A Newly Discovered Verotoxin Variant, VT2g, Produced by Bovine Verocytotoxigenic *Escherichia coli*

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A new verotoxin (VT) variant, designated vt2g, was identified from a bovine strain of verocytotoxigenic *Escherichia coli* (VTEC) serotype O2:H25. When vt2g was aligned with published sequences of vt2 and vt variants, it exhibited the highest DNA sequence homology with vt2 and vt2c. However, vt2g was not detected by vt2-specific primers and probes, although it was partially neutralized by an antiserum to the VT2A subunit. VT2g was cytotoxic for Vero and HeLa cells and was not activated by mouse intestinal mucus. The vt2g gene was detected in 3 of 409 (0.7%) bovine VTEC strains, including serotypes O2:H25, O2:H45 and Ont:H⁺.

Verotoxin (VT), also known as Shiga toxin (Stx), is a cytotoxin produced by strains of verocytotoxigenic *Escherichia coli* (VTEC), an enteric pathogen associated with bloody diarrhea and the hemolytic-uremic syndrome (3, 17). VTs are divided into two groups. VT1 is highly homologous to Stx of *Shigella dysenteriae* type I. VT2 shares approximately 55% amino acid homology with VT1 but is relatively more heterogeneous. Apart from sequence variation, variants of VT2 differ from each other in terms of their affinity for glycolipid receptors, Vero cell cytotoxicity, animal pathogenicity, and response to activation by intestinal mucus (9, 11).

The techniques used to detect and quantify VTs include assays for cytotoxicity, enzyme immunoassays, and PCR assays, with the PCR assay the most popular because of its speed and relative simplicity. The increasing number of reported vt variants, however, has led to a need for more PCR primers for their specific detection. Nevertheless, not all vt variants are detectable with the existing primers. We previously reported a VTEC strain, named 7v, of serotype O2:H25 that was isolated from the feces of healthy cattle and was vt positive when examined with the degenerative PCR primers MK1 and MK2 (5). However, the PCR product obtained from this strain did not hybridize with probes specific for vt1, vt2, or vt2e. Moreover, the variant was not detected with primers specific for vt2c (7), although the cytotoxic activity of strain 7v was partially neutralized by an antiserum specific for subunit A of VT2 (6). In this study, we show that the vt gene of strain 7v is a novel variant, which we have designated vt2g.

The VTEC serotype O2:H25 used for sequencing the vt2g gene and the other 423 VTEC strains isolated in Hong Kong (409 bovine, 10 porcine, and 4 human strains) have been described previously (7). A total of 51 human clinical VTEC isolates from Australia were also examined (Table 1). Standard

VTEC strains ATCC 43889 (vt2⁺), ATCC 43890 (vt1⁺), and S1191 (vt2e⁺) were used as controls.

The vt2g gene of strain 7v was amplified by using the primers and protocol described by Paton et al. (12). The sequences of the forward (177U) and reverse (45D) primers were 5'-GAT GGC GGT CCA TTA TC-3' and 5'-AAC TGA CTG AAT TGT GA-3', respectively. The 1,493-bp PCR product (Fig. 1) was purified on a QIAquick Mini Column (QIAGEN, Inc., Valencia, Calif.) and sequenced with the 177U and 45D primers and with the internal oligonucleotide primers 7VF (5'-CGA CCT CTC TTG AGC AT-3') and 7VR (5'-TCT TCT TCA TGC TTA ACT CC-3') with an ABI Prism BigDye Terminator cycle sequencing kit and an ABI 310 automated sequencer (Applied Biosystems Inc., Norwalk, Conn.). Analysis of this sequence (Fig. 1) revealed two open reading frames, the first from nucleotide position 191 to position 1150 (320 amino acids), encoding the putative subunit A, and the second from

TABLE 1. Serotypes of the VTEC strains used in this study

Serotype ^a	No. of strains	Source
Human clinical VTEC		
O15	3	Australia
O26	7	Australia
O111	14	Australia
O113	7	Australia
O157	20	Australia
O157	3	Hong Kong
OR	1	Hong Kong
Porcine VTEC		
Ont	6	Hong Kong
O108	1	Hong Kong
O20	1	Hong Kong
O91	1	Hong Kong
O121	1	Hong Kong
Bovine VTEC		
O157	8	Hong Kong
Non-O157	401	Hong Kong

^a OR, rough; Ont, O nontypable.

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FIG. 1. Shaded letters represent nucleotide differences between the sequences of *vt2g* and *vt2*. Open reading frames are shown in uppercase letters. Nucleotides in boldface type indicate start or stop codons. Solid arrows indicate the forward, reverse, and internal primers used to sequence *vt2g*. Dotted arrows indicate the primers for PCR screening of *vt2g*. The MK1 and MK2 primers are indicated by double underlining. Boxed sequences indicate the PCR primers described by Pollard et al. (13). The solid bar indicates the region recognized by the 428-II oligonucleotide probe.

nucleotide position 1163 to position 1432 (90 amino acids), encoding the putative subunit B.

As the most recently published VT variant was named *stx2f* (14), we designated the variant reported in this study *vt2g*. Alignment of the *vt2g* sequence and comparison of the sequence with those of *vt2*, *vt2c*, *vt2d*, *stx2d*, *vt2e*, *slt2va*, and *vt2f* (GenBank accession nos. AF461165, M59432, AF043627, AF479829, M21534, M29153, and AJ010730, respectively) were performed with Megalign and EditSeq software (DNAS-

tar, Madison, Wis.). This analysis revealed that the nucleic acid sequence of subunit A of *vt2g* showed a similarity ranging from 63.0 to 94.9% to the previously reported sequences of *vt* genes. The *vt2g* sequence was most closely related to *vt2*, *vt2c*, and *stx2d*, all of which are associated with human disease. Comparison of the sequences of *vt2g* and *vt2* revealed that the most conserved regions occurred from nucleotide position 399 to position 712 (Fig. 1), which encodes the central region of the catalytic subunit. Phylogenetic analysis of the sequences re-

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vt2g 838 AGACGTGGACCTCACTCTGAACTGGGGGGAATCAGCAATGTGCTTCCGGAGTATCGGGG
vt2 960 AGACGTGGACCTCACTCTGAACTGGGGGGAATCAGCAATGTGCTTCCGGAGTATCGGGG

vt2g 898 AGAGGATAGTGTGAGAGTGGGGAGAATATCCTTTAATAATATATCAGCAATACTGGGTAC
vt2 1020 AGAGGATAGTGTGAGAGTGGGGAGAATATCCTTTAATAATATATCAGCAATACTGGGTAC

<-----781R----->
vt2g 958 TGTAGCGGTTATCTGAATTGCCATCATCAGGGGACGCGTTCCGTTTCGCTACGTGAATGA
vt2 1080 TGTAGCGGTTATCTGAATTGCCATCATCAGGGGACGCGTTCTGTTCGCGCGGTGAATGA

vt2g 1018 AGAGATGCAACCAGAATGTCAGATAAGTGGCGACAGGCCAGTTATAAAAAATAACAATAC
vt2 1140 AGAGATGCAACCAGAATGTCAGATAAGTGGCGACAGGCCAGTTATAAAAAATAACAATAC

vt2g 1078 ATTATGGGAAAGTAATACAGCAGCAGCCTTTCTGAACAGAAAGTCTCAGTCAATTATATAC
vt2 1200 ATTATGGGAAAGTAATACAGCAGCAGCCTTTCTGAACAGAAAGTCAAGTCTTTATATATAC

7VR
vt2g 1138 AACCGGTGAATGAaaggagtttaagcATGAAGAAGATGTTTATGGCGGTTTTATTTCGATT
vt2 1260 AACCGGTGAATGAaaggagtttaagcATGAAGAAGATGTTTATGGCGGTTTTATTTCGATT
Start of B-subunit

vt2g 1198 GGTTTCTGTTAATGCAATGGCGCGGATTGTGCTAAAGGTAAATGAGTTTTCCAAATA
vt2 1319 AGTTTCTGTTAATGCAATGGCGCGGATTGTGCTAAAGGTAAATGAGTTTTCCAAATA

vt2g 1258 TAATGGGGATAACACATTTACTGTAAAGGTTGACGGGAAAGAATACTGGACTAACCGGTG
vt2 1379 TAATGAGGATGACACATTTACTGTAAAGGTTGACGGGAAAGAATACTGGACTAACCGGTG

vt2g 1318 GAATTGCAACCGTTCTTACAAAGTGCAAGTTAACAGGAATGACCGTACAAATCAAATC
vt2 1439 GAATTGCAACCGTTCTTACAAAGTGCAAGTTAACAGGAATGACCGTACAAATCAAATC

vt2g 1378 CAATACCTGTGAATCAGGCTCTGGATTTGCTGAAGTGCAAGTTAATAATGAactgaagaat
vt2 1499 CAGTACCTGTGAATCAGGCTCTGGATTTGCTGAAGTGCAAGTTAATAATGAactgaagaat

45D
vt2g 1438 aacctgattcgtggtatgtgggtaaca-gtgaatctgtgtcacaattcagtcagtt
vt2 1559 aacctgattcgtggtatgtgggtaacaagtgaatctgtgtcacaattcagtcagttgac

vt2 1619 agttgcctgtcagactgagcatttgttaaaaaatttcgcatggtgaatccccctgtgtg

vt2 1679 gaggggagactggtgaaaaatccttgcctgtgattcattatcgacacggg

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FIG. 1—Continued.

vealed two distinctive clusters: one comprising *vt2*, *vt2c*, *vt2d*, *stx2d*, *vt2e*, and *vt2g*, and the other comprising *stx2f* and *slt2va* (2).

Although there was no sequence variation within the annealing regions for primers MK1 and MK2 (5), there was a 4-bp difference within the region for the *vt2*-specific oligonucleotide probe, 428-II (4) (Fig. 1). This difference is sufficient to account for the negative hybridization reaction observed previously in the PCR product of strain 7v with the 428-II probe (7). In addition, the *vt2g* sequence showed a 4-bp difference in the forward priming region and a 1-bp difference in the reverse priming region of the PCR primers published by Pollard et al. (13), which is sufficient to account for the failure of these primers to amplify *vt2g*. Comparison of the translated sequence of the A subunits of VT2g and VT2 showed 12 (out of 320) amino acid differences between them (Fig. 2), although, importantly, the two amino acid residues E₁₈₉ and R₁₉₂ (corresponding to E₁₆₇ and R₁₇₀ that are associated with toxin activity in *vt1* [1]) were conserved. Protein modeling with a SWISS-MODEL automated protein modeling server and the

Swiss-PdbViewer (15) predicted that the three-dimensional structures of the A subunits of VT2g and VT2 would be similar. This similarity is sufficient to explain the cross-neutralization of these toxins by an antiserum prepared against subunit A1 of VT2 (6).

The C terminus of subunit A2 of VT2g included a region (amino acid positions 310 to 319) that is identical to that of Stx2d (GenBank accession number AF479829) (16). Because activation of Stx2d by intestinal mucus is reported to be mediated by elastase cleavage of the last two amino acid residues in the C terminus of subunit A2 (10), we investigated the effect of intestinal mucus on the activity of *vt2g*. For these studies, intestinal mucus was isolated from BALB/c mice as described by Melton-Celsa et al. (10). The isolated mucus was weighed and added to Luria-Bertani (LB) broth at a final concentration of 2 mg/ml. A diluted overnight culture of VTEC strain 7v was then inoculated into mucus-supplemented LB or plain LB broth at a final concentration of 10² CFU/ml. The broth was incubated at 37°C for 20 h with shaking. After incubation, a 1-ml aliquot was removed and centrifuged at 12,000 × g for 10

VT2	1	MKCILFKWVLCLLLGFSVSVSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSV
Stx2d	1	MKCILFKWVLCLLLGFSVSVSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSV
VT2g	1	MKCILFKWVLCLLLGFSVSVSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSV
VT2	61	SVINHPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI
Stx2d	61	SVINHPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI
VT2g	61	SVINHPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI
VT2	121	SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSVSSYLALMEFSGNTMTRDASRAV
Stx2d	121	SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSVSSYLALMEFSGNTMTRDASRAV
VT2g	121	SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSVSSYLALMEFSGNTMTRDASRAV
VT2	181	LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTINWGRISNVLPEYRGEDGV
Stx2d	181	LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTINWGRISNVLPEYRGEDGV
VT2g	181	LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTINWGRISNVLPEYRGEDGV
VT2	241	RVGRISFNNISAILGTVAVILNCHHQGARSVRVNEESQPECQITGDRPVIKINNTLWES
Stx2d	241	RVGRISFNNISAILGTVAVILNCHHQGARSVRVNEESQPECQITGDRPVIKINNTLWES
VT2g	241	RVGRISFNNISAILGTVAVILNCHHQGARSVRVNEESQPECQITGDRPVIKINNTLWES
VT2	301	NTAAFLNRKSQSLYTTGK
Stx2d	301	NTAAFLNRKSQSLYTTGE
VT2g	301	NTAAFLNRKSQSLYTTGE
A-subunit		
VT2	1	MKKMFMAVLFALASVNMAMPADCAKGIKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQS
Stx2d	1	MKKMFMAVLFALVSVNMAMPADCAKGIKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQS
VT2g	1	MKKMFMAVLFALVSVNMAMPADCAKGIKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQS
VT2	61	AQLTGMTVTIKSSTCESGSGFAEVQFNND
Stx2d	61	AQLTGMTVTIKSSTCESGSGFAEVQFNND
VT2g	61	AQLTGMTVTIKSSTCESGSGFAEVQFNND
B-subunit		

FIG. 2. Amino acid sequence alignments of subunits A and B of VT2, Stx2d, and VT2g. Shaded letters indicate the differences between the two sequences. The boxed letters in subunit A indicate the amino acid residues E₁₈₉ and R₁₉₂ associated with toxin activity. The boxed letters in subunit B indicate the asparagine at position 16.

min. The supernatant was then examined for cytotoxicity for Vero cells as described below (7). Our results showed that the cytotoxicity of strain 7v was not influenced by growing strain 7v in the presence of mouse intestinal mucus. This finding can be explained by the fact that subunit B is also involved in mucus-induced toxin activation of Stx2d (10) and that there were seven amino acid differences between the B subunits of VT2g and Stx2d (Fig. 2).

Comparison of the nucleotide sequence of subunit B of VT2g with that of other *vt* genes revealed homologies ranging from 76.7 to 90.7% and indicated that subunit B of VT2g is most similar to that of VT2. The amino acid similarity between the two B subunits ranged from 80 to 93%. Importantly, the asparagine at amino acid position 16 that is responsible for the high levels of in vitro cytotoxicity of VT2 is conserved in VT2g (8) (Fig. 2). As was the case with subunit A, we observed two phylogenetic clusters of subunit B, with *vt2g* clustering with *vt2*, *vt2c*, and *vt2d*. The homology between *vt2g* and *vt2* is such that their amplification with primers GK3 and GK4, described by Schmidt et al. (14), yields amplicons of a similar size. These

PCR products could be discerned, however, by digestion with *FokI*, which generates 131- and 155-bp fragments from *vt2* but not from *vt2g*.

Assays for the cytotoxic activity of VT and VT2g were performed as described previously (7). Briefly, for each test or standard VTEC strain, five colonies were picked from LB agar and cultured in LB broth for 18 h. One milliliter of broth culture was then centrifuged at $12,000 \times g$ for 10 min. A twofold-dilution series of the supernatant was prepared, after which 50 μ l of each dilution was mixed with 100 μ l of Eagle's minimal essential medium and added to monolayers of Vero and HeLa cells in 96-well microtiter plates. The plates were incubated at 37°C overnight, after which cells were examined for cytopathic effect. The highest dilution producing a cytopathic effect that was $\geq 50\%$ of the monolayer was considered to be the median cytotoxic dose. The findings indicated that the median cytotoxic doses of culture supernatants from VTEC strains 7v (*vt2g*⁺) and ATCC 43889 (*vt2*⁺) for both Vero and HeLa cells were the same, namely, 1024 and 512, respectively.

In order to investigate the prevalence of *vt2g* in VTEC isolates from animals and humans, the forward primer 209F, 5'-GTT ATA TTT CTG TGG ATA TC-3' (nucleotide positions 399 to 418), and the reverse primer 781R, 5'-GAA TAA CCG CTA CAG TA-3' (nucleotide positions 955 to 971) (Fig. 1), that are specific for two variable regions of *vt2g* were designed to amplify a 573-bp product from strain 7v but not from any of the other VTEC or control strains. A total of 474 VTEC isolates that were PCR positive with primers MK1 and MK2 were screened for the presence of *vt2g*. To detect the *vt2g* gene in VTEC isolates, bacterial DNA was extracted and prepared as described previously (7). The 25- μ l PCR mixture consisted of 3 μ l of DNA extract, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 160 μ M dNTP, 400 μ g of bovine serum albumin/ml, 20 pmol of primers 209F and 781R, and 1 U of *Taq* DNA polymerase (Applied Biosystems Inc., Norwalk, Conn.). The reaction mixture was heated at 94°C for 5 min and then subjected to 30 cycles of amplification, each consisting of 1 min at 94°C, 1 min at 46°C, and 1 min at 72°C, followed by a final extension period of 7 min at 72°C. Amplified products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gels.

Besides strain 7v, only 2 of the 409 bovine VTEC strains tested were found to harbor *vt2g*. None of the human or porcine VTEC strains examined was positive in the PCR assay for *vt2g*. Of the two *vt2g*-positive strains, one was serotype O2:H45 with a *vt1,2* genotype; the other was Ont:H⁻ with a *vt2* genotype. Both strains were *eae* negative, as was strain 7v. The *vt2g* sequence of the other two bovine VTEC isolates was determined (results not shown) and was found to be identical to that of strain 7v. Although two of the *vt2g*⁺ strains belonged to the O2 serogroup, they were clonally diverse as shown by their different H antigens. In addition, all three *vt2g*⁺ strains were isolated from cattle feces during separate abattoir visits, and the cattle were imported from different provinces in mainland China.

In summary, we have identified a new *vt* variant which we have named *vt2g*. This variant occurred at a rate of 0.7% (3 of 409) in bovine VTEC strains but was not detected in any of 10 porcine or 51 human VTEC strains studied. The low prevalence of *vt2g* suggests that it may be a newly emerged variant that has not yet extensively spread among cattle. Findings of the present study add further information to the global epidemiological picture of VTEC strains. Our findings showed that *vt2g* has high nucleic acid homology with *vt* sequences associated with human diseases. Moreover, VT2g shows conservation of the active sites for verocytotoxicity and cytotoxicity for HeLa and Vero cells that is comparable to that of VT2. For these reasons, the pathogenic potential of VT2g cannot be neglected. Importantly, this *vt* variant is not detected by *vt2*-specific primers, which are the major primers used for routine VTEC screening. Our finding that *vt2g* was detected by broad-spectrum PCR primers and cross-neutralizing antibody to

VT2A but not by specific *vt* primers indicates the need to use a range of different diagnostic tools to screen bacteria for the presence of VT.

Nucleotide sequence accession number. The *vt2g* sequence of VTEC strain 7v has been deposited in the GenBank database under the accession number AY286000.

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