# Evaluation of immunochromatographic test of Shiga toxin 2e in enrichment cultures of swine edema disease clinical samples

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**ABSTRACT.** To simplify the diagnosis of swine edema disease, overnight culture supernatants of swine clinical samples were assayed using immunochromatographic test strips we developed previously. Small-intestinal contents, mesenteric lymph nodes, and fecal samples were cultured in casamino acid-yeast extract broth overnight, after which supernatants were loaded onto immunochromatographic test strips to determine whether they could detect Shiga toxin 2e (Stx2e). Among 23 clinical samples in which PCR-identified *stx2e*-positive *E. coli* were isolated, samples from seven of ten small-intestinal contents, one of three mesenteric lymph nodes and six of ten fecal samples showed Stx2e-positive reactions in the protein-based immunochromatographic test. Additionally, one small-intestinal content sample, in which *stx2e*-positive *E. coli* were not isolated, showed an Stx2e-positive reaction. Furthermore, the immunochromatographic test results of the samples were associated with the toxin concentration determined by sandwich ELISA and cytotoxicity assay results on Vero cells. The toxin concentration range of the samples with positive and negative reactions were 2.1–196.2 ng/ml and 0–12.8 ng/ml, respectively. The sensitivity and specificity of this immunochromatographic test strip calculated from all clinical samples analyzed in this study were 60.9% and 94.4%, respectively. Our immunochromatographic test strip has strong potential for simple and accurate diagnosis for edema disease by detecting toxin expression, complementing the PCR method.

**KEY WORDS:** edema disease, immunochromatography, sandwich ELISA, shiga toxin 2e

Edema disease (ED) is caused by Shiga toxin-producing *Escherichia coli* (STEC) in post-weaning piglets. Typical clinical symptoms of ED include edema of various organs, mainly the eyelids, as well as neurological dysfunction. Frequently, sudden death occurs without premonitory clinical signs [4, 10]. Although the incidence of ED is sporadic, its mortality is very high [7, 10]. Furthermore, the bacteria are excreted in the feces, thereby prolonging the contamination of farms. This leads to recurrence of ED and the economic damage of ED to farmers is serious [12]. Therefore, rapid diagnosis and treatment are needed to prevent farms from spreading ED and to prevent residual STEC contamination.

The major causative factor of ED is Shiga toxin 2e (Stx2e). In addition, most STEC strains isolated from piglets with ED have F18 fimbriae, which are associated with colonization of the small intestine [5]. Therefore, current practical ED diagnostic methods use PCR to detect the Stx2e gene (*stx2e*) and F18 fimbriae gene (*fedA*) [8]. However, gene detection does not reflect the toxin production level. In addition, because PCR is occasionally influenced by inhibitors derived from sample materials, a bacterial isolation step using deoxycholate hydrogen sulfide lactose (DHL) agar and blood agar plates for clinical samples (particularly for small-intestinal contents and mesenteric lymph nodes, according to the manual for pathological appraisal) is needed prior to PCR. Thus, simple toxin detection would be the most practical method. However, there are no commercially available Stx2e-detection tools.

Previously, we developed a rapid and simple Stx2e detection method utilizing an immunochromatographic (IC) test based on sets of monoclonal antibodies [1]. The detection limit of these test strips against purified Stx2e was 10 ng/ml (i.e., 1.5 ng/150 µl), and we also confirmed the detection of Stx2e in culture supernatants of clinically isolated *stx2e*-positive strains. However, it is ideal for test samples to be prepared simply from crude materials in order to save labor and time-consuming isolation of *E. coli* from samples.

Accordingly, we evaluated whether our test strip can detect Stx2e in enrichment cultures derived from various types of clinical samples. In addition, to confirm the validity of the IC results, we conducted sandwich ELISA to quantify the concentration of Stx2e in each sample.

## MATERIALS AND METHODS

*Preparation of test samples*

Clinical swine samples were obtained from domestic livestock hygiene service centers. Twenty-two small-intestinal contents, 6 feces, and 4 mesenteric lymph node samples were collected from piglets that were dissected for pathological appraisal. The clinical symptoms of piglets diagnosed with ED were sudden death in most cases and ataxia and edema in some cases. Twenty-seven other fecal samples excreted in the environment were collected from piglet houses. All samples used in this study had been checked in advance for the presence or absence of *stx2e*-positive *E. coli* by the suppliers using DHL agar and blood agar plates [9] and subsequently conducting PCR for the *stx2e* gene [11]. Information about each sample used in this study is shown in Supplementary Table 1. Five to 25 mg of these samples were inoculated into 3 ml of casamino acid-yeast extract (CAYE) broth [3] and cultured overnight at 37°C. Each crude bacterial culture was treated with polymyxin B sulfate (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.5 mg/ml (approximately 5,000 U/ml) for 30 min at 37°C, after which the supernatant was collected following centrifugation at 15,000 × *g* for 10 min. Each supernatant was filtered with a 0.45 µm syringe filter (Minisart RC4; Sartorius, Göttingen, Germany) and then stored at −20°C until use.

*IC test*

The IC test strips were prepared according to previously described methods [1]. The monoclonal antibody (mAb) set mAb45B2 (A subunit-specific) and mAb55D1 (B subunit-specific) were used as gold colloid-conjugated and capture antibodies, respectively [1]. After loading 150 µl of each sample onto the test strip within 30 min, the appearance of the band on the test line was evaluated.

*Sandwich ELISA*

One hundred microliters of mAb65B2 (1 µg/ml in PBS) were plated into 96-well plates (MaxiSorp; Nunc A/S, Roskilde, Denmark). This mAb has been characterized as a B subunit-specific antibody [1]. After overnight immobilization at 4°C, each well was blocked with 200 µl of PBS containing 5% skim milk for 90 min, followed by reaction with 100 µl of the culture supernatants which were serially diluted with PBS for 60 min. Stx2e, which was purified according to a previously described method [2], was used in this step to prepare a standard curve. All wells were reacted with 100 µl of horseradish peroxidase (HRP)-conjugated mAb45B2, which was diluted with PBS, for 60 min. HRP-conjugation of the mAb was conducted using the EZ-Link Maleimide Activated Horseradish Peroxidase Kit (Thermo Fisher Scientific, Rockford, IL, USA). All wells were reacted with 100 µl of citrate buffer (pH 5.0) containing 0.4 mg/ml *o*-phenylenediamine and 0.02% hydrogen peroxide for 30 min. The reaction was stopped by the addition of 100 µl of 1 M $H_2SO_4$ and the absorbance at 492 nm was measured using the xMark$^{TM}$ Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA). All reaction steps were conducted at 37°C, and each well was washed with 300 µl of PBS containing 0.05% Tween20 three times prior to the reactions. In this sandwich ELISA, a linear dose response curve was obtained between 0 and 4 ng/ml. Based on the formula prepared from the dose response curve, the Stx2e concentration of each sample was calculated.

*Vero cell assay*

One hundred microliters of Vero cells were plated into each well of a 96-well culture plate (AGC Techno Glass, Shizuoka, Japan) at a concentration of $1 \times 10^5$ cells/ml in Eagle's minimum essential medium containing 10% fetal calf serum with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine and then incubated overnight at 37°C. After removing the supernatant, 100-fold dilutions of each bacterial culture supernatant were added, after which, the cells were incubated for 48 hr at 37°C. After removing the supernatant, which contained damaged cells, all wells that contained surviving cells were stained with crystal violet, according to the method [6]. The results were indicated as a survival index, which was calculated as the absorbance ratio at 595 nm of the well treated with each sample relative to that of a non-treated well.

*Ethical approval*

Samples were from pigs that had already died on pig farms and been sent for dissection at domestic livestock hygiene service centers, and fecal samples were collected from the environment. Thus, approval from an ethical committee was not required for this study.

## RESULTS

*Immunochromatographic detection of Stx2e in the enrichment culture supernatant of clinical samples*

First, we investigated whether our test strip could detect Stx2e in the enrichment culture supernatant of clinical samples. In the case of intestinal contents (Fig. 1A), seven samples showed a Stx2e-positive reaction among ten samples in which *stx2e*-positive *E. coli* were isolated. Additionally, one sample showed a Stx2e-positive reaction among twelve samples in which *stx2e*-positive *E. coli* were not isolated. In the case of mesenteric lymph nodes (Fig. 1B), one sample showed a Stx2e-positive reaction, from among three samples in which *stx2e*-positive *E. coli* were isolated. In the case of feces (Fig. 1C), six samples showed a Stx2e-positive reaction from among ten samples in which *stx2e*-positive *E. coli* were isolated. Although one sample also showed a Stx2e-positive reaction from among 23 fecal samples in which *stx2e*-positive *E. coli* were not isolated, this result was judged to be a false-positive reaction for reasons described later.

*Confirmation of Stx2e by quantification of protein concentration and detection of biological activity*

Based on the results of the IC differed from those of PCR for some samples, we also quantified the concentration of Stx2e in each sample by sandwich ELISA. Additionally, to confirm the existence of Stx2e in the same samples based on their biological activity, a Vero cell assay was also conducted.

The results of the Stx2e concentration and survival index based on the Vero cell assay of each sample are plotted in Fig. 2. Based on sandwich ELISA, the range of Stx2e concentrations in the IC-positive samples was 2.1–196.2 ng/ml with a mean (SD) of 40.7 (53.3) ng/ml, whereas that of the IC-negative samples was 0–12.8 ng/ml with a mean (SD) of 0.63 (2.0) ng/ml. Based on the Vero cell assay, the range of the survival index among the IC-positive samples was 0.262–0.617 with a mean (SD) of 0.431 (0.086), whereas those of the IC-negative samples were 0.622–1.477 with a mean (SD) of 0.969 (0.172). Because the Stx2e concentration and survival index of the one IC-positive fecal sample from which *stx2e*-positive *E. coli* were not isolated (marked by an asterisk in Fig. 1) were 0 ng/ml and 0.865, respectively, we judged only this sample to be a false-positive reaction. On the other hand, we detected an IC-positive reaction in one small-intestinal content sample in which *stx2e*-positive *E. coli* were not detected (Fig. 1). Because the Stx2e concentration estimated by sandwich ELISA was at a detectable level (2.1 ng/ml) and the survival index was also low (0.525), we judged this sample to have a low-positive reaction.
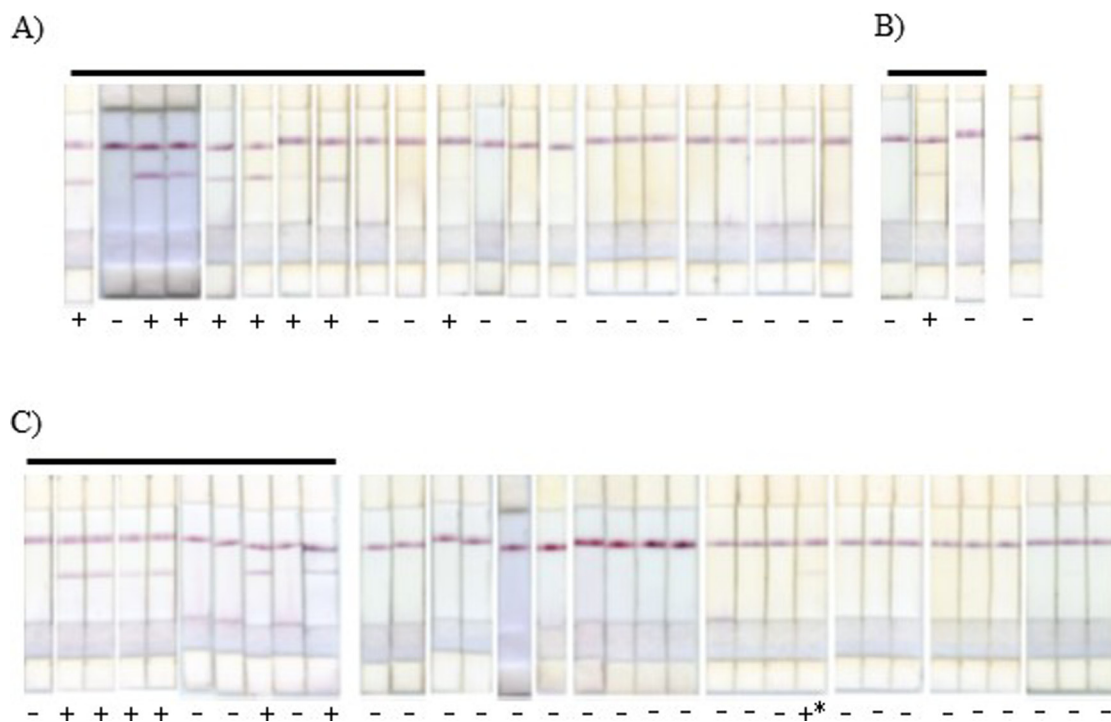


**Fig. 1.** Results of immunochromatographic detection of Stx2e in the enrichment culture of swine **(A)** small-intestinal contents, **(B)** mesenteric lymph nodes (MLN), and **(C)** feces. The images of the test strips below the black bars show the results of samples from which *stx2e*-positive *E. coli* had been isolated. Positive and negative detection of Stx2e with the strips is indicated by "+" and "-", respectively. One positive sample with an asterisk was determined to have had a false-positive reaction based on the result of other Stx2e detection methods, as shown in Fig. 2.

**Fig. 2.**   Relationship among the immunochromatographic test, sandwich ELISA, and Vero cell assay results for each sample. Black and white symbols indicate positive and negative immunochromatographic results, respectively, for each of the samples. The Vero cell assay and sandwich ELISA results are shown as the survival index (*y*-axes) and Stx2e concentration (*x*-axes), respectively. Lower survival index values (below 1.0) indicate stronger toxicity of the sample. Asterisk indicates the value of the sample that was determined to be a false-positive reaction, as mentioned in Fig. 1.

## DISCUSSION

In this study, we confirmed that our IC test strips could detect Stx2e even in the enrichment cultures derived from small-intestinal contents, feces, and mesenteric lymph nodes. However, Stx2e was not detected in some samples from which *stx2e*-positive *E. coli* were isolated. Stx2e was hardly detected in these samples by both sandwich ELISA and Vero cell assay, suggesting that these results were not false-negative reactions of the IC test. Although a *stx2e*-positive strain that does not produce toxin has been reported [13], this is not necessarily an explanation in our case because some samples were collected from the same farms in which Stx2e-producing strains were also detected by our IC test. One possible cause is the degree of STEC distribution in the bacterial flora of each clinical sample. We cultured each clinical sample in CAYE broth without any antibiotics to avoid overlooking the real causative bacteria. However, if the number of STEC in the sample is considerably low, it might be difficult to detect Stx2e even if the *stx2e* is at a level detectable by PCR amplification. The sensitivity of this IC test strip as calculated by the ratio of the IC-positive samples against all *stx2e*-positive clinical samples was 60.9% (14/23) in this study. On the other hand, specificity of the IC test strip calculated as the ratio of the number of IC-negative samples to that of all *stx2e*-negative samples was 94.4% (34/36). However, there was one small-intestinal content sample for which all assays showed Stx2e-positive reactions, but *stx2e*-positive *E. coli* were not isolated. This piglet died with edema and ataxia, and *stx2e*-gene positive *E. coli* were isolated from another piglet on the same farm (farm E shown in Supplementary Table 1), suggesting that this piglet might have been infected with STEC that was not isolated from DHL and/or blood agars. Although the ideal diagnosis method is to directly detect Stx2e in the clinical samples, the possibility of the detection is thought to depend on the amount of the toxin in the clinical samples, because

the detection limit of Stx2e using this IC is 10 ng/ml. However, our IC test strip has considerable potential value in the simple and accurate diagnosis of ED from enrichment cultures of dissected samples as well as in the environmental monitoring of pig houses for STEC pollution utilizing feces, complementing the PCR-based method.

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