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.

KEYWORDS: 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 Trypto 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...
were resuspended in 250 µl 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 × 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'-AGAGCATATGGCCGATTTGTGCTAAAGGTAATAATG-3' containing the NdeI restriction site and the downstream primer 5'-CGCTCGAGGTTAAACTTCACCTGCGCAAAG-3' containing the XhoI 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 DH5α 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 µg/animal) conjugated to the keyhole limpet hemocyanin (KLH) carrier in complete Freund's adjuvant. The Gb3-ELISA plates were prepared as described previously with some modifications [18]. Gb3 from human erythrocytes (Sigma-Aldrich, St. Louis, MO, U.S.A.) was diluted to 10 µg/ml in methanol and used to coat the Immunopure Polysorp 96-well plates (Nalgene Nunc International, Penfield, NY, U.S.A.). The plates were left open to allow methanol evaporation, and each well was then blocked with 200 µl of 1% BSA in PBS for 1 hr at room temperature. The plates were washed three times with 400 µl of PBS and incubated with the test sample (50 µl/well) at 37°C for 30 min. After the plates were washed three times with 400 µl of 0.05% Tween-20 in PBS (Tween-PBS), they were incubated with the rabbit anti-Stx2eB serum (50 µl/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 µl/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 µl/well) (KPL Inc., Gaithersburg, MD, U.S.A.) at 37°C for 15 min. Reactions were stopped by the addition of 100 µl 2N H2SO4, and the absorbance was read at 450 nm (A450) using a plate reader (Sunrise, Tecan, Salzburg, Austria).

The results indicate that our Gb3-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, A450 as determined by Gb3-ELISA increased linearly in the Stx2eB concentration range from 20 ng/ml to 2,500 ng/ml (A450 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 samples, it showed an A450 value ranging from 0.24 to 1.15, whereas for 25 Stx2e genotype-negative strains, the A450 value was 0.00 (Table 1). In comparison, the commercial RPLA assay could detect only two of five Stx2e genotype-positive strains; the A450 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 A450 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 A450 values of not less than 0.24, while all Stx2e genotype-negative strains had A450 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 A450 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.

Gb4 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, 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
The sensitivity of the Gb₄-ELISA is lower than Bead-ELISA. Thus, the Gb₄-ELISA method can be easily performed.

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