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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,

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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 enteropathogenic *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 (Sifin 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 (Sifin Diagnostics, Berlin, Germany). Positive agglutination in a polyvalent antiserum was followed by agglutination in the relevant monovalent antiserum (either Sifin 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	Duplex real-time PCR no.			
				lacY +	*ipaH* +	EIEC (%)	*Shigella* (%)
E. coli	EIEC	ONT†	47	5	47	**5 (10.6%)**	**42 (89.4%)**
		O121	15	15	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	ONT:H11, O145:H8	2	2	0	0 (0%)	0 (0%)
	EAEC	O104:H4, ONT	2	2	0	0 (0%)	0 (0%)
	ETEC	O6, ONT (2)	3	3	0	0 (0%)	0 (0%)
	Non-enteropathogenic	–	1	1	0	0 (0%)	0 (0%)
Shigella spp.	*S. sonnei*	–	13	0	13	**0 (0%)**	**13 (100%)**
	S. flexneri	1, 2, 3, 4, 6, and x variant	15	0	15	**0 (0%)**	**15 (100%)**
	S. dysenteriae	1, 2, 3, 4, 7, and 9	13	0	13	**0 (0%)**	**13 (100%)**
	S. boydii	2, 4, 8, 10, 14, and 18	12	0	12	**0 (0%)**	**12 (100%)**
Salmonella enterica spp.	*S.* Typhimurium	4, 5, 12:i:1, 2	1	0	0	0 (0%)	0 (0%)
	S. Kedougou	–	1	0	0	0 (0%)	0 (0%)
Yersinia spp.	*Y. enterocolitica*	O:3	1	0	0	0 (0%)	0 (0%)
		O:9	1	0	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 53 *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,

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	ACCAGACCCAGCACCAGATAAG	59	104		[19]
	lacY-R	TTCTGCTTCTTTAAGCAACTGGC	58.9			Modified from [19]
	lacY-MGB-p1	CATACATATTGCCCGCCAGTA	70		FAM	Modified from [19]
	lacY-MGB-p2	CATACATATGCCCGCCAGA	70		FAM	Modified from [19]
ipaH	ipaH-F	GACGGACAACAGAATACACTCCATC	59.8	108		Modified from [22]
	ipaH-R	ATGTTCAAAAGCATGCCATATCTGT	59.8			[22]
	ipaH-MGB-p	CGGAAAACAAACAATCTGATGT	69		VIC	Modified from [22]

*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
			1	EIEC	C
	EIEC	O121 and O124	17	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-X-2-NA-NA; II, 4-2-NA-X-X-X-X-2-NA-NA; III, 5-2-NA-X-X-X-X-X-X-NA; IV, 6-NA-NA-X-X-X-X-X-X-NA; V, 6-2-NA-X-3-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 copy 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-X-0-0; C, X-5-5-5-4-0-0; D, 5-X-5-5-X-0-0; E, X-X-0-5-5-0-X; F, X-X-5-5-3-0-0; and G, X-5-X-5-4-X-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

Ethical considerations

At the NIPH, all *Shigella* spp. and EIEC strains are routinely collected for disease surveillance and outbreak detection. The current study is descriptive of a bacterial collection and microbiological characteristics are not combined with clinical data. Ethical approval was therefore not required. Also, the Norwegian Act relating to control of communicable diseases (https://lovdata.no/dokument/NL/lov/1994-08-05-55?q=Smittevernloven) obliges the NIPH to monitor the *Shigella* spp. and EIEC populations within the country on a regular basis. For these reasons, consent was not obtained from the patients to analyze the bacterial samples for this research project.

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

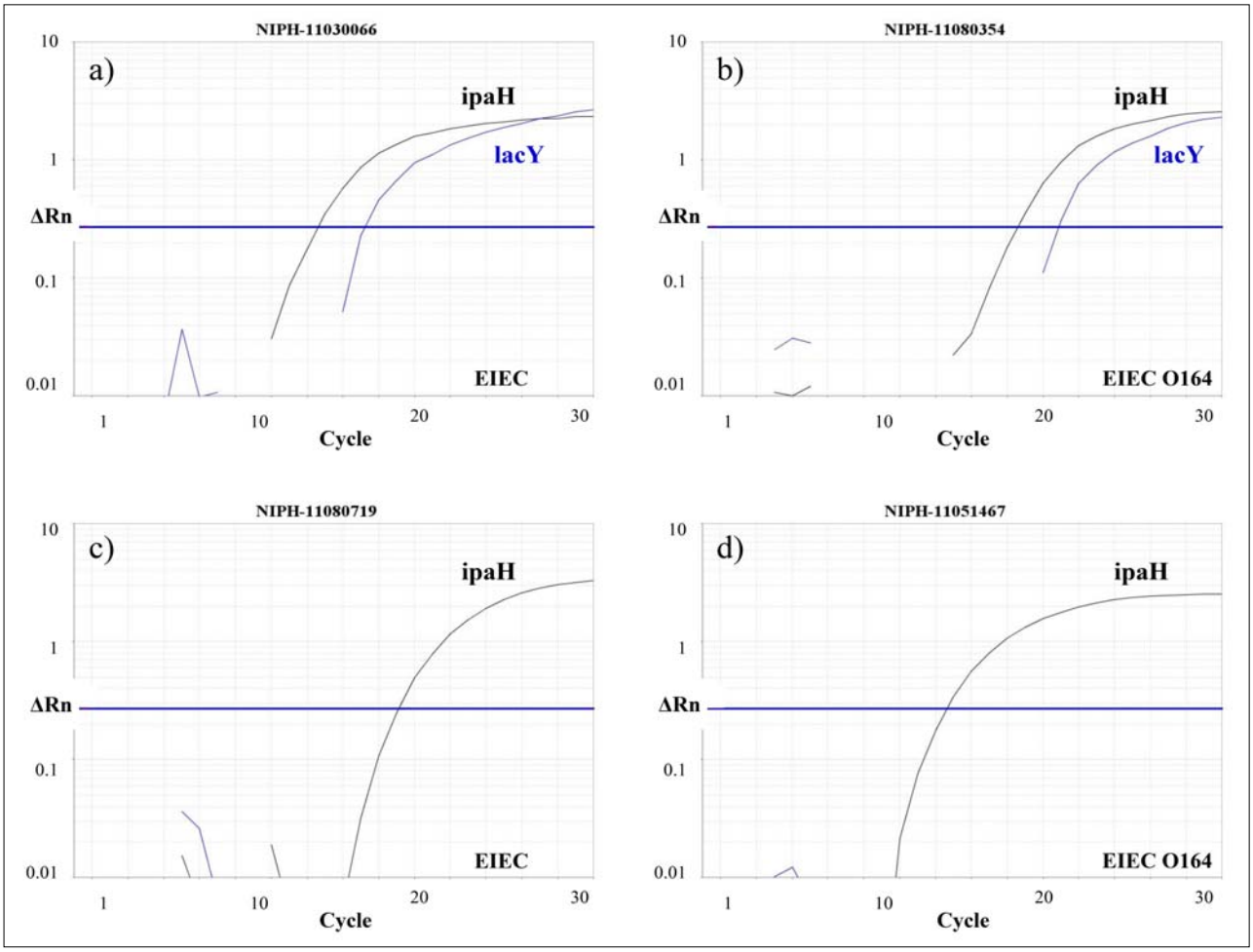

Fig. 1. Four strains phenotypically determined as EIEC O164 were either classified as EIEC (*lacY* positive) or *Shigella* (*lacY* negative) by the real-time PCR. a) NIPH-11030066 and b) NIPH-11080354 carried *lacY* and *ipaH* and were classified as EIEC. However, c) NIPH-11080719 and d) NIPH-11051467 harbored *ipaH* only and, thus, designated *Shigella* by the real-time PCR. Phenotypically, except for the lactose fermentation in NIPH-11080354, they could not be distinguished

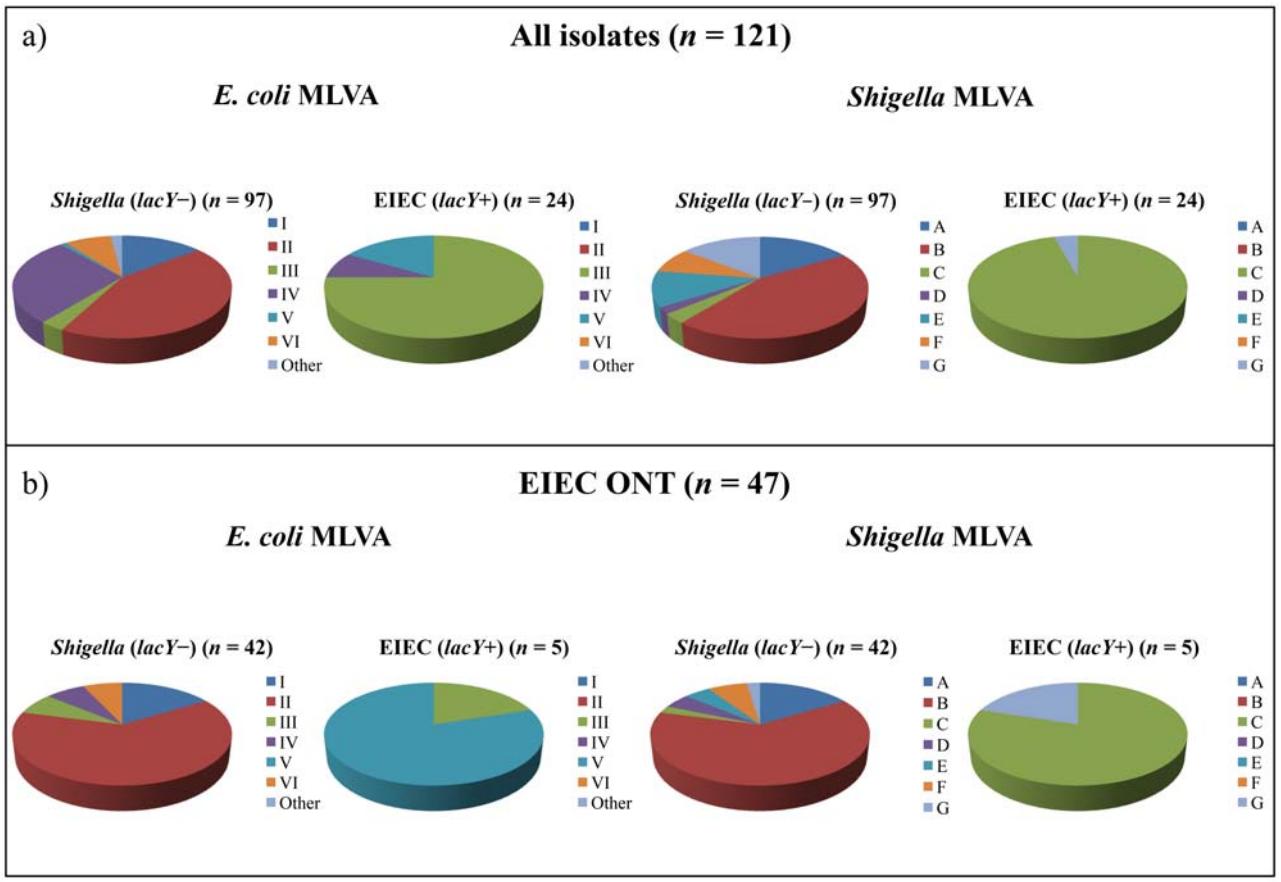

Fig. 2. Generic *E. coli* MLVA and *Shigella* MLVA groups compared with duplex real-time PCR results. a) All *Shigella* spp. and EIEC isolates (n = 121) were included. *E. coli* MLVA groups I, II, and VI and *Shigella* MLVA groups A, B, E, and F were exclusively detected in isolates classified as *Shigella* (*lacY*–) by real-time PCR. On the other hand, *E. coli* MLVA groups III and V and *Shigella* MLVA group C were preferentially associated with strains classified as EIEC (*lacY*+) by the real-time PCR. b) Only EIEC ONT isolates (n = 47) were included. Interestingly, a) and b) showed comparable patterns, indicating that the duplex real-time PCR was suited to classify the phenotypically challenging EIEC ONT group

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 unambiguous, 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 *Shigella* MLVA group C, the EIEC O164 isolate was defined as *Shigella* (*lacY* negative) by real-time PCR. The last *E. coli* MLVA group (VI) included eight isolates, five *Shigella*, and three EIEC ONT, all found as *Shigella* (*lacY* negative) by real-time PCR and all belonging to *Shigella* MLVA group F *(Table 3)*. In conclusion, in 94.7% (114/121) of the cases, MLVA profiles both from the generic *E. coli* and *Shigella* assays supported the findings achieved by duplex real-time PCR. *E. coli* MLVA groups I, II, and VI, and *Shigella* MLVA groups A, B, E, and F were exclusively seen in isolates defined as *Shigella* (*lacY* negative) by the real-time PCR. On the other hand, *E. coli* MLVA groups III and V, and *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 *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 *E. coli* MLVA profiles belonging to group III or V, and all but one clustered within *Shigella* MLVA group C, supporting the finding of these isolates as EIEC *(Fig. 2)*. Moreover, 39/42 (92.9%) EIEC ONT defined as *Shigella* (*lacY* negative) showed MLVA profiles associated with *Shigella* spp., indicating that the real-time PCR classification was correct (*Table 3* and *Fig. 2*).

Discussion

Discrimination of *Shigella* spp. from EIEC has been challenging using phenotypical typing methods and molecular typing techniques [16–19, 25]. However, due to clinical differences between *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*. *Shigella* spp. do not ferment lactose or do so slowly due to *lacY* deficiency or presence of a *lacY* pseudogene [10, 15]. Although *S. sonnei* and *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 *S. sonnei* and *S. dysenteriae* serotype 1 isolates examined. This is in concordance with previous reports demonstrating the absence of *lacY* in *Shigella* spp. [19, 27]. Thus, it is tempting to speculate that the structural changes at the 5′ end of the *lacY* pseudogene described in *S. sonnei* and *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 *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 *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 *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 *Shigella* spp. were examined and they did not include more than two uncharacterized *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 *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 *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 *Shigella* by real-time PCR, the MLVA assays disagreed with this classification. Interestingly, these three EIEC ONT isolates agglutinated with *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 *E. coli* and *Shigella* [11, 14]. These EIEC ONT isolates might be precursors of "full-blown" *Shigella* and, thus, were either classified as *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 *Shigella* spp., and this complicates serological differentiation [11, 14, 32]. Cross-reactivity between O-antigens from EIEC O164 and *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 *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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