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Research Note

Evaluation of Seven Different Commercially Available Real-Time PCR Assays for Detection of Shiga Toxin 1 and 2 Gene Subtypes

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ABSTRACT

Following the recent outbreak of Shiga toxin–producing *Escherichia coli* (STEC) O104:H4 infection in Germany, the demand for fast detection of STEC has again increased. Various real-time PCR-based methods enabling detection of Shiga toxin genes (*stx*) have been developed and can be used for applications in food microbiology. The present study was conducted to evaluate the reliability of seven commercially available real-time PCR systems for detection of stx_1 and stx_2 subtypes. For this purpose, pure cultures of 18 STEC strains harboring all known stx_1 and/or stx_2 subtypes were tested. Only one of the seven real-time PCR systems detected all known stx_1 and stx_2 subtypes. Six systems failed to detect the stx_{2f} subtype. One system missed stx_2 subtypes reported in association with severe human disease. Because the presence of certain *stx* genes (subtypes) is considered an important indicator of STEC virulence, systems differentiating between the stx_1 and stx_2 gene groups provide added value. Reliable and fast detection of *stx* genes is of major importance for both diagnostic laboratories and the food industry.

Shiga toxin–producing *Escherichia coli* (STEC), also called verocytotoxin-producing *E. coli* (VTEC), strains are recognized as a major cause of foodborne illnesses. STEC strains are responsible for a number of human diseases, including diarrhea, hemorrhagic colitis, and the life-threatening hemolytic uremic syndrome (HUS) *(4)*. Since the STEC O104:H4 infection outbreak in Germany in 2011, the demand for reliable and fast detection of STEC has increased *(9)*. STEC strains are characterized by the production of one or more Shiga toxins. Based on cytotoxicity neutralization assays and sequence analysis of Shiga toxin genes, two main Stx groups (Stx1 and Stx2) have been described *(6)*. According to new nomenclatures, the Stx1 group consists of the Stx1a, Stx1c, and Stx1d subtypes. The Stx2 group is more heterogeneous and includes the Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g subtypes. STEC strains pathogenic for humans tend to produce Stx2 and have other virulence traits such as the adhesion factor intimin. In STEC strains from HUS patients, genes encoding Stx2a or Stx2c (mainly in combination with *eae* genes encoding intimin) are frequently found, and Stx2d-producing strains also have been isolated from HUS patients *(5)*.

Testing for Shiga toxin genes (*stx*) is used for diagnosis of STEC infections in humans and for detection of STEC in foods, although *Hafnia alvei* and *Citrobacter freundii* may also harbor *stx*. Nevertheless, the presence of *stx* and in particular certain *stx* subtypes (especially in association with other virulence factors such as intimin) is considered a better indicator for STEC virulence than is the serotype. Persson et al. *(5)* were not able to find an association between HUS and individual O groups after investigating 20 human STEC infection cases in Denmark. Boerlin et al. *(1)* found that stx_2-positive strains were five times more likely to be linked with severe illness than were stx_2-negative isolates of the same serotype. The aim of the present study was to evaluate the reliability of seven commercially available real-time PCR systems for detection of all known stx_1 and stx_2 subtypes. For this purpose, pure cultures of 18 STEC strains harboring various stx_1 and/or stx_2 subtypes were tested. The convenience of the performance of the seven real-time PCR protocols for application in food microbiology also was assessed.

MATERIALS AND METHODS

STEC strains. Eighteen target strains carrying various stx_1 and/or stx_2 subtypes were used (Table 1). Sixteen strains were obtained from the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* in Denmark (Statens Serum Institute, Copenhagen, Denmark), and these strains have been used recently in the 2nd International External Quality Assurance program. The two additional strains were obtained from the collection of the Institute for Food Safety and Hygiene (Vetsuisse Faculty University of Zurich, Zurich, Switzerland). Strains were grown on plate count agar (Oxoid AG, Pratteln, Switzerland) for 24 h at 37°C.

Real-time PCR systems evaluated. The following commercially available real-time PCR systems were evaluated: GeneDisc STEC and *E. coli* O157 applied on the GeneDisc Cycler (Pall

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TABLE 1. *Detection of the* stx_1 *and* stx_2 *subtypes by the seven evaluated real-time PCR assays*[a]

Strain	*stx* subtype(s)	Assurance GDS STEC Genetic Detection System		ABI Custom TaqMan VT1/VT2 Assay		LightMix Kit EHEC		GeneDisc STEC and *E. coli* O157 (*stx*)	iQ Check STEC VirX (*stx*)	BAX System PCR Assay (*stx*)	Mericon VTEC stx1/2 Assay (*stx*)
		stx_1	stx_2	stx_1	stx_2	stx_1	stx_2				
C147-10	1c, 2b	+	+	+	+	−[b]	−[b]	+	+	+	+
C148-10	1a	+	−	+	−	−	+[c]	+	+	+	+
C153-10	2f	−	−	−	+	−	−	−	−	−	−
C151-10	2c, 2d	−	+	−	+	−	+	+	+	+	+
C152-10	1a, 2a	+	+	+	+	+	−	+	+	+	+
C154-10	1c, 2b	+	+	+	+	−[b]	−[b]	+	+	+	+
C157-10	1a	+	−	+	−	−	+[c]	+	+	+	+
C158-10	1a	+	−	+	−	−	+[c]	+	+	+	+
C159-10	1d	+	−	+	−	−	+	+	+	−	+
C160-10	2c	−	+	−	+	−	+	+	+	+	+
Sf133-1	1c	+	−	+	−	−[b]	−	+	+	+	+
D3428	2b	−	+	−	+	−	+	+	+	−	+
D2587	2b, 2c	−	+	−	+	−	+	+	+	+	+
D3435	2d	−	+	−	+	−	+	+	+	+	+
D3648	2e	−	+	−	+	−	+	+	+	+	+
D3509	2g	−	+	−	+	−	−	+	+	+	+
1093-00	1a, 2a	+	+	+	+	−[b]	−[b]	+	+	+	+
001-06	2b	−	+	−	+	−	+	+	+	−	+

[a] +, positive, −, negative. If the first run yielded negative results for certain *stx* subtypes, the run was repeated with a reduced lysate concentration.
[b] Melting curve had one peak at 60°C instead of different peaks for stx_1 (melting point of 55.3°C) and stx_2 (melting point of 66.3°C).
[c] Strains harboring stx_{1a} produced positive results for stx_2.

GeneDisc Technologies, Bruz, France), the Assurance GDS STEC applied on the GDS Rotor Gene (BioControl, Bellevue, WA), the Mericon VTEC stx1/2 applied on the Qiagen Rotor-gene Q (Qiagen, Hombrechtikon, Switzerland), the LightMix Kit EHEC applied on the LightCycler 2.0 (Roche Diagnostics AG, Rotkreuz, Switzerland), the iQ Check STEC VirX (Bio-Rad, Marnes-la-Coquette, France) applied on the MiniOpticon, the BAX System STEC Suite Screening Assay applied on the BAX System Q7 (DuPont, Qualicon, Wilmington, DE), and the ABI Custom TaqMan VT1/VT2 Assay (Applied Biosystems, Carlsbad, CA) applied on the 7500 Fast real-time PCR system.

Detection of stx_1 and stx_2 subtypes. Pure culture experiments were performed to evaluate the reliability of the seven real-time PCR systems for detection of stx_1 and/or stx_2 subtypes. Boiled lysates prepared from colonies of the 18 STEC strains grown on plate count agar were used as templates. A colony was added to 450 μl of lysis buffer containing 20 ml of 1 M Tris HCl (pH 8.5), 100 μl of Tween 20 (Merck, Darmstadt, Germany), 48 mg of Proteinase K (Sigma-Aldrich, Buchs, Switzerland), and distilled water to 200 ml. Suspensions were heated for 40 min at 60 ± 1°C and then for 20 min at 95 ± 1°C in a heating block. The amount of lysate used in each assay was adapted according to the required template volume and diluted with lysis or resuspension buffer supplied with the kits. PCR assays were performed according the manufacturer's instructions. When the first run yielded negative results for certain *stx* subtypes, the run was repeated with a reduced lysate concentration.

Convenience in performance. Because the evaluated real-time PCR systems are intended to be used by diagnostic laboratories, convenience in performance is also of importance. Thus, the systems were evaluated with regard to sample preparation time, running time for STEC detection, and ease of data analysis.

RESULTS AND DISCUSSION

Detection of stx_1 and stx_2 subtypes. The Assurance GDS STEC, LightMix Kit EHEC, and ABI Custom TaqMan VT1/VT2 assays report results itemized for the stx_1 and stx_2 groups. The GeneDisc STEC and *E. coli* O157, Mericon VTEC stx1/2, BAX STEC Suite Screening, and iQ Check STEC VirX assays do not allow differentiation between the stx_1 and stx_2 groups. With exception of the LightMix Kit EHEC system, all tested systems included an internal control. Results for detection of the different stx_1 and stx_2 subtypes by the seven evaluated commercial real-time PCR-based systems are shown in Table 1.

The ABI Custom TaqMan VT1/VT2 Assay correctly detected all stx_1 and stx_2 subtypes in the first run. All other systems yielded negative results for at least stx_{2f}. The iQ Check STEC VirX, GeneDisc STEC and *E. coli* O157, and Mericon VTEC stx1/2 assays yielded correct positive results in the first run for strains harboring all stx_1 and stx_2 subtypes except stx_{2f}. However, in the Mericon VTEC stx1/2 Assay, the color compensation was not ideal because negative results were occasionally indicated as uncertain. The Assurance GDS STEC assay did not detect the stx_{2b} subtype in the first run, but in the second run with a decreased amount of template, all stx_1 and stx_2 subtypes (except stx_{2f}) were detected. The LightMix Kit EHEC assay was unable to detect stx_{2a}, stx_{2f}, and stx_{2g}, and some of the results obtained were inconsistent, especially with regard to

strains harboring stx_{1a} and stx_{2b} (Table 1). This system should separately detect stx_1 and stx_2 subtypes by yielding different peaks for the stx_1 (55.3°C) and stx_2 (66.3°C) melting curves. Despite performing several runs with different concentrations of lysate as template and diluted template, for some strains and *stx* subtypes (C147-10, stx_{1c} and stx_{2b}; C154-10, stx_{1c} and stx_{2b}; Sf133-1, stx_{1c}; 1093-00, stx_{1a} and stx_{2a}) it was not possible to obtain a definite result because the respective melting curves showed only one peak at 60°C. In addition, three strains (C148-10, C157-10, C158-10) harboring only stx_{1a} were indicated as positive for stx_2. Because this system does not include an internal toxin, it was not possible to detect potential PCR inhibitions. The BAX System STEC Suite Screening system was not able to detect stx_{1d}, stx_{2b}, and stx_{2f} in any of three replicated runs.

Five of the seven evaluated real-time PCR assays detected all stx_1 subtypes (stx_{1a}, stx_{1c}, and stx_{1d}) and stx_2 subtypes stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2g}. Because strains harboring stx_{2a}, stx_{2c}, and stx_{2d} have been reported in association with outbreaks and severe human illness *(2, 3, 5),* the ability of a system to detect these subtypes is of great importance. Six of the seven evaluated real-time PCR assays met this requirement. However, stx_{2a} was always detected in strains also harboring stx_{1a}. The lack of reactivity for the stx_{2f} subtype has been reported previously and explained by divergence of the stx_{2f} nucleotide sequence *(2).* The Stx2f toxin is also not detected by some immunological kits. STEC strains harboring stx_{2f} have been isolated from pigeons *(7),* but such strains seem to be only rarely associated with severe human diseases *(8).*

An additional desirable trait in an STEC detection system is differentiation between the stx_1 and stx_2 groups. The presence of stx_2 in STEC strains significantly increases the likelihood of disease progression to HUS, whereas STEC isolates carrying stx_1 are mainly found in healthy humans or patients with uncomplicated diarrhea *(1).*

Convenience in performance. For testing of pure cultures, all kits could be used with lysates from a simple heating step. The LightMix Kit EHEC system can be run on a standard real-time PCR machine, whereas for the Assurance GDS STEC, the BAX System STEC Suite Screening, the iQ Check STEC VirX, the GeneDisc STEC and *E. coli* O157, and the ABI Custom TaqMan VT1/VT2 assays the respective instrument must be purchased. However, these tests can also be used for the analysis of several other microorganisms. Another advantage of assays such as the BAX System STEC Suite Screening assay or the Assurance GDS STEC system is that no separate work station or room for DNA extraction is needed. Hands-on times also are shorter, even though there are differences in the running time for PCR amplification and detection.

Reliable and fast detection of STEC is of major importance for diagnostic laboratories. Only one of the seven commercially available real-time PCR-based assays evaluated in the present study detected all known stx_1 and stx_2 subtypes and therefore could detect STEC strains with all combinations of *stx* subtypes. One system missed stx_2 subtypes reported in association with severe human disease. Because the presence of certain Shiga toxin genes (subtypes) is considered an important indicator of STEC virulence, systems differentiating between the stx_1 and stx_2 groups provide added value.

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