103:H2 (4, 9, 21).

Possession of markers of O island 122 (OI-122) and OI-43/48 and VT production levels have been used to differentiate VTEC strains into seropathotypes and to distinguish human disease-associated VTEC strains from those that present a low zoonotic risk, based on the notion that these markers may be significantly associated with serotypes incriminated in severe human disease or outbreaks (13, 14, 22). Since only a small number or no VTEC O103:H2 strains were included in these studies, the objective of the present study was to evaluate the association of the same markers with strains of human and bovine origins in a large number of strains of this serotype. The expectation was that a marker that was associated with virulent strains would be present at a higher frequency in human than bovine strains. The strains used in this study were previously characterized for *vt1* and *vt2*, *eae*, and the plasmid-encoded virulence markers *ehxA* (enterohemorrhagic *E. coli* [EHEC] hemolysin), *hlyA* (alpha hemolysin), *katP* (catalase peroxidase), *espP* (extracellular serine protease), and *etp* (type two secretion system) (12).

VTEC O103:H2 strains. A total of 91 VTEC O103:H2 strains from frozen (−70°C) culture collections at the University of Guelph and the Laboratory for Foodborne Zoonoses in Guelph were examined. The strains had been isolated between 1991 and 2002 in three countries in Europe (Germany, Switzerland, and Belgium) and in North America (the United States and Canada). Forty-six strains were of human origin,

and 45 were isolated from cattle. All of the strains from cattle were from healthy animals in Canada, while the 46 human strains consisted of 23 isolated in North America and 23 from continental Europe, all from patients with clinical disease.

PCR for OI marker genes in VTEC O103:H2. DNA was extracted from all of the strains by the boiling method, and a PCR was used to identify the OI marker genes. The primers and cycling parameters used have been described previously (13, 16, 23, 24). The Z4321, Z4326, Z4332, and Z4333 genes were examined as OI-122 markers, and *terC*, *iha*, and *ureC* were examined as OI-43/48 markers. PCR was performed in a 25-μl reaction mixture containing 2.5 μl of DNA, 2.5 μl of 10× PCR buffer, 1.5 or 2 mM MgCl₂, 200 μM each deoxynucleoside triphosphate, 2 U of *Taq* DNA polymerase, and water. VTEC O157:H7 strain EDL933 was used as a positive control. PCR mixtures without template DNA were used as a negative control for all reactions.

Test for urease production and resistance to potassium tellurite. Urease activity and resistance to potassium tellurite were tested by standard methods (2, 6). *E. coli* strains EDL933 and C600 were the positive and negative controls, respectively.

VT production in low- and high-iron media. VT1 production in 23 cattle strains and 22 human VTEC O103:H2 strains that were randomly selected was measured following growth of the VTEC in syncase broth (2) or syncase broth plus iron (25). Single colonies were grown overnight with shaking in one tube with syncase broth and one with syncase broth plus FeCl₃ (10 μg/ml). A 3-ml volume of the culture from each tube was sonicated (Heat Systems-Ultrasonics, Farmingdale, NY) on ice to achieve complete lysis. The toxin concentration in each lysate was measured by an enzyme-linked immunosorbent assay (1). Eight duplicate serial twofold dilutions containing 0 to 250 pg of purified VT1 per well were processed by enzyme-linked immunosorbent assay (1), and a standard curve obtained by plotting the optical densities was used to generate quantitative data. To evaluate the reproducibility of the results, the experiments were repeated with 10 strains selected to cover the range of toxin concentrations in the lysates.

VT production in the presence of mitomycin C. A 0.5-ml volume of the overnight syncase broth culture was transferred to a tube containing 5 ml of fresh syncase medium and incubated with shaking to an optical density at 600 nm of 0.6,

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TABLE 1. Distribution of gene markers for OI-122 and OI-43/48 in VTEC O103:H2 strains

Gene or O island	% (no.) of strains positive for gene or O island			
	All strains (n = 91)	North American cattle strains (n = 45)	North American human strains (n = 23)	European human strains (n = 23)
Z4321	31.8 (29)	26.6 (12)	47.8 (11)	26 (6)
Z4326	100	100	100	100
Z4332	100	100	100	100
Z4333	100	100	100	100
<i>terC</i> ^{a,b}	67 (61)	86.6 (39)	69.5 (16)	26 (6)
<i>iha</i> ^{a,b}	45 (41)	64.4 (29)	43.4 (10)	8 (2)
<i>ureC</i> ^b	68.1 (62)	86.6 (39)	73.9 (17)	26 (6)
OI-43/48 ^{a,b}	41.9 (39)	62.2 (28)	39.1 (9)	8 (2)
OI-122	31.8 (29)	26.6 (12)	47.8 (11)	26 (6)

^a Statistically significant difference between North American cattle and human strains.

^b Statistically significant difference between North American and European human strains.

followed by the addition of mitomycin C (2 µg/ml) (27), and incubated for 3 h. Sonication and measurement of toxin concentrations were done as described above.

Statistical analysis. Fisher's exact test was used to determine the statistical significance of differences ($P < 0.05$) between the proportions of OI marker genes and phenotypes in the groups of VTEC O103:H2 strains. P values of <0.05 were considered significant. Toxin concentrations were compared by transforming them into logarithmic values and conducting an analysis of variance (SAS Institute Inc., Cary, NC), followed by a paired t test. Median values and interquartile ranges (IR) of toxin concentrations obtained under different treatments with the three groups of strains were also determined.

Distribution of OI-encoded genes. The frequencies of the four OI-122 marker genes in VTEC O103:H2 were 32.2% for Z4321 and 100% for Z4326, Z4332, and Z4333 (Table 1). The full complement of OI-122 markers was present in 31.8% of the VTEC O103:H2 strains tested. There were no significant differences among the three groups of strains with regard to the distribution of the OI-122 markers or possession of a complete OI-122.

The frequencies of detection of the OI-43/48 markers were 67% for *terC*, 68% for *ureC*, and 45% for *iha* (Table 1). The *terC* gene was present in 86.6% of the North American cattle strains, 69.5% of the North American human strains, and 26% of the European human strains. The *terC* gene was more prevalent in North American strains of cattle origin than in those of human origin ($P = 0.03$) and was more frequent in human strains from North America than in those from Europe ($P = 0.02$). There was no significant difference ($P = 0.06$) in the distribution of *ureC* in North American strains of bovine origin compared with those of human origin. However, *ureC* was more prevalent in human strains from North America than in those from Europe ($P = 0.0007$). The *iha* gene was more prevalent in North American strains of cattle origin than in those of human origin ($P = 0.02$) and in human strains from North America than in those from Europe ($P = 0.02$). The full complement (*terC*, *iha*, and *ureC*) of OI-43/48 markers was present in 41.9% of the strains and was more prevalent in

North American strains from cattle than in those from humans ($P = 0.0003$) and in North American human strains than in European strains ($P = 0.02$). Only three strains had the full complement of both OI-122 and OI-43/48 markers.

Distribution of tellurite resistance and urease production phenotypes. Tellurite resistance was detected in 67% of the strains and was shown by 86.6% of the North American cattle strains and 69.5% of the North American human strains but by only 26.6% of the European human strains. These differences between cattle and human North American strains and between human strains from North America and Europe were significant ($P = 0.03$ and $P = 0.02$, respectively). Urease was produced by a single strain.

VT production by O103:H2 strains. VT1 concentrations in lysates of strains grown in low-iron syncase medium (median = 17,320 ng/ml; IR = 2,340 to 42,921) were higher ($P < 0.0001$) than in lysates of strains grown in syncase medium plus iron (median = 993 ng/ml; IR = 529 to 2,869). There were increases of fivefold or greater for 29 strains (64.4%) and less than fivefold for 7 strains (15.5%), no change for 8 strains (17.7%), and a decrease for 1 strain (2%). VT1 concentrations were also higher ($P < 0.0001$) for strains grown in syncase medium than for strains cultured in syncase medium and exposed to mitomycin C (median = 3,080; IR = 721 to 11,684). There were no significant differences between the toxin concentrations of cattle strains and those of human strains under the three treatments. Following attempted induction with mitomycin C, there was no significant increase in toxin production by 9 of the 45 strains and a decrease in the remaining 36 strains.

The full complement of OI-122 markers was more frequent in North American human than in North American cattle strains and European human strains. Karmali et al. (13) proposed that the presence of all four markers be an indication of a complete OI-122 and noted that a complete OI-122 was present in most strains of seropathotype B, which are frequently incriminated in hemolytic-uremic syndrome and to which VTEC O103:H2 belongs. Both Karmali et al. (13) and Wickham et al. (26) investigated the same three O103:H2 VTEC strains and reported a lack of the Z4321 gene in strains of this serotype. The present study, involving 91 strains, showed that 32.2% of the O103:H2 strains possessed the Z4321 gene and a complete OI-122. Thus, serotype O103:H2 appears to deviate markedly from the norm for seropathotype B, in which 60% of the strains had a complete OI-122 (13).

Our findings are largely in agreement with other studies which detected *terC*, *ureC*, and *iha* in the majority of human clinical isolates of EHEC, including O26:H21 and O157:H7, with the difference that OI-43/48 markers were more prevalent (100% or close to 100%) in these serotypes (5, 6, 10, 16, 18, 19). These significant differences in the distribution of OI-43/48 markers in VTEC O103:H2 strains are a reflection of the mosaic structure of pathogenicity islands, which are generated through recombination events resulting in DNA deletions or acquisition. Differences in the acquisition of some OI-43/48 markers between cattle and human strains and North American and European strains most probably reflect differences in the selective pressures to which these strains have been exposed in their respective environments.

The possession of *terC* correlated perfectly with tellurite

resistance, but urease was expressed in only 1 of the 62 *ureC*-positive VTEC O103:H2 isolates. This is consistent with various reports on *terC* and resistance to potassium tellurite in EHEC O157:H7 and O26:H11/H-/NM strains (6, 18, 24). The very low frequency of expression of urease activity in the majority of the VTEC strains which were *ureC* positive is in agreement with previous studies on other EHEC strains, including serotype O157:H7, O26:H11/H-/NM, and O111 strains (5, 10, 19).

Consistent with other reports (8, 22, 25), VT1 concentrations in low-iron medium were significantly higher than those in high-iron medium and in the presence of mitomycin C. The absence of lysis of the cultures and of a significant increase in toxin concentration indicates that there was a failure of induction. These findings are in agreement with those of Ritchie et al. (22), who observed that mitomycin C was ineffective as a VT1 inducer. Mühldorfer et al. (15) and Ritchie et al. (22) have suggested that prophage induction might not be as critical for VT1 production as it is for VT2 and have concluded that "additional processes" (22) regulate and enable VT1 release. Based on a study of VT2 production, Orth et al. (20) concluded that it is the type of VT rather than the amount produced in vitro which determines whether a VTEC strain will produce mild or severe disease. However, further studies are needed to confirm whether this also applies to VT1-positive VTEC strains such as serotype O103:H2 strains.

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