A globotetraosylceramide (Gb₄) receptor-based ELISA for quantitative detection of Shiga toxin 2e

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ABSTRACT. Currently, no simple assays are available for routine quantitative detection of *Escherichia coli*-produced Shiga toxin 2e (Stx2e) that causes porcine edema disease. Here, we present a novel quantitative detection method for Stx2e based on the measurement of Stx2e binding to the specific globotetraosylceramide (Gb₄) receptor by ELISA (Gb₄-ELISA). No cross-reactivity was found with the other Shiga toxins Stx1 and Stx2, indicating high specificity. When the recombinant Stx2e B subunit (Stx2eB) was used, the absorbance measured by Gb₄-ELISA increased linearly with Stx2eB concentration in the range of 20–2,500 ng/ml. The Gb₄-ELISA method can be easily performed, suggesting that it would be a useful diagnostic tool for porcine edema disease.

KEY WORDS: ELISA, globotetraosylceramide, porcine edema disease, Shiga toxin 2e

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are important pathogens that cause diarrhea and hemorrhagic colitis in humans and pigs [12, 13, 15]. The Stx family of toxins, considered to be the cardinal virulence factors of STEC, comprises Stx1 and its variants (Stx1c and Stx1d) and Stx2 and its variants (Stx2c, Stx2d, Stx2e, Stx2f and Stx2g) [2, 8, 10]. The toxins are composed of an enzymatic A subunit and five receptor-binding B subunits. After the binding of the B subunits to the glycolipid receptors, globotriaosylceramide (Gb₃) or globotetraosylceramide (Gb₄), on mammalian cells, the A subunit is activated, cleaving a specific adenine residue in the 28S rRNA and inhibiting new protein synthesis [5].

STEC producing Stx1, Stx2 and Stx2e variants have been isolated from pigs; among them, Stx2e-producing strains are the most frequent [7]. Stx2e has been identified as the key factor involved in the pathogenesis of porcine edema disease characterized by subcutaneous and submucosal edema, hemorrhagic lesions, neurological impairment and death [11]. Stx2 and Stx2e have a high degree of nucleotide and amino acid homology in their A and B subunits (94% and 84%, respectively) [9, 14, 16].

A number of functional (cytotoxicity) and serological tests have been developed for the detection of Stx. Tests for routine Stx diagnostics should ideally be rapid and easy to perform. The Vero cell toxicity test enables determination of

the type and amount of toxin in the sample, but the method is cumbersome and time-consuming. The commercially available Vero toxin enzyme immunoassay kit (Ridascreen Verotoxin enzyme immunoassay, R-Biopharm AG, Darmstadt, Germany) and the reversed passive latex agglutination (RPLA) assay kit (VTEC-RPLA “SEIKEN”, Denka Seiken Ltd., Tokyo, Japan) are easier to use, but have low sensitivity for Stx2e [19]. Furthermore, these three major detection methods are unable to discriminate Stx2e from other Stx variants. In a previous study, the Bead-ELISA method, which can discriminate Stx2e from other Stx variants, has been developed. The method is highly sensitive and specific, but requires multi-step purification of the anti-Stx2e antibody [3]. Thus, currently, there are no rapid assays available for routine quantitative detection of Stx2e.

A previous study has reported a rapid Stx1 detection method based on the binding to the Stx1-specific Gb₃ receptor and measurement of the bound toxin by the enzyme-linked immunosorbent assay (ELISA) [1]. In this study, we established a novel quantitative detection method for Stx2e based on the measurement of Stx2e binding to its preferential receptor Gb₄ by ELISA (Gb₄-ELISA).

Thirty *E. coli* strains were used in this study: five Stx2e genotype-positive clinical isolates from pigs, one Stx1-Stx2 genotype-positive reference *E. coli* strain (ATCC 35150), one Stx1-Stx2 genotype-positive isolate from a deer, one Stx1 genotype-positive reference *E. coli* strain (ATCC 43890), one Stx genotype-negative reference *E. coli* strain (ATCC 25922) and 21 Stx genotype-negative isolates from pigs (wild strains). The genotypes of clinical isolates were determined by polymerase chain reaction (PCR) [6, 17]. Each *E. coli* strain was inoculated in 1 ml of Tryptic Soy Broth (Eiken-Chemical Co., Ltd., Tokyo, Japan) and grown at 37°C overnight with shaking at 100 rpm. The cultures were centrifuged at 6,000 × g for 15 min; then, cell pellets

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were resuspended in 250 μ l of PBS and treated with polymyxin B (12,500 U/ml) (Pfizer K. K., Tokyo, Japan) for 1 hr at 37°C to extract toxins. Cell lysates were centrifuged at 6,000 $\times$ g for 15 min to remove bacterial debris, and the supernatants were used as test samples. Stxs in the test samples were determined by the reversed passive latex agglutination (RPLA) assay. The assay was performed using the VTEC-RPLA "SEIKEN" test kit (Denka Seiken Ltd.) according to the manufacturer's instructions.

The recombinant Stx2e B subunit (Stx2eB) was amplified from a Stx2e genotype-positive *E. coli* isolated from a pig that died of edema disease. The *stx2eB* gene without a leader sequence was amplified using the upstream primer 5'-AGAG-CATATGGCGGATTGTGCTAAAGGTAAAATTG-3' containing the *Nde*I restriction site and the downstream primer 5'-CGCTCGAGGTAAACTTCACCTGGGCAAAG-3' containing the *Xho*I restriction site. The amplified gene was cloned into the pET23b vector (Novagen, Gibbstown, NJ, U.S.A.) to add a 6-His tag to the Stx2eB N-terminus. The expression plasmid was introduced into competent *E. coli* DH5 α cells (Life Technologies, Rockville, MD, U.S.A.); the sequence of the recombinant *stx2eB* gene was confirmed by automatic DNA sequencing. Expression of the recombinant Stx2eB protein was induced with 1 mM isopropyl- β -D-thiogalactopyranoside at 30°C for 3 hr. The cells were collected by centrifugation and disrupted in 6 M guanidine buffer (pH 8.0) using an ultrasonic disintegrator. After the cells were centrifuged, the clarified lysate was subjected to immobilized cobalt-based metal affinity chromatography (TALON; Clontech, Mountain View, CA, U.S.A.) according to the manufacturer's instructions. The bound proteins were eluted with 200 mM imidazole under denaturing conditions and dialyzed against PBS at 4°C overnight. Since most of the recombinant proteins were precipitated during dialysis, the precipitate was dissolved in 1% SDS after brief centrifugation. The dissolved recombinant proteins were analyzed by SDS-PAGE, and the proteins were confirmed to be homogeneous by Coomassie Brilliant Blue staining. The concentration of the recombinant protein was determined by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc., Waltham, MO, U.S.A.) using bovine serum albumin (BSA) as a standard.

Rabbit antiserum against Stx2eB was raised by repeated injections of the Stx2eB recombinant protein (100 μ g/animal) conjugated to the keyhole limpet hemocyanin (KLH) carrier in complete Freund's adjuvant. The Gb₄-ELISA plates were prepared as described previously with some modifications [18]. Gb₄ from human erythrocytes (Sigma-Aldrich, St. Louis, MO, U.S.A.) was diluted to 10 μ g/ml in methanol and used to coat the Immunopure Polysorp 96-well plates (Nalge Nunc International, Penfield, NY, U.S.A.). The plates were left open to allow methanol evaporation, and each well was then blocked with 200 μ l of 1% BSA in PBS for 1 hr at room temperature. The plates were washed three times with 400 μ l of PBS and incubated with the test sample (50 μ l/well) at 37°C for 30 min. After the plates were washed three times with 400 μ l of 0.05% Tween-20 in PBS (Tween-PBS), they were incubated with the rabbit anti-Stx2eB se-

rum (50 μ l/well) diluted 1:1,000 in 0.1% BSA-containing Tween-PBS at room temperature for 1 hr and washed as above. The peroxidase-conjugated goat anti-rabbit IgG (50 μ l/well) (Millipore, Billerica, MA, U.S.A.) diluted 1:10,000 in 0.1% BSA-containing Tween-PBS was added following incubation at room temperature for 1 hr. The plates were washed and developed with tetramethylbenzidine (100 μ l/well) (KPL Inc., Gaithersburg, MD, U.S.A.) at 37°C for 15 min. Reactions were stopped by the addition of 100 μ l 2N H₂SO₄, and the absorbance was read at 450 nm (A_{450}) using a plate reader (Sunrise, Tecan, Salzburg, Austria).

The results indicate that our Gb₄-ELISA was highly reproducible, had a very low background (<0.08 absorbance at 450 nm) and displayed minimal variation among three independent measurements of recombinant Stx2eB concentration with maximum standard error (SE) of 0.05. When the recombinant Stx2eB protein was used in the assay, A_{450} as determined by Gb₄-ELISA increased linearly in the Stx2eB concentration range from 20 ng/ml to 2,500 ng/ml (A_{450} was 0.08 at 20 ng/ml and 0.72 at 2,500 ng/ml) (Fig. 1).

When Gb₄-ELISA was used to analyze five Stx2e genotype-positive strains, it showed an A_{450} value ranging from 0.24 to 1.15, whereas for 25 Stx2e genotype-negative strains, the A_{450} value was 0.00 (Table 1). In comparison, the commercial RPLA assay could detect only two of five Stx2e genotype-positive strains; the A_{450} values for the detected strains (titer >2 by the RPLA assay) were 0.38 and 1.15 by Gb₄-ELISA. At the same time, the A_{450} values of the strains negative by the RPLA assay were 0.24, 0.25 and 0.29 by Gb₄-ELISA. When the RPLA assay was used to analyze Stx1 or Stx2 genotype-positive strains, it showed titers ranging from 16 to 128.

Stx2e, an important toxin in the Stx family, is the cause of porcine edema disease. In this study, we detected Stx2e in polymyxin B-treated *E. coli* extracts based on the binding to the Stx2e-preferred receptor Gb₄, which was measured by ELISA. Using our Gb₄-ELISA method, we were able to detect Stx2e in all five Stx2e genotype-positive *E. coli* strains with A_{450} values of not less than 0.24, while all Stx2e genotype-negative strains had A_{450} values of 0.00. According to the protocol for the RPLA kit, Stx1 or Stx2 at the concentration of 100 ng/ml should demonstrate titers ranging from 64 to 128. In this study, Stx1 and Stx2 genotype-positive samples had maximum titers of 64 and 128, respectively, indicating a concentration of about 100 ng/ml for Stx1 or Stx2. When the Gb₄-ELISA was used to analyze these samples, it showed an A_{450} value of 0.00, demonstrating that Gb₄-ELISA did not detect 100 ng/ml Stx1 and Stx2. Thus, the Gb₄-ELISA displayed no cross-reactivity with Stx1 and Stx2, suggesting high specificity for Stx2e.

Gb₃ is a common glycolipid receptor for the Stx family; however, Stx2e exhibits higher affinity for Gb₄ than for Gb₃. Previous studies have shown that Gb₄ is specifically recognized by Stx2e, but not by any other Stx family members [4]. In this study, although we used crude anti-Stx2eB polyclonal serum with relatively low concentration of Stx2e-specific antibodies, the Gb₄-ELISA demonstrated high specificity for the detection of Stx2e-Gb₄ binding in the polymyxin

Fig. 1. Detection of recombinant Stx2eB by Gb₄-ELISA. Each serial two-fold dilution of recombinant Stx2eB was assayed in triplicate; the results are presented as the mean $\pm$ SE. The line is an approximated curve.

Table 1. Detection of Stx2e, Stx1 and Stx2 by Gb₄-ELISA

Strain	DNA (PCR)	RPLA (titer)		Gb ₄ -ELISA (A ₄₅₀)
		Stx1	Stx2	
S15-32	Stx2e	ND ^{a)}	<2	0.25
S15-55	Stx2e	ND	4	0.38
S17-30	Stx2e	ND	32	1.15
S17-50	Stx2e	ND	<2	0.24
S19-181	Stx2e	ND	<2	0.29
S17-31	Stx1, Stx2	16	128	0
ATCC35150	Stx1, Stx2	32	64	0
ATCC43890	Stx1	64	ND	0
ATCC25922	Stx (-)	ND	ND	0
Wild strains	Stx (-)	ND	ND	0.00 ^{b)}

Stx (-), Stx-negative; A₄₅₀, absorbance at 450 nm; Wild strains, strains isolated from pigs (n=21); a) ND, not determined; b) 0.00, absorbance for 21 samples.

B extracts of *E. coli*. There have been no reliable methods for the sensitive and quantitative detection of Stx2e. In this study, the RPLA kit could detect only two of five Stx2e genotype-positive strains, indicating low sensitivity. The comparison of the performance of our Gb₄-ELISA with that of the RPLA kit showed that Gb₄-ELISA had higher sensitivity than the RPLA kit. The experiments with the recombinant Stx2eB protein demonstrated linear correlation of A₄₅₀ with Stx2eB concentration in the range from 20 to 2,500 ng/ml, suggesting that the Gb₄-ELISA can be used to measure Stx2e concentration in *E. coli* extracts.

The sensitivity of the Gb₄-ELISA is lower than Bead-ELISA [3]. However, the sensitivity is enough to detect Stx2e in polymyxin B-treated *E. coli* extracts. The Gb₄-ELISA method does not require highly purified anti-Stx2e antibodies, as required in Bead-ELISA. Thus, the Gb₄-ELISA method can be easily performed.

Genetic diagnosis by PCR is a popular and highly reliable method for routine STEC detection; however, the evaluation of Stx protein production is more applicable for the diagnostics of the STEC-related infection. Previous findings have demonstrated that Stx2e genotype-positive STEC strains significantly differ with regard to Stx2e production and that some of them do not synthesize Stx2e protein [19]. In this study, all the tested Stx2e genotype-positive strains produced the toxin; however, Stx2e protein levels varied among the isolates. Quantitative assessment of Stx2e production by STEC strains is important for the accurate diagnosis of porcine edema disease, and the results of this study suggest that Gb₄-ELISA may be a useful tool for the reliable diagnosis of this disease.

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