MOLECULAR DIFFERENTIATION OF SHIGELLA SPP. FROM ENTEROINVASIVE E. COLI

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A real-time polymerase chain reaction (PCR) assay, amplifying the genes encoding lactose permease (lacY) and invasion plasmid antigen H (ipaH), was run on 121 isolates phenotypically classified as Shigella spp., enteroinvasive Escherichia coli (EIEC), or EIEC O nontypable (ONT). The results were compared with data from a generic E. coli multiple-locus variable-number of tandem repeat analysis (MLVA) and a Shigella MLVA.

The real-time PCR verified all Shigella spp. (n = 53) as Shigella (lacY negative) and all EIEC O121 (n = 15) and EIEC O124 (n = 2) as EIEC (lacY positive). However, the real-time PCR typed EIEC O164 as either EIEC (n = 2) or Shigella (n = 2) and, thus, was not suited for classifying this group of isolates. Interestingly, the majority (42/47, 89.4%) of the EIEC ONT were classified as Shigella (lacY negative) by the real-time PCR, and in nearly all cases, (92.9%, 39/42) data from both MLVA assays supported these findings. Overall, in 94.7% (114/121) of the isolates, the results from the real-time PCR were substantiated by the results from the MLVA assays.

In conclusion, the real-time PCR assay was fast and accurate in differentiating Shigella spp. from EIEC, with the exception of the EIEC O164 group. This molecular assay was particularly pragmatic for the challenging EIEC ONT group.

Keywords: EIEC, Shigella, real-time PCR, molecular differentiation, MLVA

Abbreviations: EIEC, enteroinvasive E. coli; ipaH, invasive plasmid antigen H; lacY, lactose permease; MLVA, multiple-locus variable-number of tandem repeat analysis; ONT, O nontypable; spp., species; Stx, shiga toxin

Introduction

Shigella is a gram-negative, lactose-negative, facultative intracellular pathogen, closely related to Escherichia coli (E. coli). It was recognized as the etiologic agent of bacillary dysentery or shigellosis in the 1890s, and in the 1950s, Shigella was adopted as a genus and subgrouped into four species (spp.): Shigella dysenteriae, Shigella flexnerii, Shigella boydii, and Shigella sonnei [1]. Shigellosis remains a major cause of morbidity and mortality among children in developing countries, in which S. flexneri is the dominating species. These bacteria are also important causes of morbidity in the industrialized part of the world where S. sonnei is the most common [2]. Shiga toxins (Stx) carrying S. dysenteriae serotype 1 and, to a lesser extent, S. flexneri, are the Shigella spp. responsible for most severe diseases. Recently, Stx2, the Stx subtype associated with hemolytic uremic syndrome in patients infected with Stx-producing E. coli (STEC), was described in an S. sonnei isolate [3]. Shigella infection spreads by the fecal–oral route, and the infectious dose is low [4]. Rapid identification of Shigella spp. is thus important for outbreak control purposes. In Norway, shigellosis is a rare disease, with 100–200 cases annually. S. sonnei is the dominating species, and the majority of the cases are infected abroad (http://www.msis.no/). However, some domestic outbreaks of shigellosis have been detected in Norway, mainly associated with imported vegetables, meat, or herbs [5–8].

In the 1970s, the first invasive strains of E. coli causing Shigella-like dysentery were described [9]. Thereafter,
several studies have shown that *Shigella* spp. and enteroinvasive *E. coli* (EIEC) form a single pathovar of *E. coli* [10–13]. In spite of this, discrimination between *Shigella* spp. and EIEC is essential due to clinical differences and also for epidemiological purposes [14]. However, the close relatedness between *Shigella* spp. and EIEC makes the distinction difficult if based on biochemical, serological, or molecular characteristics [11]. Most *Shigella* spp. are lactose negative, whereas EIEC isolates display variable ability to utilize lactose. It has been suggested that *Shigella* spp. lack the lactose permease gene (*lacY*), one of three genes constituting the *lac* operon important for lactose fermentation, or carry a *lacY* pseudogene. On the other hand, EIEC, as do all *E. coli*, harbor this particular gene [10, 14, 15]. Even though various molecular methods developed in the past few years presumably allow differentiation between *Shigella* spp. and EIEC, the discrimination between the two still represents a challenge [4, 16–19]. Therefore, in the present study, we aimed at establishing a rapid and reliable duplex real-time polymerase chain reaction (PCR) able to differentiate *Shigella* spp. from EIEC based on the presence or absence of *lacY*. Second, we wanted to substantiate these results by comparing them with genotyping data from two multiple-locus variable-number of tandem repeat analysis (MLVA) assays: one designed for *E. coli* and one for *Shigella* spp.

**Materials and methods**

**Phenotypical characterization and *E. coli* pathotype PCR**

Clinical microbiology laboratories throughout Norway mandatory forwarded presumptive *Shigella* and entero-pathogenic *E. coli* isolated from stool specimens to the National Reference Laboratory for Enteropathogenic Bacteria at the Norwegian Institute of Public Health (NIPH). At NIPH, the received isolates were routinely subjected to a broad panel of single tube biochemical tests, and the results were evaluated according to established criteria [20]. Based on the biochemical findings, the isolates were tested for agglutination with either polyvalent anti-*S. flexneri*, anti-*Shigella* II and III (Sinf Diagnostics, Berlin, Germany), and anti-*S. boydii* 14–18 (Difco by Becton and Dickinson, Franklin Lakes, New Jersey), or polyvalent *E. coli* antisera, Anti-Coli I, II, and III (Sinf Diagnostics, Berlin, Germany). Positive agglutination in a polyvalent antiserum was followed by agglutination in the relevant monovalent antiserum (either Sinf or from noncommercial production at NIPH). Isolates not clearly defined as either *Shigella* spp. or EIEC by phenotypic typing were denoted EIEC O nontypable (ONT). Presumptive *E. coli* isolates were classified into well-known pathotypes by running a multiplex PCR including, among other genes, *ipaH* [21].

### Table 1. Bacterial isolates examined and results achieved using the duplex real-time PCR

| Pathogen               | Pathotype* | Serotype                        | No. analyzed | lacY+ | ipaH+ | EIEC (%) | Shigella (%) |
|------------------------|------------|---------------------------------|--------------|-------|-------|----------|-------------|
| *E. coli*              | EIEC       | ONT†                            | 47           | 5     | 47    | 5 (10.6%)| 42 (89.4%)  |
|                        |            | O121                            | 15           |       |       | 15 (100%)| 0 (0%)     |
|                        |            | O124                            | 2            | 2     | 2     | 2 (100%) | 0 (0%)     |
|                        |            | O164                            | 4            | 2     | 4     | 2 (50%)  | 2 (50%)    |
| STEC                   |            | O103:H2, O26:H11                 | 2            | 2     | 0     | 0 (0%)  | 0 (0%)     |
| aEPEC                  |            | O104:H4, ONT                     | 2            | 2     | 0     | 0 (0%)  | 0 (0%)     |
| EAEC                   |            | O6, ONT (2)                      | 3            | 3     | 0     | 0 (0%)  | 0 (0%)     |
| ETEC                   |            | Non-enteropathogenic             | 1            | 1     | 0     | 0 (0%)  | 0 (0%)     |
| *Shigella* spp.        |            |                                 |              |       |       |          |             |
| *S. sonnei*            |            | –                               | 13           | 0     | 13    | 0 (0%)  | 13 (100%)  |
| *S. flexneri*          |            |                                 | 15           | 0     | 15    | 0 (0%)  | 15 (100%)  |
| *S. dysenteriae*       |            |                                 | 13           | 0     | 13    | 0 (0%)  | 13 (100%)  |
| *S. boydii*            |            |                                 | 12           | 0     | 12    | 0 (0%)  | 12 (100%)  |
| *Salmonella enterica*  |            |                                 |              |       |       |          |             |
| *S. Typhimurium*       |            |                                 | 4, 5, 12:i:1,2| 1     | 0     | 0 (0%)  | 0 (0%)     |
| *S. Kedougou*          |            |                                 |              |       |       |          |             |
| *Yersinia* spp.        |            |                                 |              |       |       |          |             |
| *Y. enterocolitica*    |            |                                 | O:3          | 1     | 0     | 0 (0%)  | 0 (0%)     |
|                        |            |                                 | O:9          | 1     | 0     | 0 (0%)  | 0 (0%)     |

*The pathotype was phenotypically determined for *Shigella* spp., *Salmonella* spp., and *Yersinia* spp.; however, for *E. coli*, the pathotype was determined running an 11-plex PCR [21]

†ONT: O nontypable
Bacterial isolates

A total of 121 isolates from 121 patients infected within the period 2006 to 2014 were obtained from the national strain collection at NIPH. The selection was based on phenotypical findings and comprised 33 Shigella spp. (13 S. sonnei, 15 S. flexneri, 12 S. boydii, and 13 S. dysenteriae), 21 EIEC of known serotype (15 O121, four O164, and two O124), and 47 EIEC ONT. All isolates, except two S. sonnei and two S. dysenteriae serotype 2, were sporadic cases. To ensure the specificity of the real-time PCR method, the following strains were added: STEC (n = 2), enteropathogenic E. coli (EPEC) (n = 2), enteroaggregative E. coli (EAEC) (n = 2), enterotoxigenic E. coli (ETEC) (n = 3), non-diarrhea/commensal E. coli (n = 1), Salmonella Typhimurium (n = 1), Salmonella Kedougou (n = 1), and Yersinia enterocolitica (serogroups 3 and 9, respectively) (n = 2) (Table 1).

Growth conditions and extraction of DNA

All isolates were recultivated from stabbing agar on nutrient broth agar at 37 °C overnight. Suspensions of bacterial cells were boiled for 15 min and used directly as template in the real-time PCR after a brief 3 min centrifugation at 13,000 rpm.

Primer and probe design

Two primer-probe sets were used in the duplex real-time PCR (Table 2). The primer set for lacY was modified from Pavlovic et al., 2011 [19], whereas the primer set for the internal amplification control, ipaH, was adapted from Barletta et al., 2013 [22], with minor modifications. The probes for lacY [19] were modified to minor groove binder (MGB) format, and an MGB probe for ipaH was designed using PrimerExpress 3.0 (LifeTechnologies). To check the specificity of both primer pairs and the probes, a BLAST search on NCBI was performed.

Conventional PCR and sequencing

Two conventional PCRs, including either the lacY or the IpaH primer set, were conducted to verify the expected PCR product size and to check the specificity of each primer set. EIEC O121 (lacY and ipaH positive) and S. dysenteriae (lacY negative, but ipaH positive) were used as positive controls in each run. PCR was performed using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany), as described by the manufacturer. The PCRs were run in a GeneAmp 9700 machine (Life Technologies, Carlsbad, California, USA) with a temperature profile as indicated for the Qiagen Multiplex PCR kit and an annealing temperature of 58 °C. PCR products were diluted 1:10 prior to capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). DNA 1000 LabChip kit series II was prepared and loaded with samples as recommended by the manufacturer (Agilent Technologies, Santa Clara, California, USA). The specificity of each primer pair was verified by direct sequencing of the PCR product of the positive control.

Real-time PCR, efficiencies and detection limits

For each primer-probe set, a 20× primer-probe mix was prepared with a final concentration of 2.5 μM of the respective primers and probes. Each reaction mix consisted of 10 μl 2× QuantiTect Multiplex RT-PCR Rox Mastermix (Qiagen, Hilden, Germany), 1 μl of 20× primer-probe mix for ipaH and/or lacY, 4 μl template DNA diluted 1:10, 1 μl of 2× QuantiTect Multiplex RT-PCR Rox Mastermix (Qiagen, Hilden, Germany), 1 μl of 20× primer-probe mix for lacY, and 3 μl of 2× QuantiTect Multiplex RT-PCR Rox Mastermix (Qiagen, Hilden, Germany), 1 μl of 20× primer-probe mix for ipaH and/or lacY, 4 μl template DNA diluted 1:10.

Table 2. ipaH and lacY primers and probes used in the present study

| Gene | Primer or probe | Sequence (5′–3′) | Melting point (°C) | PCR product (bp) | Fluorochrome (5′ end) | Reference |
|------|----------------|------------------|-------------------|-----------------|----------------------|-----------|
| lacY | lacY-F         | ACCAGACCCCGCACCCAGATAAG | 59 | 104 | FAM | [19] |
|      | lacY-R         | TTCTGCTTTTATAGCAACTTGCC | 59.8 | | | Modified from [19] |
|      | lacY-MGB-p1    | CATACTATTGCCCGAGTAGA | 70 | | FAM | Modified from [19] |
|      | lacY-MGB-p2    | CATACTATGGAGCCAGAAG | 70 | | FAM | Modified from [19] |
| ipaH | ipaH-F         | GACGGACAACAGAATACACTCCATC | 59.8 | 108 | | Modified from [22] |
|      | ipaH-R         | ATGGTTCAAAAGCATGCCATATCTG | 59.8 | | VIC | Modified from [22] |
|      | ipaH-MGB-p     | CCGAAGAACCAAAATCTGAGT | 69 | | | |

*All probes were conjugated with minor groove binder (MGB) and had a “Black Hole Quencher” at the 3′ end

*Due to sequence variation in the lacY gene of certain EIEC strains, two different lacY probes were used to detect all EIEC strains [19]
and sterile PCR grade water (Qiagen, Hilden, Germany) to bring the final volume to 20 μl. Real-time PCR was run in a StepOnePlus machine (Life Technologies, Carlsbad, California, USA) with the following PCR program: initial activation step of 15 min at 95 °C followed by 30 cycles of denaturation for 60 s at 94 °C and annealing/extension for 60 s at 58 °C. DNA from EIEC O121 was used as template, and a dilution series ranging from 50 ng/μl to 0.5 pg/μl was measured. Triplicates of the dilution series were run, and PCR efficiencies were calculated as described previously [23].

**MLVA typing**

All 121 isolates were examined by a 10-loci E. coli generic MLVA assay (GECM10) as described by Løbersli et al. [24] and an MLVA specific for *Shigella* spp. as described by Rawal et al. [8].

### Table 3. *E. coli* MLVA and *Shigella* MLVA profiles in concordance with the duplex real-time PCR results

| E. coli MLVA group | Pathotype | Serotype | No. analyzed | Duplex real-time PCR | Shigella MLVA group |
|-------------------|-----------|----------|-------------|----------------------|---------------------|
| I                 | *S. boydii* | 18       | 1           | Shigella             | A                   |
|                   | *S. dysenteriae* | 3, 4, and 9 | 6       | Shigella             | A/B                  |
|                   | EIEC      | ONT      | 7           | Shigella             | A                   |
| II                | *S. boydii* | 2, 4, 8, 10, 14, and 16 | 12     | *Shigella*           | B                   |
|                   | *S. dysenteriae* | 7       | 1           | Shigella             | B                   |
|                   | *S. flexneri* | 6       | 3           | Shigella             | B                   |
|                   | EIEC      | ONT      | 27          | Shigella             | B                   |
| III               | EIEC      | ONT      | 3           | *Shigella*           | C (n = 1)/D          |
|                   |           |          |             | EIEC                 | C                   |
|                   |           |          |             | EIEC                 | C                   |
| IV                | *S. flexneri* | 1, 2, 3, 4, and x variant | 11     | *Shigella*           | A/E                  |
|                   | *S. sonnei* | –        | 13          | Shigella             | G                   |
|                   | EIEC      | O164     | 1           | *Shigella*           | C                   |
|                   |           | O164     | 2           | EIEC                 | C                   |
|                   |           | ONT      | 2           | Shigella             | E                   |
| V                 | EIEC      | O164     | 1           | *Shigella*           | C                   |
|                   |           | ONT      | 4           | EIEC                 | C/G (n = 1)          |
| VI                | *S. dysenteriae* | 2       | 5           | Shigella             | F                   |
|                   | EIEC      | ONT      | 3           | Shigella             | F                   |
| Other MLVA profiles not seen in EIEC | *S. dysenteriae* | 1 | 1 | Shigella | G |
|                   | *S. flexneri* | 4       | 1           | Shigella             | B                   |

*Six main groups of *E. coli* MLVA profiles are defined; each group was given a Roman numeral (I–VI). Within each group, different copy number profiles are seen: I, 4-NA-NA-X-NA-X-2-NA-NA; II, 4-2-NA-X-X-X-2-NA-NA; III, 5-2-NA-X-X-X-X-NA; IV, 6-2-NA-X-X-X-X-NA; and VI, 11-2-NA-9-X-X-5-2-NA-NA. The repeat number of each allele is designated as suggested by ref. [24]; however, absence of PCR product is designated with NA instead of a negative number (−2). X assign the presence of a PCR product; however, different allele numbers of the specific locus exist.*

*The pathotype was phenotypically determined for *Shigella* spp.; however, for *E. coli*, the pathotype was determined running an 11-plex PCR [21].

*The MLVA group for *Shigella* spp. is designated by letters (A–F). Seven different MLVA groups were defined: A, X-X-0-5-4-0-0; B, X-5-0-X-0-0; C, X-5-5-4-0-0; D, 5-X-5-5-0-0; E, X-X-0-5-5-0-0; F, X-X-5-5-3-0-0; and G, X-5-5-3-0-0. The allele number of each locus is designated as suggested by ref. [8]. Within each letter variation of MLVA, profiles exist, but each letter has from four to five identical loci. X assigns the presence of a PCR product; however, different allele numbers of the specific locus exist. Absence of PCR product is designated zero (0).*

*ONT: O nontypable

*Bold indicate isolates (7/121, 5.8%) showing disagreement between the real-time PCR method and one or both MLVA assays. In total, 94.2% (114/121) of the strains showed concordance when comparing these molecular methods. 
Results

Duplex real-time PCR: efficiencies, detection limits, sensitivity, and specificity

The NCBI BLAST search confirmed that the lacY primers were absent in published sequences of Shigella spp. but present in E. coli. The ipaH primers were exclusively seen in Shigella spp. and EIEC. By conventional PCR, both PCR products showed expected base pair sizes and no scatter bands were observed. Sequencing of the PCR products confirmed the correct sequences (data not shown). The PCR efficiencies for lacY primer-probes were 106.3% in singleplex PCR and 93.1% in duplex PCR, whereas the values for ipaH primer-probe were 109.4% and 90.4%, respectively. The detection limit for both genes was 5 pg/μl. All E. coli isolates, except the majority of the EIEC ONT group and two EIEC O164 isolates, were positive for lacY. On the other hand, the Shigella spp., Salmonella spp., and Yersinia spp. were all negative for this specific gene (Table 1). As expected, ipaH was detected in all EIEC and Shigella spp. isolates, but in no other pathogens. Thus, the duplex real-time PCR had a high sensitivity and specificity.

Evaluating the duplex real-time PCR with other typing methods

A 100% (53/53) concordance between phenotypic typing and the duplex real-time PCR was seen for all Shigella spp. isolates (Table 1). Similar results were observed for...
EIEC O121 and O124 (100%, 17/17), whereas only 10.6% (5/47) of the isolates phenotypically determined as EIEC ONT were confirmed as EIEC by duplex real-time PCR. Furthermore, of the four EIEC O164 isolates, two were verified as EIEC (lacY positive) and two were identified as Shigella (lacY negative) (Fig. 1). In total, disagreement between the real-time PCR and the phenotypic typing was observed in 36.4% (44/121) of the isolates examined, and the majority of the discrepant cases was seen within the EIEC ONT group (42/44, 95.5%).

Results from generic E. coli MLVA and Shigella MLVA showed six main groups of E. coli MLVA profiles (I–VI) and seven groups of Shigella MLVA profiles (A–G) (Table 3, Fig. 2). E. coli MLVA group I included seven Shigella spp. and seven EIEC ONT isolates. All these 14 isolates were classified as Shigella (lacY negative) by the duplex real-time PCR, and they belonged to one of two Shigella MLVA groups (A and B). The second E. coli group (II) constituted 16 Shigella spp. and 27 EIEC ONT. The real-time PCR assay identified all 43 isolates as Shigella (lacY negative), and they all fell into Shigella MLVA group B (Table 3). E. coli group III included 17 EIEC with known O groups (15 EIEC O121 and two EIEC O124) and four isolates phenotypically defined as EIEC ONT. Of these, 18/21 (85.7%) were verified as EIEC (lacY positive) by real-time PCR and they belonged to Shigella MLVA group C. The three last isolates, all EIEC ONT, were classified as Shigella (lacY negative) and were assigned to one of two Shigella MLVA groups (C or D) (Table 3). Interestingly, these three latter isolates, although not unambiguously, were phenotypically typed as EIEC ONT, but agglutinated with S. boydii serotype 9 (2/3) or S. dysenteriae serotype 3. The fourth E. coli MLVA group (IV) harbored 24 Shigella spp., three EIEC O164, and two EIEC ONT isolates. All Shigella spp. were verified as Shigella (lacY negative) by real-time PCR, and they were placed in Shigella MLVA groups A, E, or G. However, only two EIEC O164 were confirmed as EIEC (lacY positive), whereas the last EIEC O164 was classified as Shigella (lacY negative). All three EIEC O164 belonged to Shigella MLVA group C. Both EIEC ONT were lacY negative and clustered within Shigella MLVA group A, supporting the real-time PCR results (Table 3). Within E. coli MLVA group V, one EIEC O164 and four EIEC ONT were defined. The four EIEC ONT were determined as EIEC (lacY positive), and all but one belonged to Shigella MLVA group C. Although clus-
tering within \textit{Shigella} MLVA group C, the EIEC O164 isolate was defined as \textit{Shigella} (lacY negative) by real-time PCR. The last \textit{E. coli} MLVA group (VI) included eight isolates, five \textit{Shigella}, and three EIEC ONT, all found as \textit{Shigella} (lacY negative) by real-time PCR and all belonging to \textit{Shigella} MLVA group F (Table 3). In conclusion, in 94.7\% (114/121) of the cases, MLVA profiles both from the generic \textit{E. coli} and \textit{Shigella} assays supported the findings achieved by duplex real-time PCR. \textit{E. coli} MLVA groups I, II, and VI, and \textit{Shigella} MLVA groups A, B, E, and F were exclusively seen in isolates defined as \textit{Shigella} (lacY negative) by the real-time PCR. On the other hand, \textit{E. coli} MLVA groups III and V, and \textit{Shigella} MLVA profile C, were associated with isolates defined as EIEC (lacY positive). Overall, a discrepancy between the real-time PCR and the MLVA assays was seen for the O164 EIEC group (n = 4) and in three EIEC ONT isolates (Table 3). Repeated biochemical analyses of the four EIEC O164 isolates showed that one of two was verified as EIEC (lacY positive) by real-time PCR fermented lactose, whereas no other biochemical differences among the isolates were revealed. All four EIEC O164 agglutinated weakly in monovalent antiserum against \textit{S. dysenteriae} serotype 3. Of the 47 EIEC ONT examined, only five were defined as EIEC (lacY positive) by real-time PCR. All five showed \textit{E. coli} MLVA profiles belonging to group III or V, and all but one clustered within \textit{Shigella} MLVA group C, supporting the finding of these isolates as EIEC (Fig. 2). Moreover, 39/42 (92.9\%) EIEC ONT defined as \textit{Shigella} (lacY negative) showed MLVA profiles associated with \textit{Shigella} spp., indicating that the real-time PCR classification was correct (Table 3 and Fig. 2).

**Discussion**

Discrimination of \textit{Shigella} spp. from EIEC has been challenging using phenotypical typing methods and molecular typing techniques [16–19, 25]. However, due to clinical differences between \textit{Shigella} spp. and EIEC and also from an epidemiological point of view, discriminating the two is essential [13, 14, 19, 26]. The lac operon, responsible for fermentation of lactose, consists of three functional genes: lacZ, lacY, and lacA. \textit{Shigella} spp. do not ferment lactose or do so slowly due to lacY deficiency or presence of a lacY pseudogene [10, 15]. Although \textit{S. sonnei} and \textit{S. dysenteriae} serotype 1 carry the lacY pseudogene [10, 15], this is not detected by our lacY primers since no match was observed during the NCBI BLAST search and no positive results were seen in the \textit{S. sonnei} and \textit{S. dysenteriae} serotype 1 isolates examined. This is in concordance with previous reports demonstrating the absence of lacY in \textit{Shigella} spp. [19, 27]. Thus, it is tempting to speculate that the structural changes at the S’ end of the lacY pseudogene described in \textit{S. sonnei} and \textit{S. dysenteriae} serotype 1 inhibited binding of the lacY primers [28]. Considering EIEC, previous studies have suggested the presence of lacY in this bacterium [19, 29]. A probe based real-time PCR assay detecting all known variants of lacY, using uidA (encoding the β-glucuronidase) as an internal amplification control, has previously been developed and shown to differentiate \textit{Shigella} spp. from EIEC [19]. In the current study, this assay was established but with some modifications. Surprisingly, 25\% (3/12) of the strains initially examined (1 EIEC O164, 1 EIEC ONT, and 1 \textit{S. boydii} serotype 13) did not amplify uidA using these uidA primers (data not shown). Thus, uidA was replaced by ipaH, a gene known to be present in all \textit{Shigella} spp. and EIEC isolates [13]. Additionally, to ensure the specificity of the lacY and ipaH probes, these were redesigned to MGB format [30]. In the study by Pavlovic et al. [19], only 11 EIEC and 18 \textit{Shigella} spp. were examined and they did not include more than two uncharacterized \textit{Shigella} spp. [19]. The latter group, defined as EIEC ONT in our study, is the most challenging and cumbersome in a phenotypical diagnostic perspective. Therefore, a molecular method rapidly classifying these isolates as either \textit{Shigella} or EIEC was sought. In the present study, as many as 47 EIEC ONT strains were examined. Interestingly, most of these strains were detected as \textit{Shigella} by the duplex real-time PCR, and the two MLVA assays supported our findings in the majority of the cases. This indicated that the real-time PCR was able to classify the challenging EIEC ONT group. However, for three EIEC ONT isolates typed as \textit{Shigella} by real-time PCR, the MLVA assays disagreed with this classification. Interestingly, these three EIEC ONT isolates agglutinated with \textit{Shigella} antisera. Nonetheless, they were phenotypically defined as EIEC due to biochemical characteristics [31]. It has been suggested that EIEC is an intermediate stage between noninvasive \textit{E. coli} and \textit{Shigella} [11, 14]. These EIEC ONT isolates might be precursors of “full-blown” \textit{Shigella} and, thus, were either classified as \textit{Shigella} or EIEC depending on the characteristics examined. Furthermore, the EIEC O164 group was not unambiguously classified molecularly, although being so by phenotypical typing. It is well known that some EIEC O antigens are identical to O antigens present in \textit{Shigella} spp., and this complicates serological differentiation [11, 14, 32]. Cross-reactivity between O-antigens from EIEC O164 and \textit{S. dysenteriae} serotype 3 has been described [32, 33], an observation also detected in our study. Therefore, based on the present knowledge, we cannot conclude on the molecular classification of the EIEC O164 group. Whole genome sequencing of the EIEC O164 strains, as well as the three EIEC ONT strains, is in progress and will hopefully help us understand the discrepancies observed.

Culture-independent assays for detecting gastrointestinal pathogens at clinical microbiological laboratories are increasingly used. These multiplex PCR assays particularly focus on ipaH and, therefore, do not distinguish \textit{Shigella} spp. from EIEC. Hence, after isolation of ipaH positive bacteria, the herein described real-time PCR will be an important supplement for fast and reliable molecular differentiation of these two entities.
Conclusion

A high correlation between the real-time PCR method, the two MLVA assays (generic E. coli MLVA and Shigella MLVA), and phenotypical typing was achieved. This indicated that the real-time PCR was well suited for discriminating Shigella spp. from EIEC and especially fruitful for the challenging EIEC ONT group. Phenotypical typing methods distinguishing Shigella spp. from EIEC are labor intensive and sometimes nonconclusive. Thus, implementing the herein described real-time PCR method is advantageous for a fast and reliable discrimination between Shigella spp. and EIEC.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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