

# Real-Time Multiplex PCR for Detecting Shiga Toxin 2-Producing *Escherichia coli* O104:H4 in Human Stools

Wenlan Zhang,<sup>a</sup> Martina Bielaszewska,<sup>a</sup> Andreas Bauwens,<sup>a</sup> Angelika Fruth,<sup>b</sup> Alexander Mellmann,<sup>a,c</sup> and Helge Karch<sup>a,c</sup>

Institute for Hygiene and the National Consulting Laboratory for Hemolytic Uremic Syndrome, University of Münster, Münster, Germany<sup>a</sup>; National Reference Center for Salmonella and Other Bacterial Enteric Pathogens, Robert Koch Institute, Wernigerode, Germany<sup>b</sup>; and Interdisciplinary Center of Clinical Research (IZKF), University of Münster, Münster, Germany<sup>c</sup>

**A real-time multiplex PCR targeting *stx*<sub>2</sub>, *wzy*<sub>O104</sub>, and *fliC*<sub>H4</sub> of enterohemorrhagic *Escherichia coli* (EHEC) O104:H4 correctly determined the presence or absence of these genes in 253 EHEC isolates and enrichment cultures of stool samples from 132 patients. It is a rapid, sensitive, and specific tool for detecting EHEC O104:H4 in human stools.**

A large outbreak caused by Shiga toxin 2 (Stx<sub>2</sub>)-producing enterohemorrhagic *Escherichia coli* (EHEC) O104:H4 that involved >4,000 cases, including 855 patients with hemolytic-uremic syndrome (HUS) and 53 fatalities, occurred in Germany in May to July 2011 (6, 14). Early in the outbreak, we and others developed PCRs targeting typical molecular features of the outbreak strain, including the *stx*<sub>2</sub> gene and the *wzy*<sub>O104</sub> and *fliC*<sub>H4</sub> genes, components of the O104 and H4 antigen synthetic gene clusters, respectively (3), or *stx*<sub>2</sub> plus *aggC* and *aggD*, components of the gene cluster encoding aggregative adherence fimbria I (13). Here, we extended this approach by developing a real-time multiplex PCR (rtMPCR) and evaluating its utility for the detection of EHEC O104:H4 in human stools.

The rtMPCR was performed in a CFX96 real-time PCR system (Bio-Rad, Munich, Germany) in a 20- $\mu$ l volume containing 10  $\mu$ l of 2 $\times$  Fast EvaGreen SuperMix (Bio-Rad), each primer in a concentration optimized (Table 1) to obtain melting peaks of similar intensities, and 1  $\mu$ l (~30 ng) of genomic DNA extracted using InstaGene Matrix (Bio-Rad). The cycling protocol included initial denaturation at 98°C for 2 min, as recommended by the manufacturer, followed by 35 cycles of denaturation (98°C, 10 s), annealing (55°C, 10 s), and extension (72°C, 20 s). Fluorescence was recorded at the end of each extension. Melting curves were generated from 65°C to 95°C with increments of 0.2°C/s. Results were analyzed using Bio-Rad CFX Manager version 1.6. Positive (EHEC O104:H4), negative (*E. coli* K-12 C600), and no-template (distilled water) controls were included in each run.

A total of 253 EHEC isolates, including 91 O104:H4 outbreak isolates, 42 strains of the HUSEC (HUS-associated *E. coli*) collection (10) ([www.ehec.org](http://www.ehec.org)), which includes prototypic strains of all EHEC serotypes associated with HUS in Germany (see Table S1 in the supplemental material), and 120 EHEC strains that represent the whole spectrum of diarrhea-associated EHEC serotypes present in our collections (see Table S2 in the supplemental material) were tested to determine the specificity of the rtMPCR. Serotyping, *stx* typing, and multilocus sequence typing (MLST) of the strains were performed as described previously (2, 4, 10, 12). All 91 EHEC O104:H4 outbreak isolates produced all three amplicons, with melting peaks at 75.2°C for *wzy*<sub>O104</sub>, 80.2°C for *stx*<sub>2</sub>, and 83.6°C for *fliC*<sub>H4</sub> (Fig. 1). In the HUSEC collection, these three peaks were produced only by strain HUSEC041 (*stx*<sub>2</sub>-harboring EHEC O104:H4) (Fig. 1; see also Table S1 in the supplemental material). Another strain, HUSEC037 (*stx*<sub>2</sub>-harboring EHEC

O104:H21), yielded amplicons for *wzy*<sub>O104</sub> and *stx*<sub>2</sub> but not for *fliC*<sub>H4</sub> (Fig. 1; see also Table S1 in the supplemental material). In addition, strain HUSEC038, which belongs to serotype Ont (non-typeable O antigen):H21 and to the same multilocus sequence type (ST672) as HUSEC037, also produced the *wzy*<sub>O104</sub> but not the *fliC*<sub>H4</sub> amplicon (see Table S1 in the supplemental material). The specificity of the *wzy*<sub>O104</sub> amplification in HUSEC038 was confirmed by partial (627-bp) sequencing of the *gnd* locus, which is adjacent to the O104 antigen-encoding cluster and is serogroup specific (7). The partial *gnd* sequences of HUSEC038 and HUSEC037 were 100% identical to that of HUSEC041, demonstrating that HUSEC038 indeed belongs to serogroup O104. Among the 120 EHEC strains associated with diarrhea, the rtMPCR amplified *wzy*<sub>O104</sub> in the strain of serotype O104:H21 and *fliC*<sub>H4</sub> in all strains with the H4 antigen (serotypes O8:H4, O68:H4, O113:H4, and O119:H4) as well as in two nonmotile strains (O74:H<sup>-</sup> and O78:H<sup>-</sup>), each of which contained *fliC*<sub>H4</sub>, as demonstrated by *fliC* restriction fragment length polymorphism analysis (19) (see Table S2 in the supplemental material). In all HUSEC strains and the diarrhea-associated EHEC strains, the *stx*<sub>2</sub> primer pair detected *stx*<sub>2</sub> and its variants *stx*<sub>2c</sub>, *stx*<sub>2d</sub>, *stx*<sub>2dactivatable</sub>, and *stx*<sub>2e</sub> but not *stx*<sub>2f</sub> (see Tables S1 and S2 in the supplemental material), which has a low degree of sequence homology to *stx*<sub>2</sub> (15); however, that allele is only rarely present in human clinical EHEC (16).

To determine the utility of the rtMPCR for the detection of EHEC O104:H4 in human stools, we applied the test to 132 stool samples from patients with HUS or diarrhea, which were selected from 1,207 stool samples received in our laboratory between 23 May and 31 July 2011; 412 of these stools were positive for the outbreak strain, as determined by culture. For the rtMPCR, every 5th culture-positive sample and every 15th culture-negative stool sample were used. To prepare PCR templates, the stool samples

Received 27 December 2011 Returned for modification 24 January 2012

Accepted 6 February 2012

Published ahead of print 15 February 2012

Address correspondence to Helge Karch, [hkarch@uni-muenster.de](mailto:hkarch@uni-muenster.de).

Supplemental material for this article may be found at <http://jcm.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.06817-11

**TABLE 1** Primers for real-time multiplex PCR to identify *stx*<sub>2</sub>-harboring *E. coli* O104:H4

Primer <sup>a</sup>	Sequence (5'–3')	Concn (nM) per reaction <sup>b</sup>	Target	Amplicon size (bp)
O104wzy-f	GGTTATGTTCTTGTCTTTFGC	225	<i>wzy</i> <sub>O104</sub>	154
O104wzy-r	CTAATACTTGTCTGATACGG			
RT-stx2F	CGACCCCTCTTGAACATA	100	<i>stx</i> <sub>2</sub>	106
RT-stx2R	TAGACATCAAGCCCTCGTAT			
fliCH4-a	GGCGAAACTGACGGCTGCTG	75	<i>fliC</i> <sub>H4</sub>	201
fliCH4-b	GCACCAACAGTTACCGCCGC			

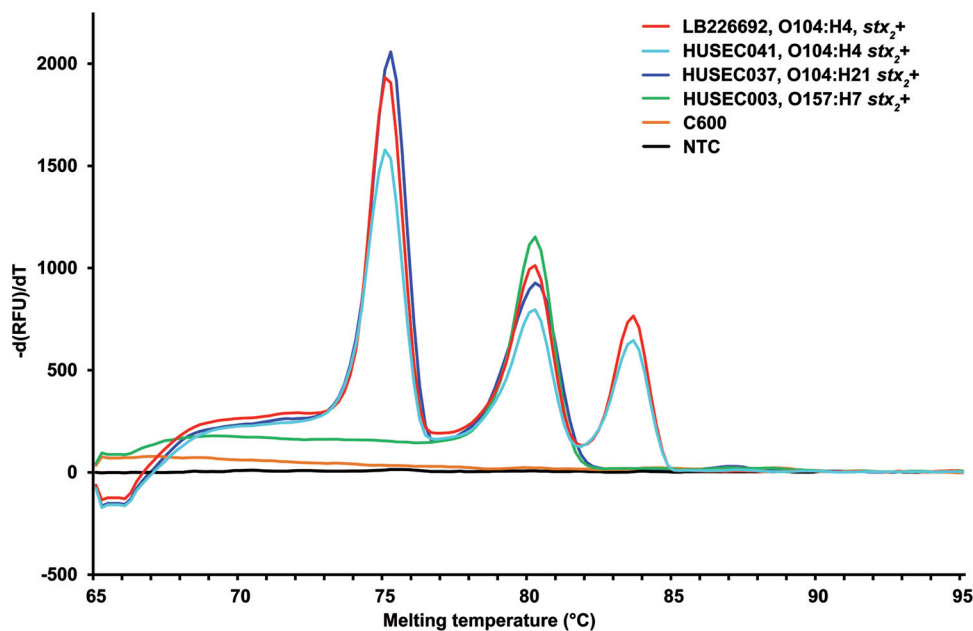
<sup>a</sup> Primers for *wzy*<sub>O104</sub> and *stx*<sub>2</sub> were designed in this study. Primers for *fliC*<sub>H4</sub> were described previously (3).

<sup>b</sup> The primer concentrations were optimized to obtain melting peaks of similar intensities.

were enriched for 4 h in GN broth Hajna (Difco Laboratories, Detroit, MI) and a 100- $\mu$ l volume was plated on individual plates of sorbitol MacConkey agar (Becton Dickinson, Heidelberg, Germany), enterohemolysin agar (Sifin, Berlin, Germany), and extended-spectrum  $\beta$ -lactamase agar (chromID ESBL; bioMérieux, Nürtingen, Germany). The overnight growth from the plates was washed into 1 ml of 0.9% NaCl solution and boiled for 10 min; 1  $\mu$ l of the total extracted DNA (diluted 1:10 in sterile water) was used per 20  $\mu$ l of rtMPCR volume. Eighty-three (62.9%) of the 132 stool enrichment cultures contained the EHEC O104:H4 outbreak strain, as demonstrated by conventional multiplex PCR (3) and subsequent isolation of the strain, which was used as the gold standard to which results of the rtMPCR were compared. Each of the 83 cultures produced all three amplicons (*wzy*<sub>O104</sub>, *stx*<sub>2</sub>, and *fliC*<sub>H4</sub>) in the rtMPCR. The remaining 49 enrichment cultures lacked the outbreak strain in both the conventional multiplex PCR and culture on ESBL agar. Thirty-eight of them yielded none of the three amplicons, whereas 11 yielded the *stx*<sub>2</sub> amplicon only;

various *stx*<sub>2</sub>-positive non-O104:H4 *E. coli* strains were subsequently isolated from these 11 samples. The detection limit of the rtMPCR for identification of EHEC O104:H4 in stool cultures was determined by spiking three different human O104:H4-negative stools enriched for 4 h in GN broth Hajna with 10-fold dilutions ( $10^1$  to  $10^{10}$  CFU/ml) of EHEC O104:H4 outbreak strain LB226692 (3, 11), growing 100  $\mu$ l of the mixtures on ESBL agar and Luria-Bertani agar plates at 37°C overnight, extracting total DNA from bacteria washed from the plates by boiling for 10 min, and using 1  $\mu$ l of the DNA in rtMPCR. The detection limit of the rtMPCR was  $7 \times 10^3$  (range,  $1 \times 10^3$  to  $1 \times 10^4$ ) CFU/ml of EHEC O104:H4 on the background of  $4.2 \times 10^7$  (range,  $7 \times 10^6$  to  $6 \times 10^7$ ) CFU/ml of normal coliform intestinal flora. The detection limit of the test for EHEC O104:H4 strain LB226692 in pure culture was  $1.6 \times 10^2$  CFU/ml.

The rtMPCR developed here has 100% specificity and 100% sensitivity for the detection of EHEC O104:H4 in human stool samples compared to culture (i.e., isolation of the strain) and for identification of EHEC O104:H4 isolates compared to serotyping. Although the EHEC O104:H4 outbreak is over, this assay can be utilized in diagnostic laboratories in Germany, in particular, in those specialized for detection of EHEC, because sporadic cases of infection with the outbreak strain still rarely occur in this country (our unpublished data). Also, the rtMPCR represents a rapid and reliable tool for epidemiological studies to determine the prevalence of EHEC O104:H4 in the human population, which is considered the major (if not the only) reservoir of this pathogen (1). Moreover, because the rtMPCR detects *stx*<sub>2</sub> and its variants present in HUS-associated as well as diarrhea-associated EHEC (see Tables S1 and S2 in the supplemental material), it will also detect non-O104:H4 EHEC causing human disease. Thus, stool samples positive only for *stx*<sub>2</sub> in the rtMPCR, as was the case for the sam-



**FIG 1** Real-time multiplex PCR for the detection and identification of EHEC O104:H4. Data represent amplification of *wzy*<sub>O104</sub>, *stx*<sub>2</sub>, and *fliC*<sub>H4</sub> in prototypic EHEC O104:H4 outbreak isolate LB226692 (3, 11), HUSEC041 (O104:H4), HUSEC037 (O104:H21), and HUSEC003 (O157:H7) by the use of the real-time MPCR. Melting peaks of the *wzy*<sub>O104</sub>, *stx*<sub>2</sub>, and *fliC*<sub>H4</sub> amplicons at 75.2°C, 80.2°C, and 83.6°C, respectively, are shown. C600, *E. coli* K-12 C600; NTC, no-template control.

ples from the 11 patients described above, need to be further investigated for EHEC of other serotypes in order to detect both known and possibly new, emerging EHEC strains. Preliminary information about the presence of an EHEC strain in the stool within 24 h, as provided by the rtMPCR, is critical for epidemiological purposes, in particular, for “real-time” monitoring of spread of the infection and tracing it back to the source. From the therapeutic standpoint, rapid detection of evidence of EHEC O104:H4 infection may provide a basis for applying, in addition to general therapeutic strategies used for EHEC infections (increasing the volume of intravenous fluids and avoiding antibiotic administration) (9, 17), additional, more specialized approaches such as were successfully used during the EHEC O104:H4 outbreak (8). The method described here extends the real-time multiplex PCRs available for detecting EHEC O104:H4 in food (5, 18) for the first time to rapid detection of the strain in human stools. rtMPCRs for detecting other members of the HUSEC collection are under development.

#### ACKNOWLEDGMENTS

This study was supported by grants from the Interdisciplinary Center of Clinical Research (IZKF) Münster (Me2/021/12) and the Medical Faculty of the University of Münster (BD9817044).

We thank Ralph Fischer and Andrea Lagemann for technical assistance.

#### REFERENCES

1. Beutin L, Martin A. 2012. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *J. Food Prot.* 75:408–418.
2. Bielaszewska M, Friedrich AW, Aldick T, Schürk-Bulgrin R, Karch H. 2006. Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. *Clin. Infect. Dis.* 43:1160–1167.
3. Bielaszewska M, et al. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect. Dis.* 11:671–676.
4. Bielaszewska M, et al. 2009. Shiga toxin, cytolethal distending toxin, and hemolysin repertoires in clinical *Escherichia coli* O91 isolates. *J. Clin. Microbiol.* 47:2061–2066.
5. EUReference Laboratory for *E. coli* (EU-RL VTEC). 2 June 2011, posting date. Detection and identification of verocytotoxin-producing *Escherichia coli* (VTEC) O104:H4 in food by real time PCR. Istituto Superiore di Sanità, Rome, Italy. [http://www.iss.it/binary/vtec/cont/Lab\\_Proc\\_VTEC\\_O104.pdf](http://www.iss.it/binary/vtec/cont/Lab_Proc_VTEC_O104.pdf).
6. Frank C, et al. 2011. Epidemic profile of Shiga toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *N. Engl. J. Med.* 19:1771–1780.
7. Gilmour MW, Olson AB, Andrysiak AK, Ng L, Chui L. 2007. Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing *Escherichia coli* serogroup lineages. *J. Med. Microbiol.* 56:620–628.
8. Greinacher A, et al. 2011. Treatment of severe neurological deficits with IgG depletion through immunoabsorption in patients with *Escherichia coli* O104:H4-associated haemolytic uraemic syndrome: a prospective trial. *Lancet* 378:1166–1173.
9. Hickey CA, et al. 2011. Early volume expansion during diarrhea and relative nephroprotection during subsequent hemolytic uraemic syndrome. *Arch. Pediatr. Adolesc. Med.* 165:884–889.
10. Mellmann A, et al. 2008. Analysis of collection of hemolytic uraemic syndrome-associated enterohemorrhagic *Escherichia coli*. *Emerg. Infect. Dis.* 14:1287–1290.
11. Mellmann A, et al. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS One* 6:e22751.
12. Prager R, Strutz U, Fruth A, Tschäpe H. 2003. Subtyping of pathogenic *Escherichia coli* strains using flagellar H-antigens: serotyping versus *fliC* polymorphisms. *Int. J. Med. Microbiol.* 292:477–486.
13. Qin J, et al. 2011. Identification of the Shiga toxin-producing *Escherichia coli* O104:H4 strain responsible for a food poisoning outbreak in Germany by PCR. *J. Clin. Microbiol.* 49:3439–3440.
14. Robert Koch Institute. 2011. Report: final presentation and evaluation of epidemiological findings in the EHEC O104:H4 outbreak, Germany 2011. Robert Koch Institute, Wernigerode, Germany. [http://www.rki.de/clin\\_226/nn\\_217400/EN/Home/EHEC\\_final\\_report,templateId=raw,property=publicationFile.pdf/EHEC\\_final\\_report.pdf](http://www.rki.de/clin_226/nn_217400/EN/Home/EHEC_final_report,templateId=raw,property=publicationFile.pdf/EHEC_final_report.pdf).
15. Schmidt H, et al. 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl. Environ. Microbiol.* 66:1205–1208.
16. Sonntag AK, Zenner E, Karch H, Bielaszewska M. 2005. Pigeons as a possible reservoir of Shiga toxin 2f-producing *Escherichia coli* pathogenic to humans. *Berl. Munch. Tierarztl. Wochenschr.* 118:464–470.
17. Tarr PI, Gordon CA, Chandler WL. 2005. Shiga toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 365:1073–1086.
18. Tzschoppe M, Martin A, Beutin L. 2012. A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. *Int. J. Food Microbiol.* 152:19–30.
19. Zhang W, et al. 2007. Structural and functional differences between disease-associated genes of enterohaemorrhagic *Escherichia coli* O111. *Int. J. Med. Microbiol.* 297:17–26.