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 positive and potentially carry STEC are subjected to plating PCR assays specific for the 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 strains that carried the various Stx subtypes to examine 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 subtypes to examine specificity of the various assays. In this study, we used a panel of reference strains that carried various Stx subtypes to examine specificity of these PCR assays for stx subtypes that was positive 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 stx2 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.

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 stx1, stx2, ehaA (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 stx1 and stx2 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 stx1 and stx2 (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...
to screen for O157:H7 in foods. This assay, which targets the pathotype A/B PCR; PrE, Premier EHEC ELISA; PrST, ProSpectT Shiga toxin (E. coli) microplate assay ELISA.

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 titters of the supernatant were determined in inoculate a tube of Casamino acids-yeast extract (CA-YE) plate that was used to prepare the PCR templates was used to age inserts. For VTEC-RPLA, growth from the same TSA colonies grown on tryptic soy agar (TSA) plates. All the sero-

### TABLE 1. Results of various PCR and serological assays for Stx and stx subtypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>stx subtype(s)</th>
<th>5P (stx1, stx2)</th>
<th>BAM (stx1, stx2)</th>
<th>V-R (Stx1, Stx2)</th>
<th>STAT (Stx1, Stx2)</th>
<th>AB (nt)</th>
<th>PrE (Stx)</th>
<th>PrST (Stx)</th>
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<tr>
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<td>+ +</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BB2</td>
<td>O55:H7</td>
<td>1a</td>
<td>+ +</td>
<td>+ +</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>O126a[H2]</td>
<td>2f</td>
<td>+ +</td>
<td>+ +</td>
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<td>+ +</td>
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<td>O111:[H8]</td>
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</table>

*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.

Strains were obtained from the STEC Center, Michigan State University.

Strain was obtained from the Staten Serum Institute.

### Notes

- The results in Table 1 show that the 5P PCR did not detect the stx1d and stx2f subtypes. Similar specificities were observed for the BAM RT-PCR, except that the stx2f subtype was also not detected by this assay (Table 1). The seropathotype A/B PCR did not detect the stx1c, stx2c, and stx2g subtypes, and in addition, its specificity for stx2b is also uncertain. This PCR uses a single primer pair to detect both stx1 and stx2, so the positive signal obtained for strains AA1 and FF6 that have stx2b may be due to the stx1c 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 stx2c gave positive although low titers with this assay (11). Similarly, one study (4) showed that VTEC-RPLA does not detect the Stx2f 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 stx1d and stx2f subtypes and, depending on
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 stx2b subtype was proposed to designate a subtype of stx2e that is not found in STEC strains causing HUS (18) and, therefore, has not been implicated in severe illness. STEC carrying the stx2c subtype are commonly associated with pig edema disease, but the stx2c subtype is seldom found in human STEC and has not been implicated in diarrhea or severe illnesses (2). Similarly, the stx2e 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 stx2g 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 stx2g 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.

We thank the STEC Center at Michigan State University for providing some of the strains used in this study.

REFERENCES


