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Specificity of PCR and Serological Assays in the Detection of *Escherichia coli* Shiga Toxin Subtypes[▽]

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Specificity analysis for *stx* or Stx subtypes in *Escherichia coli* showed that the PCR assays we tested did not detect stx_{1d} and stx_{2f}, and some also missed stx_{2b} and stx_{2g}. Most of the serological assays examined did not detect Stx2c, Stx2e, Stx2f, and Stx2g, and some strain-to-strain variation in reactivity was observed for Stx2b.

The production of Shiga toxin (Stx) is a characteristic trait of Shiga toxin-producing *Escherichia coli* (STEC), of which there are several hundred known serotypes, many of which have not been implicated in illness. A subset of STEC, referred to as enterohemorrhagic *E. coli* (EHEC), is comprised of pathogenic strains and includes serotype O157:H7, a recognized pathogen worldwide, as well as others, such as O26:H11, O111:H8, and O103:H2, that also cause human infections (15). The U.S. Centers for Disease Control and Prevention implemented a nationwide surveillance to test all suspect clinical samples simultaneously for O157:H7 and STEC. Similarly, some regulatory agencies have also started to look at the prevalence of STEC in foods and the environment and to assess the public health significance of STEC in foods.

Regardless of the sample type, almost all STEC testing methods screen for Stx using commercial serological assays or PCR assays specific for the *stx* gene. Any samples that are positive and potentially carry STEC are subjected to plating and isolation methods, with the pure culture isolates being retested by serology and/or PCR to confirm either the production of Stx or the presence of *stx*. Occasionally in our analyses, a STEC isolate determined to carry *stx* using one PCR assay is not confirmed to have *stx* using another PCR assay. Similarly, some STEC isolates that were found to be positive for *stx* by PCR fail to show serological reactivity with anti-Stx or vice versa (5). For instance, in the characterization of STEC isolated from produce, some strains were found to carry stx_2 but did not react with antibody to Stx2 (8). The lack of serological Stx confirmation of a strain that was positive by *stx*-specific PCR may be due to the absence of Stx expression (22), or the Stx level produced may be below the sensitivity of the assays. Serological assays for Stx can vary greatly in sensitivity (13), and some STEC strains have been found to produce low levels of Stx that are not detectable by tissue culture or serological tests (3, 23). However, another possible cause of these discrepancies may be differences in the specificities of anti-Stx antibodies or *stx* PCR primers for the various Stx subtypes. According to the subtyping nomenclature proposals and discussions held in 2009 at the 7th International Symposium on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections in Buenos Aires, there are currently three Stx1 subtypes (Stx1a, Stx1c, and Stx1d) and seven Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g), all of which might not be necessarily detected by the various assays. In this study, we used a panel of reference strains that carried various Stx subtypes to examine three *stx*-specific PCR assays routinely used in our laboratory for analysis and identification of STEC from foods. The specificity of these PCR assays for *stx* subtypes has not been tested previously. In contrast, the subtype specificity of some Stx immunoassays has been tested, although not with a panel of reference strains; consequently, four anti-Stx kits were also included in the study.

The bacterial isolates used in this study were obtained from the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* in Denmark. These strains were used in the recent 2nd International External Quality Assurance (EQA) program funded by the European Centre for Disease Prevention and Control (ECDC), where a total of 45 laboratories from 38 countries participated to evaluate the performance of their assays. This panel of strains consisted of STEC serotypes that carried the various stx_1 and stx_2 subtypes either individually or in combinations. In addition, 3 other strains that carried the stx_{2d}, stx_{2e}, and stx_{2g} subtypes were also included in the study (Table 1).

Among the PCR assays examined was the 5P multiplex PCR that is used in our laboratory to confirm O157:H7 isolates. The 5P assay targets O157:H7 markers, including stx_1, stx_2, *ehxA* (enterohemolysin), the γ-intimin (*eae*) allele, and the +93 *uidA* single-nucleotide polymorphism (SNP) that is unique to O157:H7 (9). The *stx* primers used in the 5P were designed for the detection of stx_1 and stx_2 in O157:H7 in 1995 (7), a time when little information was available on Stx subtypes. Also examined was the seropathotype A/B multiplex PCR which is used in the characterization of non-O157 STEC isolates from foods. This assay detects most *eae* alleles, the O type-specific genes of 6 major EHEC serotypes, and a single primer pair to detect both stx_1 and stx_2 (17). The specificity of this PCR assay has never been tested against specific Stx subtypes. The third assay tested is a real-time (RT) PCR described in the FDA's *Bacteriological Analytical Manual* Online (BAM) (10) and used

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TABLE 1. Results of various PCR and serological assays for Stx and *stx* subtypes[a]

Strain	Serotype	Stx subtype(s)	5P		BAM		V-R		STAT		AB (*stx*)	PrE (Stx)	PrST (Stx)
			stx_1	stx_2	stx_1	stx_2	Stx1	Stx2	Stx1	Stx2			
AA1	O174:H8	1c, 2b	+	+	+	−	+	−	+	−	+	+	+
BB2	O55:H7	1a	+	−	+	−	+	−	+	−	+	+	+
CC3	O128ac[H2]	2f	−	−	−	−	−	+	−	−	−	−	−
DD4	O177:[H25]	2c, 2d	−	+	−	+	−	+	−	+	+	+	+
EE5	O111:[H8]	1a, 2a	+	+	+	+	+	+	+	+	+	+	+
FF6	O113:H4	1c, 2b	+	+	+	−	+	+	+	−	+	+	+
GG7	O103:H2	1a	+	−	+	−	+	−	+	−	+	+	+
HH8	O26:H11	1a	+	−	+	−	+	−	+	−	+	+	+
II9	O41:H26	1d	−	−	−	−	+	−	+	−	−	+	+
JJ10	O157:H7	2c	−	+	−	+	−	−	−	−	+	−	−
05622	O138[b]	2e	−	+	−	+	−	−	−	−	+	−	−
B2F1	O91:H21[b]	2d	−	+	−	+	−	+	−	+	+	+	+
D3509	O2:H25[c]	2g	−	+	−	+	−	−	−	−	−	−	−

[a] Assays: 5P, multiplex PCR; BAM, *Bacteriological Analytical Manual* RT-PCR; V-R, VTEC-RPLA; STAT, ImmunoCard STAT! lateral flow device; AB, seropathotype A/B PCR; PrE, Premier EHEC ELISA; PrST, ProSpectT Shiga toxin (*E. coli*) microplate assay ELISA.
[b] Strains were obtained from the STEC Center, Michigan State University.
[c] Strain was obtained from the Staten Serum Institute.

to screen for O157:H7 in foods. This assay, which targets the +93 *uidA* SNP, stx_1, and stx_2, will also detect other STEC, and as with the other 2 assays, its specificity for Stx subtypes was unknown.

The 4 immunoassays for Stx examined include the VTEC-RPLA "Seiken" (Denka Seiken, Japan), which is a reverse passive latex agglutination assay (RPLA) used to determine Stx1 and Stx2 titers, the ImmunoCard STAT! EHEC (Meridian Biosciences, Cincinnati, OH), which is a lateral flow device that detects Stx1 and Stx2, the Premier EHEC (Meridian Bioscience), which is often used to screen for Stx in clinical samples, and the ProSpectT Shiga toxin (*E. coli*) microplate assay (Remel, Lenexa, KS). The latter 2 assays are enzyme-linked immunosorbent assay (ELISA)-based methods that detect Stx; however, they will not distinguish between Stx1 and Stx2. The Stx subtype specificity of some of these assays has been reported but has not been tested using a panel of reference strains.

The PCR assays were performed as described previously (9, 10, 17), using as the template a boiled lysate prepared from colonies grown on tryptic soy agar (TSA) plates. All the serological assays were done according to the manufacturers' package inserts. For VTEC-RPLA, growth from the same TSA plate that was used to prepare the PCR templates was used to inoculate a tube of Casamino acids-yeast extract (CA-YE) medium and incubated at 37°C for 18 to 24 h. After a brief centrifugation, the titers of the supernatant were determined out to a 1:8 dilution and assayed with the respective anti-Stx latex beads. For the other 3 serological assays, growth from the TSA plates was used to inoculate MacConkey broth, incubated at 37°C for 18 to 24 h, and tested as specified by the manufacturers. All assays were repeated to verify results and reproducibility.

The results in Table 1 show that the 5P PCR did not detect the stx_{1d} and stx_{2f} subtypes. Similar specificities were observed for the BAM RT-PCR, except that the stx_{2b} subtype was also not detected by this assay (Table 1). The seropathotype A/B PCR did not detect the stx_{1d}, stx_{2f}, and stx_{2g} subtypes, and in addition, its specificity for stx_{2b} is also uncertain. This PCR uses a single primer pair to detect both stx_1 and stx_2, so the positive signal obtained for strains AA1 and FF6 that have stx_{2b} may be due to the stx_{1c} subtype that is also carried by both strains (Table 1).

Serological analysis showed that the anti-Stx1 antibodies used in both the VTEC-RPLA and the ImmunoCard STAT! assay effectively detected all 3 Stx1 subtypes. The specificity of VTEC-RPLA for Stx1c is consistent with previous findings, which showed that most strains carrying Stx1c gave a positive but low-titer reaction with this assay (12). The anti-Stx2 antibody of the STAT assay did not detect Stx2b, Stx2c, Stx2e, Stx2f, and Stx2g (Table 1). The VTEC-RPLA assay was the only assay examined that detected Stx2f, but it did not detect Stx2c, Stx2e, and Stx2g and some of the specificity observed was inconsistent with the results of other studies. For example, the Stx2c subtype was negative with VTEC-RPLA, but another study showed that 23 strains that had stx_{2c} gave positive although low titers with this assay (11). Similarly, one study (4) showed that VTEC-RPLA does not detect the Stx2e subtype, which is consistent with our data, while other studies showed that some Stx2e-producing strains will give a low titer with VTEC-RPLA (11, 22). The specificity of VTEC-RPLA for Stx2b is also uncertain. In our analysis of the 2 strains that had *stx2b*, it was negative with AA1 but gave a weak positive with FF6. Perhaps AA1 produces low levels of Stx2b that are below the detection sensitivity of the assay or, as in the situation for Stx2e, not all the strains that carried the gene expressed the toxin or showed serological reactivity (22).

The 2 ELISAs examined had identical specificities in detecting all Stx1 subtypes, but neither detected Stx2c, Stx2e, Stx2f, and Stx2g, and their specificity for Stx2b is also uncertain. Analogous to the situation with the seropathotype A/B PCR, these assays will not differentiate between Stx1 and Stx2, and therefore, the positive ELISA obtained for AA1 and FF6 that carried Stx2b could be due to the Stx1c subtype (Table 1).

The *stx* genes and Stx subtypes that are not detected by the various assays are summarized in Table 2. The results showed that all three PCR assays routinely used in our laboratories will consistently miss the stx_{1d} and stx_{2f} subtypes and, depending on

TABLE 2. Summary of *stx* and Stx subtypes that are not detected by the various assays

Format and assay	*stx* or Stx subtypes not detected
PCR	
5P	stx_{1d}, stx_{2f}
BAM RT-PCR	stx_{1d}, stx_{2b}, stx_{2f}
Seropathotype A/B[a]	stx_{1d}, stx_{1c}, stx_{2b}, stx_{2f}, stx_{2g}
Serology	
VTEC-RPLA	Stx2b, Stx2c, Stx2e, Stx2g
ImmunoCard STAT!	Stx2b, Stx2c, Stx2e, Stx2f, Stx2g
Premier EHEC[a]	Stx1c, Stx2b, Stx2c, Stx2e, Stx2f, Stx2g
ProsSpectT[a]	Stx1c, Stx2b, Stx2c, Stx2e, Stx2f, Stx2g

[a] Reactivities to Stx1c and Stx2b and their respective genes are uncertain. These assays detect *stx* or Stx without differentiation, and the strains on the panel carried both Stx1c and Stx2b.

the assay, might also fail to detect stx_{2b} and stx_{2g}. This observation is consistent with a sequence alignment of the alleles identifying the annealing sites of the different primers that shows these primers lack the specificities for the various subtypes not detected. The lack of reactivity for the stx_{2f} subtype is also in agreement with the findings that the nucleotide sequence of stx_{2f} is sufficiently divergent that it is not detected by many stx_2-specific PCR primers (20).

The ELISAs we examined did not detect many of the Stx2 subtypes; however, some of these results were inconsistent with previous findings. For instance, Stx2f was not detected by the Premier ELISA in our study, although Schmidt et al. (20) showed that 2 of 6 Stx2f strains tested were positive and, more importantly, that all 6 were positive if the strains were induced with mitomycin C. Since induction was not specified in the kit protocol, none of the strains tested in this study were induced. The Premier ELISA also failed to detect Stx2c (strain JJ10) and Stx2e (strain 05622) in this study, but another study showed that both subtypes could be detected regardless of whether the strains were induced or not (20). In that study, however, not all the strains carrying these subtypes were detected by the assay. Similar results were observed in the recent EQA program, where only 9 of 17 (53%) participating laboratories that tested for Stx subtypes were able to phenotypically detect Stx2c in the O157:H7 strain JJ10. It has been reported that some strains may have a weak upstream promoter that can cause weak expression of some Stx subtypes (1). Our results are consistent with reports from others that there can be strain-to-strain variations in serological reactivity with some Stx subtypes (4, 11, 22).

Most of the serological assays we tested detected subtypes Stx2a and Stx2d but failed to detect Stx2b, Stx2c, Stx2e, Stx2f, and Stx2g (Table 2). The ability of assays to detect Stx2a and Stx2d is important. The Stx2a subtype is often carried by O157:H7 and other pathogenic EHEC, but these strains also have additional virulence factors, as Stx alone does not appear to be sufficient to cause severe symptoms, such as bloody diarrhea and hemolytic uremic syndrome (HUS) (14). Exceptions include strains like O113:H21 that produces only Stx2d but has caused outbreaks of HUS (6, 14). In addition to Stx2a and Stx2d, the Stx2c subtype, which, along with Stx2a, was carried by the O157:H7 strain that caused the spinach outbreak in the United States in 2006, has also emerged as an important subtype that can cause severe illness (11, 18); hence, the inability of assays to detect Stx2c may be of concern. The capacity of other Stx2 subtypes to cause severe illness remains uncertain. The stx_{2b} subtype was proposed to designate a subtype of stx_{2c} that is not found in STEC strains causing HUS (18) and, therefore, has not been implicated in severe illness. STEC carrying the stx_{2e} subtype are commonly associated with pig edema disease, but the stx_{2e} subtype is seldom found in human STEC and has not been implicated in diarrhea or severe illnesses (2). Similarly, the stx_{2f} subtype, primarily found in STEC isolated from pigeons (20), has been found in 24 Danish cases with nonsevere disease symptoms (F. Scheutz, unpublished data) and has rarely been implicated in severe human illness (21). Lastly, the stx_{2g} subtype, originally isolated from STEC in cattle (16), has been isolated from a Danish patient without diarrhea (Scheutz, unpublished) and, more recently, from some human isolates, but in most of these strains, the stx_{2g} gene was not expressed (19). Since the association of some Stx subtypes with illness remains uncertain, the inability of assays to detect some of these Stx subtypes may not be of significant public health concern.

In conclusion, the differences in specificities observed for the various assays for *stx* genes and Stx subtypes make it conceivable that, occasionally, results obtained from one assay may not be confirmed with another assay and underline the importance of additional genotypic characterization. In any case, such data, especially those of serological assays, may need to be interpreted with caution. This and other studies showed that there are differences in assay sensitivities and that there may be strain-to-strain variations, perhaps due to the absence or low levels of Stx expression, so that not all strains carrying a particular *stx* subtype will react serologically with the respective antibody.

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