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A rapid method for the discrimination of genes encoding classical Shiga toxin (Stx) 1 and its variants, Stx1c and Stx1d, in *Escherichia coli*

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Subtyping of Shiga toxin (Stx)-encoding genes by conventional polymerase chain reaction (PCR) is time-consuming. We developed a single step real-time fluorescence PCR with melting curve analysis to distinguish rapidly stx_1 from its variants, stx_{1c} and stx_{1d}. Melting temperatures (T_m) of 206 Stx-producing *Escherichia coli* (STEC) identified to harbor stx_1 or stx_{1c} were analyzed using a specific hybridization probe over the variable region. 170 of 171 stx_1-harboring STEC displayed T_m of 69°C to 70°C, whereas 34 of 35 strains containing stx_{1c} had T_m of 65°C – 66°C. This constant and reproducible difference of 4°C demonstrated that melting curve analysis is a reliable technique to differentiate stx_1 from stx_{1c}. Two isolates displayed atypical T_m. Sequence analysis showed that one of them was 100% identical to stx_{1d} within a 511 bp DNA stretch. Our data demonstrate that real-time PCR is a rapid and reliable tool to differentiate stx_1 from stx_{1c} and stx_{1d} and to detect new stx_1 variants. Because stx_1-harboring STEC cause diarrhoea and hemolytic-uremic syndrome, whereas those containing stx_{1c} are often shed asymptomatically, a rapid differentiation between stx_1 and its variants using the procedure developed here has both clinical implications and a direct significance for the risk assessment analysis of STEC isolated from foods.

Keywords: *Escherichia coli* / Melting curve analysis / Real-time fluorescence polymerase chain reaction / Shiga toxin

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1 Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 and several non-O157 serogroups have emerged worldwide as important food-borne pathogens causing diarrhea, hemorrhagic colitis and a life-threatening hemolytic-uremic syndrome (HUS) [1 – 5]. Cattle are the major reservoir of these organisms [3, 6]. The pathogenicity of STEC is related to the production of one or more Stxs. Two major toxin types, Stx1 and Stx2, have been assigned [7]. The Stx2 group is highly heterogeneous, comprising, in addition to Stx2, an increasing number of Stx2 variants including Stx2c [8], Stx2c2 [9], Stx2d [10, 11], Stx2e [12], and Stx2f [13]. The Stx1 group appears to be more homogeneous, but new variants have been emerging also here [14 – 17]. We identified a stx_{1c} allele, and developed a $stxB_1$-restriction fragment length polymorphism strategy and a stx_{1c}-specific PCR in order to differentiate stx_{1c} from stx_1 [16]. Using this approach, stx_{1c} was detected in 17% of STEC isolated from humans [16]. A prominent feature of STEC carrying stx_{1c} is lack of the *eae* gene encoding intimin, suggesting the absence of the locus of enterocyte effacement (LEE) [18]. Another stx_1 variant, designated stx_{1d}, has been recently described in a STEC strain isolated from cattle [17].

Since PCR is a highly sensitive approach to detect *stx* genes, different PCR protocols have been developed for the identification of classical stx_1 and stx_2 and their different alleles [11, 13, 16, 17, 19 – 21]. However, subtyping of *stx* genes using conventional PCR is a time-consuming procedure, because it requires repeated PCR runs, often combined with restriction analysis. Furthermore, new alleles and slight variations in DNA sequence may not be detectable using this approach. In this study, we developed a single-step real-time fluorescence PCR on a LightCycler instrument in combination with melting curve analysis to differentiate rapidly stx_1 from its variants stx_{1c} and stx_{1d}.

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Abbreviations: FRET, fluorescence energy transfer; **HUS**, hemolytic-uremic syndrome; **LC**, Light Cycler; **STEC**, Stx-producing *Escherichia coli*; **Stx**, Shiga toxin

Table 1. *stx* genotypes and serotypes of STEC strains investigated in this study

stx genotype[a)]	Other virulence factors (no. of isolates)		No. of strains	Serotype[b)]
	eae	E-*hly*		
stx_{1c}	0	8	12	O8:H19 (1), O76:H19 (1), O78:NM (3), O112:H2 (1), O113:H4 (1), O128:NM (1), O174:H8 (1), ONT:H21 (1), Orough:NM (1), Orough:HNT (1)
$stx_{1c} + stx_2$	0	1	1	ONT:NM (1)
$stx_{1c} + stx_{2d}$	0	20	22	O22:H8 (1), O75:H8 (1), O76:H19 (1), O91:NM (1), O113:H4 (3), O128:H2 (5), O128:H8 (2), O146:H21 (3), O174:H8 (1), OX176:NM (1), OX178: NM (1), ONT:H2 (1), ONT:NM (1)
stx_1	79	79	100	O3:H2 (1), O3:H10 (1), O8:NM (3), O25:NM (2), O26:H11 (15), O26:NM (5), O31:NM (1), O62:NM (1), O84:H4 (1), O84:HNT (1), O84:NM (1), O91:NM (1), O91:H14 (2), O92:H33 (1), O103:H2 (18), O103:H18 (2), O103:NM (2), O111:H2 (2), O111:NM (9), O112:NM (1), O118:NM (2), O119:H2 (1), O128:NM (1), O129:NM (1), O145:H25 (2), O145: NM (8), O145:HNT (1), O146:H20 (1), O152:H4 (1), O156:NM (1), O157:H7 (1), ONT:H14 (2), ONT:NM (3), Orough:NM (5)
$stx_1 + stx_2$	49	50	50	O4:NM (1), O26:H11 (5), O26:HNT (1), O26:NM (7), O68:H4 (1), O103:H2 (1), O111:NM (7), O118:NM (1), O145:NM (1), O157:H7 (13), O157:NM (7)
$stx_1 + stx_{2c}$	13	16	16	O75:NM (1), O104:H16 (1), O113:NM (1), O120:NM (1), O157:H7 (2), O157:NM (9)
$stx_1 + stx_{2d}$	0	2	5	O8:NM (1), O62:NM (2), O91:NM (1), O128:NM (1)

a) *stx* genotype identified by conventional PCR

b) ONT and HNT, O antigen and H antigen, respectively, are nontypeable; Orough, O antigen is not determined because of rough liposaccharide; NM, nonmotile. The numbers in the parentheses indicate numbers of isolates within serotypes.

2 Materials and methods

2.1 Bacterial strains

STEC strains used in this study are listed in Table 1. They were isolated between 1996 and 2001 at the Institute of Hygiene and Microbiology, University of Würzburg, and at the Institute of Hygiene, University Hospital of Münster, Germany, according to protocols described earlier [22]. All STEC strains originated from apparently sporadic cases of infection. They were serotyped (Table 1) with antisera against *Escherichia coli* O antigens 1 – 181 and H antigens 1 – 56 using microtitration plates [23]. The presence of *stx*, *eae*, and E-*hly* genes was detected by PCR analysis as previously described [16, 18] (Table 1). Subtyping of stx_2 and stx_{2c} was accomplished by restriction analysis using *Hae*III and *Fok*I as described by Rüssmann *et al.* [24]. For the experiments, strains were cultured on sorbitol MacConkey agar plates at 37°C for 18 h, and cells derived from a single colony (*ca.* 10^4 bacteria) were resuspended in saline (0.85% NaCl) for PCR analysis. *E. coli* strains EDL933 ($stx_1 + stx_2$), 933J (stx_1), and K-12 strain C600 were used as controls.

2.2 LightCycler (LC) PCR assay and product detection

The amplification reactions and the fluorescence energy transfer (FRET) hybridization probe melting curve analysis were carried out in a fluorescence thermal cycler (LightCycler; Roche Diagnostics, Mannheim, Germany). DNA oligonucleotide primers were synthesized by Sigma ARK (Mannheim, Germany), and hybridization probes labelled with fluorescein and LC Red 640 were from TIB Molbiol (Berlin, Germany). The nucleotide sequences of primers STEC-1 and STEC-2 [25] and the fluorogenic hybridization probes STEC-I HP-1 and STEC-I HP-2 [25] were chosen to specifically hybridize with the hypervariable gene region of stx_1. The LightCycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics) was used as the basis for the reaction mixture in the LC-PCR assay. The reaction conditions were optimized according to the manufacturer's protocol. Amplification reactions were carried out in a total volume of 20 µL at 3.0 mM magnesium chloride, 0.5 µM of each primer, and 0.2 µM of each oligonucleotide probe. Template DNA (2 µL) and 1 × LC FastStart DNA master hybridization probe buffer were added to the mixture. Reaction was started with an initial FastStart *Taq* DNA polymerase activation phase at 95°C for 10 min. Target DNA was amplified in 40 cycles of denaturation (95°C, 10 s), primer and probe annealing (50°C, 20 s), and extension (72°C, 30 s). The temperature transition rate was 20°C/s. Positive samples were identified by increasing fluorescence by the instrument compared with background fluorescence and the quantities of amplified products were monitored in the F2 mode by detection of energy emitted at 640 nm. In each set of experiments, DNA from strain 933J (stx_1) and 3117/97 (stx_{1c}) was used as positive control and DNA from *E. coli* C600 as well as water were included as negative controls.

2.3 Melting curve analysis

The hybridization probes were designed to be homologous to a highly conserved region within the stx_1 gene. The temperature at which the hybridization probes dissociated from their target sites was determined by melting curve analysis following the completion of the LC-PCR amplification using the LightCycler software. This allowed differentiating between stx_1 and stx_{1c} based on differences in the avidity of the hybridization probes for the complementary sequences in the amplified DNA. The melting curve analysis was performed by an initial denaturation step at 95°C for 10 s, followed by cooling to 45°C for 40 s. Continuous fluorescence reading was carried out at a linear temperature transition rate of 0.2°C/s by heating up to 95°C. The melting curve for each strain was analyzed manually to determine T_m. The T_m value is the peak of the curve assigned from a plot generated by the instrument of the negative derivative of fluorescence *versus* temperature ($-dF/dT$) of the melting curve for amplification products measured at 640 nm.

2.4 Nucleotide sequence analysis of PCR products

PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed with an automated 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Palo Alto, CA, USA) using PCR primers, and the ABI Prism BigDye Terminator Ready Reaction Cycle Sequencing Kit (Perkin Elmer Applied Biosystems). Nucleotide sequence analysis was performed with DNASIS program (Hitachi Software, San Bruno, CA, USA) and homology searches with the NCBI GeneBlast (http://www.ncbi.nlm.nih.gov/BLAST/).

3 Results

A total of 206 STEC strains of 33 different serogroups (Table 1) identified by conventional PCR to contain stx_1 (171 isolates) or stx_{1c} (35 isolates), alone, or in combination with stx_2, stx_{2c} or stx_{2d}, were analyzed by a real-time fluorescence PCR. Primers STEC-1 and STEC-2 [25], derived from the *stxA* subunit gene, were used to amplify 521-bp regions of *stx* genes in a single-step PCR on the LightCycler. To differentiate STEC carrying stx_1 from those harboring stx_{1c}, the hybridization probes STEC-I HP-1 and STEC-I HP-2 [25l were positioned over the variable regions of the amplicons. Melting curve analysis was then performed. The melting temperature (T_m) values were determined for each allele by plotting fluorescence *versus* temperature (Fig. 1). Clearly distinguishable T_m were obtained for STEC harboring stx_1 and those carrying stx_{1c} (Fig. 1). Whereas STEC harboring stx_1 yielded a melting peak at 69°C–70°C, stx_{1c} resulted in a substantially lower T_m of 65°C to 66°C. In at least three independent runs on the LightCycler instrument, the stx_1 and stx_{1c} alleles constantly differed in their T_m values by 4°C, demonstrating that the difference in their T_m is highly reproducible. Despite a slight movement of T_m (range of 1°C) for each of the alleles in runs performed with different batches of the LC-PCR hybridization kit, the difference of 4°C between T_m of stx_1 and stx_{1c} was conserved.

Two of the 206 strains tested, however, differed in their T_m values from both that of classical stx_1 and that of stx_{1c} (Fig. 2A). Melting peaks were 64°C for isolate 7139/96 (serotype O8:H19) and 69°C for isolate 7140/96 (serotype O8:NM) (Fig. 2A). Isolate 7139/96 harbored stx_{1c} and the isolate 7140/96 stx_1 as determined by conventional PCR. To investigate possible reasons for the atypical T_m, the nucleotide sequences of the amplicons from these two isolates were

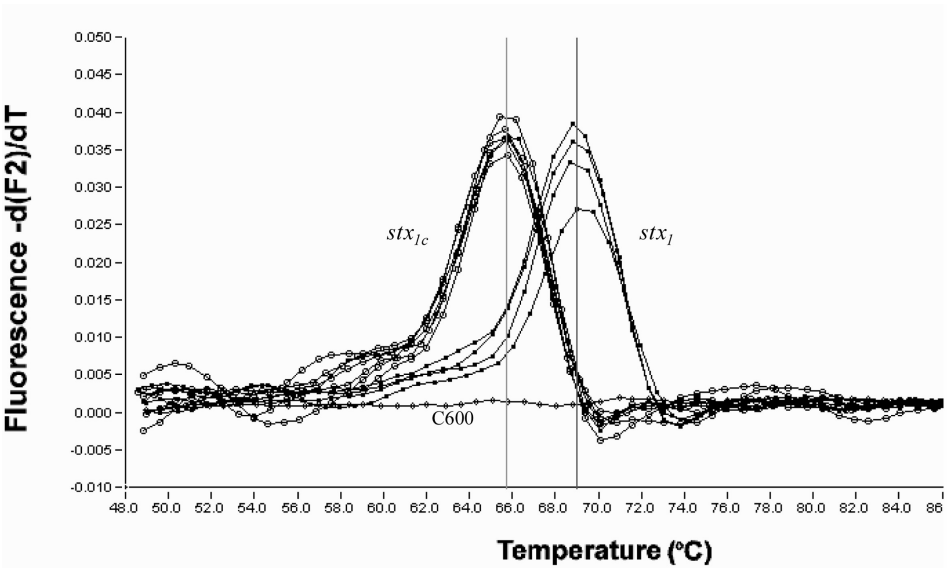
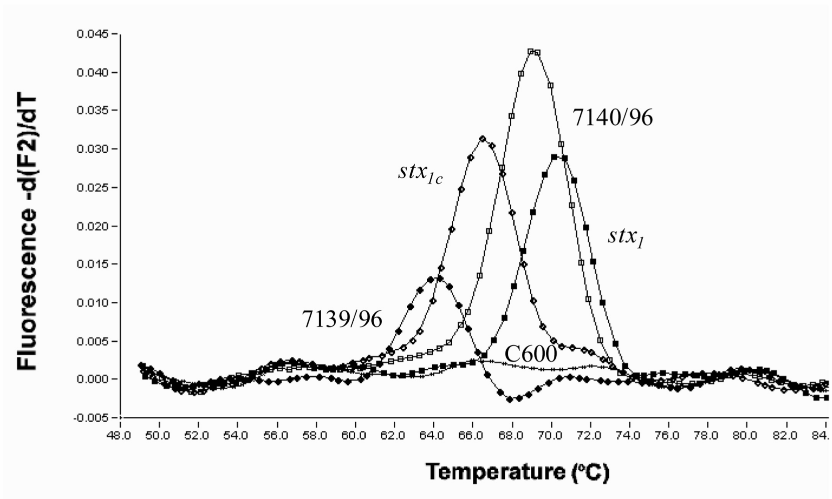


Figure 1. Melting curve analysis performed on amplification products of stx_1- and stx_{1c}-positive isolates. STEC harboring stx_1 alleles (dark circle lines) represent a melting peak of 69°C. Isolates with stx_{1c} (empty circle lines) represent a T_m of 65°C. Four STEC strains of each genotype are shown to demonstrate reproducibility of the discrimination. *E. coli* laboratory strain C600 was used as a negative control.

A



B

```
                                  (STEC-I-HP-1)
                    570                                598
                 ...T TTACGTTTTC GGCAAATACA GAGGGGATT
stx1(M19473)        - ---------- ---------- --------- /70°C
stx1c(AJ312232)     - ---------- -------T-- --------- /66°C
stx1d(AY170851)     - --G------- -C-----C-- --------- /64°C
7139/96             - --G------- -C-----C-- --------- /64°C
7140/96             - ---------- ---------- ------G-- /69°C

                                  (STEC-I-HP-2)
                    600                              627
                    T CGTACAACAC TGGATGATCT CAGTGGG...
stx1(M19473)        - ---------- ---------- ------- /70°C
stx1c(AJ312232)     - ---------- --T------- ------- /66°C
stx1d(AY170851)     - ---------- --T------- ------A /64°C
7139/96             - ---------- --T------- ------A /64°C
7140/96             - ---------- ---------- ------- /69°C
```

Figure 2. Melting curve and sequence analysis of STEC strains harboring stx_1 and its variants. (A) Melting peaks are shown for STEC strains carrying classical stx_1 gene (T_m 70°C) and stx_{1c} (T_m 66°C). Isolate 7140/96 (serotype O8:NM), identified as harboring the stx_1 gene by conventional PCR, represents a T_m of 69°C and isolate 7139/96 (serotype O8:H19) identified as carrying stx_{1c}, yielded a T_m of 64°C. (B) Sequence alignments of stx_1, stx_{1c}, stx_{1d} (GenBank numbers given in brackets) and of the genes of 7139/96 and 7140/96. Underlined sequence corresponds to the hybridization probe over the hypervariable region according to nucleotide sequence at position 570–627. Sequence identity is indicated by dashes.

analyzed. Sequence alignments demonstrated base pair exchanges over the probe binding sites (Fig. 2B). One nucleotide exchange (A → G) within the binding site of the hybridization probe in isolate 7140/96 resulted in a decrease of its T_m value to 69°C in contrast to T_m of 70°C of classical stx_1. Another two nucleotide differences were identified outside the probe-binding site resulting in three nucleotide differences of stx_1 of 7140/96 from classical stx_1. Isolate 7139/96 displayed four mutation sites within the probe-binding site (A → G; G → C; T → C; G → A) as compared to stx_{1c}. The T_m of this amplicon reached 64°C, which was 2°C lower than that of stx_{1c} (66°C) (Fig. 2A). Sequence analysis of the PCR amplicon demonstrated 100% nucleotide identity to a recently described new variant stx_{1d} [17]. This indicates that the isolate 7139/96 carries the stx_{1d} gene. In this study, stx_1 and stx_1 variant genes of all STEC strains tested could be amplified, whereas no increase in fluorescence signals was observed using the negative control template of *E. coli* C600.

4 Discussion

In this study, we characterized 206 STEC isolates harboring stx_1, stx_{1c} or stx_{1d}, as determined by conventional PCR [16] and by nucleotide sequencing, using a real-time fluorescence PCR assay on an LC instrument combined with melting curve analysis. We developed a simple and rapid one-step PCR protocol for the differentiation of allelic polymorphisms of stx_1 variants using labelled hybridization probes which allowed a specific detection based on FRET

technique [26]. The specific T_m of DNA templates were defined as temperatures at which 50% of the duplicies became single-stranded. By melting curve analysis we detected two different T_m values, which were specific for *stx*$_1$ (69°C–70°C) or *stx*$_{1c}$ (65°C–66°C). This difference of 4°C between T_m of *stx*$_1$ and *stx*$_{1c}$ was reproducible within repeated runs. Whereas it was shown previously that the melting peak is influenced by the GC content, the length and the nucleotide sequence of the amplified product [27, 28], in this experimental setup we observed that the marked difference in T_m values between *stx*$_1$ and *stx*$_{1c}$ resulted from as few as two nucleotide differences within the amplicon over the hybridization probe.

STEC strains harboring classical *stx*$_1$ or its variants often carry additional *stx* genes, like various *stx*$_2$ alleles, and/or other putative virulence genes, such as *eae* encoding an adherence factor intimin [29], EHEC-*hly* encoding an EHEC hemolysin [5, 18], *cdt* coding for cytolethal distending toxin [30], and some others [18, 31]. Moreover, such strains belong to a broad spectrum of different serotypes ([16, 18]; this study). However, neither additional virulence characteristics nor differences in their serotypes had any influence on the allele-specific T_m of the isolates. This demonstrates that the procedure developed in this study is highly discriminative for *stx*$_1$ and *stx*$_{1c}$ based on melting curve analysis, but it is independent on serotypes and other virulence markers of STEC isolates.

From the practical standpoint, we strongly advise that positive controls for both *stx*$_1$ and *stx*$_{1c}$ are used within each run of real-time PCR. This is based on our observation that minor differences in T_m values between runs performed with different batches of the hybridization kits can occur, and slight variations in nucleotide sequences might influence the T_m of the PCR product. Therefore, the presence of positive controls in each run allows minimizing these drawbacks which appear to be objective and inherent to this technique. The importance of using a positive control for each allele within one run is underlined by our finding of two isolates (7140/96 and 7139/96) the T_m of which differed from both *stx*$_1$ and *stx*$_{1c}$. Only a direct comparison of T_m of these isolates with those of each of the positive controls allowed us to suspect that the relatively small deviations in their T_m may reflect structural differences within the amplicons. In order to investigate this possibility, the PCR amplicons of both isolates with atypical T_m were sequenced. Three mutation sites in the nucleotide sequence, one of them located within the probe-binding region of the amplicon, were detected in isolate 7140/96. In isolate 7139/96, which had a T_m value of 64°C as compared to T_m of 66°C of *stx*$_{1c}$, sequence analysis resulted in four nucleotide exchanges within the amplicons over the hybridization probe compared to *stx*$_{1c}$. We presume that these changes, the significance of which for a biological activity of this *stx*$_1$ variant is presently not understood, accounted for the atypical melting peak and the T_m decrease to 64°C. Interestingly, the sequence variations in isolate 7139/96 were identical to a new *stx*$_1$ allele, *stx*$_{1d}$, identified recently in a STEC strain isolated from cattle [17]. This finding demonstrates that *stx*$_{1d}$-harboring STEC may also infect humans, and warrants further investigation of their significance in human diseases. Moreover, the previous finding of *stx*$_{1d}$-harboring STEC in cattle [17] indicates that such strains can contaminate foods of bovine origin. Therefore, the procedure developed in this study might be potentially useful for food microbiological laboratories to identify STEC harboring *stx*$_{1d}$ in foods. Moreover, it also allows to compare, in prospective studies, human *stx*$_{1d}$-harboring isolates with such strains isolated from food and from cattle to understand better the epidemiology of human infections and clonal structure of these strains. In general, a wide spectrum of serotypes identified among STEC harboring *stx*$_1$ or its variants in this study (Table 1) does not support a clonal origin of such strains and suggests that they probably rather developed as a consequence of spread of *stx* genes *via stx*-encoding bacteriophages.

While no information is presently available about the clinical significance of STEC harboring *stx*$_{1d}$, infections by STEC harboring *stx*$_{1c}$ are usually asymptomatic or manifest as mild diarrhea [16, 18]. In contrast, infections by *stx*$_1$-containing STEC can be complicated by HUS [16, 32]. Therefore, a rapid differentiation of *stx*$_1$ from its variants using the approach developed in this study has two potential practical implications. First, it allows a clinician to predict a risk of a disease development in a patient who presents with STEC infection. Second, it forms a basis for the risk assessment analysis for STEC strains isolated from foods. Taking into account the previously reported clinical significance of the differentiation between *stx*$_2$ and its variants [30], a multiplex PCR combining the approach developed in this study with that published earlier for the discrimination of *stx*$_2$ alleles [25] would provide a rapid screening procedure for stool and food samples in routine laboratories.

Altogether, our data demonstrate that a single-step real-time fluorescence PCR provides a rapid and reliable approach to identify STEC harboring different *stx*$_1$ alleles and to distinguish classical *stx*$_1$ from its variants. Therefore, it has potential implications for the risk assessment analysis in the food microbiology.

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