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A new multiplex PCR for differential identification of *Shigella flexneri* and *Shigella sonnei* and detection of *Shigella* virulence determinants

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SUMMARY

Most of the multiplex PCR (mPCR) used to identify *Shigella* do not discriminate between *Shigella* species or serotypes. We designed a mPCR to differentiate between *S. flexneri* and *S. sonnei* strains based on the detection of markers associated with the *she* pathogenicity island described in *Shigella*. In addition, specific primers were included to detect the *Shigella* virulence determinants ShET-1 and ShET-2 enterotoxin genes. The analysis of 304 *Shigella* strains from Chile and 79 *Shigella* strains from other geographic locations indicated that the mPCR described here detected all *Shigella* species and specifically differentiated *S. flexneri* and *S. sonnei*. The technique was sensitive, reproducible, specific and simple to perform, providing a new tool with the potential to be employed for epidemiological and diagnostic purposes.

Key words: *Shigella*, multiplex PCR, *she* pathogenicity island, ShET-1, ShET-2.

INTRODUCTION

Infections caused by *Shigella* continue to be a major public health problem with an estimated annual incidence of 160 million cases worldwide [1]. Several epidemiological studies indicate that *S. flexneri* 2a and *S. sonnei* are the most predominant *Shigella* isolated in both developing and industrialized countries [1, 2]. Considering the global burden of *Shigella*, the difficulties in implementing preventive and control measures and emerging antibiotic resistance, WHO has given high priority for vaccine development programmes against *Shigella* [3, 4]. However, this development requires the capacity to identify the most prevalent species and serotypes in different geographic locations.

Routine microbiological identification of *Shigella*, including serotyping, is a multiple-step technique that usually takes 3–5 days [2]. Multiplex PCR (mPCR) assays, which detect several virulence markers in a single PCR reaction, are becoming the method of choice for rapid, specific and sensitive detection of diarrhoeagenic pathogens in both developing and industrialized countries [5, 6]. For *Shigella*, several mPCR have been described, but they do not

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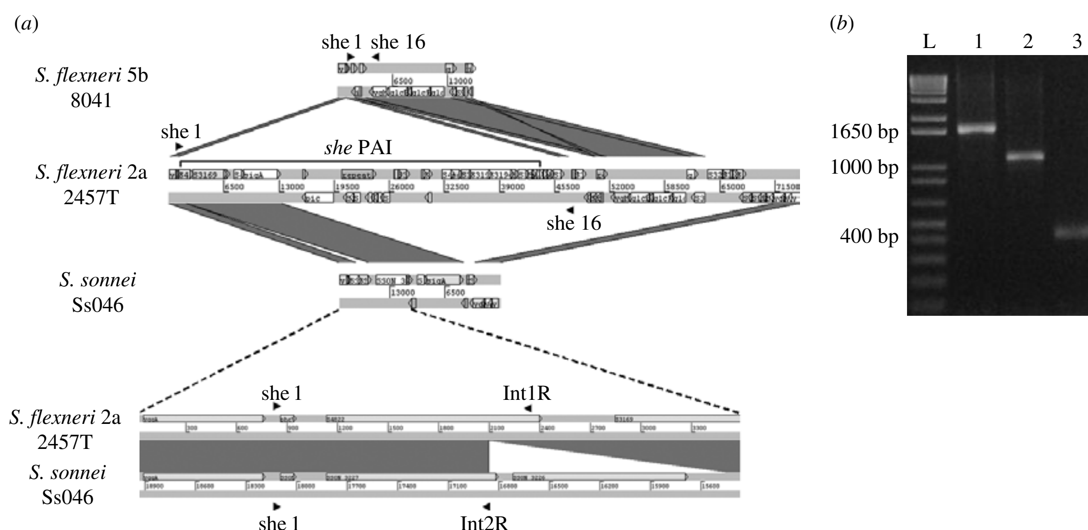

Fig. 1. *Shigella* spp. detection. (*a*) Alignment analysis comparing the *she* pathogenicity island (PAI) insertion region on the genome sequence of *S. flexneri* 2a strain 2457T with *S. sonnei* strain Ss046 and *S. flexneri* 5b strain 8401 (top panel). Arrows indicate localization of She1, She16, Int1R and Int2R primers. Magnification of the integrase gene region of *S. flexneri* 2a strain 2457T and *S. sonnei* strain Ss046, indicating the recognition site of primer Int1R and Int2R (bottom panel). (*b*) Agarose gel electrophoresis showing mPCR products obtained with the four primers described above simultaneously using *S. flexneri* 2a (lane 1, 1676-bp fragment), *S. sonnei* (lane 2, 1097-bp fragment) and *S. flexneri* non-2a (lane 3, 401-bp fragment) strains as template. L, Molecular size markers (1 kb plus ladder from Invitrogen).

discriminate between *Shigella* species or serotypes, nor do they differentiate *Shigella* from the closely related pathogen enteroinvasive *Escherichia coli* (EIEC) [7–10]. To improve the specificity of these tests, genetic markers exclusively present in *Shigella* spp. and/or serotypes need to be identified.

Pathogenicity islands (PAI) are discrete genetic elements often inserted adjacent to tRNA genes, which encode virulence genes and mobile genetic elements such as integrase genes [11, 12]. The *Shigella*-specific *she* PAI is located in the chromosome next to the *pheV* tRNA gene and has been found mostly in *S. flexneri* 2a but rarely in other serotypes [13]. This PAI encodes a P4 phage-like integrase (*int* gene), and proteins associated with *Shigella* pathogenicity such as *Shigella* enterotoxin 1 (ShET-1) and a cytopathic autotransporter protease, SigA [14, 15]. Using *in silico* analyses with available *Shigella* genome sequences, we identified that the 5′-end of the *she* PAI, which includes the *sigA* gene, but not the 3′-end is present in *S. sonnei* Ss046 genome sequence. Moreover, this analysis showed that the *she* PAI is completely absent in *S. flexneri* serotype 5b strain 8401 genome sequence (Fig. 1*a*). With this information, we designed primers to develop a mPCR that specifically differentiates *S. flexneri* and *S. sonnei*. In addition, and because of their importance as virulence factors in the development of live attenuated vaccines against *Shigella*, specific primers were included in this mPCR to detect ShET-1 and ShET-2 enterotoxin genes, *set* and *sen* genes, respectively [16, 17]. The mPCR described here provides a new tool not only useful for identification of a presumptive *Shigella* isolate in a diagnostic laboratory but also for epidemiological surveillance of the most prevalent *Shigella* serotypes and *Shigella*-associated virulence determinants.

METHODS

Bacterial strains

We evaluated 304 *Shigella* strains, corresponding to 129 strains of *S. sonnei*, 99 of *S. flexneri* 2a, 72 of *S. flexneri* non-2a (1 of *S. flexneri* 1a, 4 of *S. flexneri* 1b, 18 of *S. flexneri* 2b, 14 of *S. flexneri* 3a, 1 of *S. flexneri* 3b, 7 of *S. flexneri* 3c, 8 of *S. flexneri* 4a, 2 of *S. flexneri* 5b, 12 of *S. flexneri* 6, 1 of *S. flexneri* X and 4 of *S. flexneri* Y) and 4 of *S. boydii*. These strains were isolated from stool samples of Chilean children aged <14 years with acute diarrhoea, collected from 1995 to 2007. Bacteria were cultured and identified by conventional biochemical methods and serotyping (Denka Seiken Co., Japan). We also analysed 79 *Shigella* strains from the French National Reference Centre for *Escherichia coli* and *Shigella* Collection, Institut Pasteur, France (Table 1). These strains were mostly

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Table 1. Shigella *strains from different geographic region used in this study*

Species	Bio/serotype	No. of strains	Country of isolation or origin*
S. sonnei	g	2	France
	g (onpg-)	1	Cape Verde
S. flexneri	1a	2	Cameroon, India
	1b	4	Egypt, French Guyana, Gabon, Mauritania
	2a	4	Benin, France, French Guyana, India
	2b	7	Burkina, France, Madagascar, Mauritania, Morocco
	3a	4	France, French Guyana, India, Morocco
	3b	2	France, French Guyana
	4	3	Egypt, France, Thailand
	4a	2	France, Mali
	4 Saigonensis	3	France, India, Peru
	6 Boydii 88	3	Egypt, France, French Guyana
	6 Herfordshire	2	France, Morocco
	6 Manchester variant	1	France
	X	2	French Guyana, Senegal
	Y	3	France, French Guyana, India
S. boydii	1	2	Angola, France
	2	2	Egypt, France
	4	2	France, Mauritania
	5	1	France
	8	2	France, India
	10	1	France
	12	1	India
	14	1	France
	18	2	France, Senegal
	19	1	France
	20	2	France, Mauritania
S. dysenteriae	2	3	France, India, Madagascar
	3	3	France, Réunion Island, Senegal
	4	3	France, North Africa, Senegal
	5	1	France
	9	2	France, India
	12	1	France
	96–204†	4	Cape Verde, France

* The probable original source is associated to travellers' diarrhoea when the source is different from France.
† Serotype described by Matsushita *et al*. [18].

isolated from stools during the period 2004–2007; they represent a set of different serotypes coming from different geographic regions (Europe, Asia, Africa, South America). Serotype distribution of the 79 strains was as follow: *S. sonnei* (3), *S. flexneri* 2a (4), *S. flexneri* non-2a (38), *S. boydii* (17), and *S. dysenteriae* (17). *S. flexneri* 2a strain 2457T and *S. sonnei* ATCC 25922 strain were used as *Shigella* reference strains.

The specificity of the mPCR was tested using the following bacteria obtained from clinical samples: *Salmonella* group A (1), *Salmonella* group B (7), *Salmonella* group C (1), *Salmonella* group D (14), *Klebsiella pneumoniae* (3), *Hafnia* spp. (1), *Proteus* spp. (1), *Yersinia enterocolitica* (1), *Citrobacter freundii* (1), *Vibrio parahaemolyticus* (1), *Enterobacter cloacae* (1), *Acinetobacter baumannii* (1), 3 *Pseudomonas aeruginosa* (3), *Campylobacter jejuni* (2), and *Listeria monocytogenes* (1). We also tested the following diarrhoeagenic *E. coli* strains: EIEC (1), enterohaemorragic *E. coli* (EHEC) (4), enteropathogenic *E. coli* (EPEC) (4), enteroaggregative *E. coli* (EAEC) (3), diffuse adherent *E. coli* (DAEC) (3), Shiga toxin-producing *E. coli* (STEC) (1), and enterotoxigenic *E. coli* (ETEC) (3).

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Table 2. *Primers used in this study*

Primers	Sequences (5′-3′)	Primer concn (nM)	Amplicon size (bp)	Reference
She1	TCAACATGCTTCCAGCACTC	400		This study
Int1R	AAACGGGCTGATACCCTTCT	600	1676*	This study
Int2R	GCCAATACGCGACAAAAGTT	600	1097*	This study
She16	AAGGCCACAGTGACCAGAAG	200	401*	This study
SetF	TCCCTTCATACTGGCTCCTG	200	553	This study
SetR	AACACTCTGTGGGGAACAG	200		This study
SenF	TTGCATCAGCCTGTCCATTA	120	968	This study
SenR	AAAACGGTTCATGGGGAGAT	120		This study
VirF	AGCTCAGGCAATGAAACTTTGAC	120	607	[5]
VirR	TGGGCTTGATATTCCGATAAGTC	120		[5]

* Amplicon obtained with primer She1.

Multiplex PCR design

To develop a mPCR to specifically differentiate *S. flexneri* and *S. sonnei*, specific markers were sought at the *she* PAI. Genome sequences available for *S. flexneri* 2a strains 2457T and 301 [19, 20], *S. sonnei* strain Ss046 (GenBank accession no. CP000038) and *S. flexneri* 5b strain 8401 (GenBank accession no. CP000266.1) were compared at the *she* PAI insertion site, the *pheV* tRNA gene. This analysis indicated that a homologous *int* gene in *S. sonnei*, located at the 5′-end of the *she* PAI, can be differentiated from the *S. flexneri* 2a P4-like *int* gene using primers Int1R and Int2R. This analysis also showed that in the *S. flexneri* 5b genome sequence the *she* PAI is not present in *pheV* gene boundary and its absence can be detected using primers She1 and She16. The recognition sites of these primers are shown in Fig. 1*a*. Alignment analyses using *Shigella* genome sequences described above and the primers She1, She16, Int1R, Int2R showed that a 1676-bp and a 1097-bp fragment would be detected exclusively in *S. flexneri* 2a (with She1 and Int1R primers), and *S. sonnei* (with She1 and Int2R primers), respectively. Moreover, a predicted 401-bp fragment would be detected only in *S. flexneri* 5b (with She1 and She16 primers) indicating the absence of the *she* PAI in this reference strain. The final mPCR included the primers described above plus three sets of primers specific for *sen*, *set* and *virF* genes (Table 2). Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used for design of the primers.

Multiplex PCR protocol

One colony from a McConkey or Salmonella-Shigella (SS) agar plate was suspended in 200 μl of 1% Triton X-100 solution, and boiled for 10 min. The mPCR reaction was performed with a 25-μl mixture, containing 1 μl of boiled lysate as DNA template, 3 mm $MgCl_2$, 400 μM (each) deoxynucleoside triphosphate, the 10 primers simultaneously (Table 2), and 1 U of *Taq* polymerase (Invitrogen, USA). Optimal mPCR reaction was performed using an initial 2 min denaturation step at 95 °C, 30 cycles at 95 °C for 1 min, 56 °C for 30 s, and 72 °C for 2·5 min, with a final extension at 72 °C for 10 min. PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide.

Determination of mPCR sensitivity

Cultures of $\sim 1 \times 10^8$ colony-forming units (c.f.u.)/ml of *S. flexneri* 2a 2457T, *S. sonnei* ATCC 25922 or a clinical isolate of *S. flexneri* serotype 3 were serially diluted in phosphate buffered saline (PBS) (pH 7·4). Two hundred microlitres of each diluted culture was boiled for 10 min and 1 μl of the lysate was used as a template for the mPCR reaction. The number of c.f.u. was determined in 100 μl of each diluted culture on McConkey agar. The sensitivity of the assay was defined as the lowest c.f.u. of *Shigella* per mPCR reaction that yielded positive amplification of all the markers expected.

RESULTS

***Shigella* spp. detection**

To develop a mPCR to specifically differentiate *S. flexneri* and *S. sonnei*, we searched for specific markers at the *she* PAI. The comparative analyses between *S. flexneri* 2a strain 2457T, *S. sonnei* strain

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Table 3. *Amplification products obtained with primers She1, Int1F, Int2F and She16 using* Shigella *strains as a template*

Serotype	Total strains	1676-bp product	1097-bp product	401-bp product	No amplification
Chilean strains					
S. flexneri 2a	99	99	0	0	0
S. flexneri non-2a	72	2	0	58	12*
S. sonnei	129	0	129	0	0
S. boydii	4	0	0	0	4
Strains from other regions					
S. flexneri 2a	4	4	0	0	0
S. flexneri non-2a	38	10	0	22	6*
S. sonnei	3	0	3	0	0
S. boydii	17	0	0	0	17
S. dysenteriae	17	0	0	0	17

* All strains correspond to *S. flexneri* serotype 6.

Ss046 and *S. flexneri* 5b strain 8401 genome sequences and the location of primers Int1R, Int2R, She1 and She16 are detailed in Fig 1*a*. Using these four primers simultaneously, a band of ~1650 bp was detected exclusively in the *S. flexneri* 2a 2457T reference strain used as a template (Fig. 1*b*, lane 1). A ~1000-bp fragment was amplified only in *S. sonnei* ATCC 25922 (Fig. 1*b*, lane 2). In addition, the mPCR was tested with 10 Chilean strains of *S. flexneri* non-2a randomly selected, amplifying a ~400-bp single band in all the strains assayed (Fig. 1*b*, lane 3). Non-specific bands were not detected. The sequencing of the mPCR products amplified above corresponded to the expected size fragments obtained by the alignment analyses with *Shigella* genome sequences.

Based on the detection of *she* PAI associated markers *S. flexneri* and *S. sonnei* reference strains were differentiated using four specific primers in the same PCR reaction. Moreover, in all *S. flexneri* non-2a Chilean strains assayed a 401-bp fragment was amplified indicating lack of the *she* PAI insertion at *pheV* gene.

mPCR evaluation in *Shigella* isolates from clinical samples

To test the mPCR including primers She1, She16, Int1R and Int2R, lysates were tested from isolated colonies of 304 *Shigella* strains obtained from Chilean children previously identified by serotyping. All 99 *S. flexneri* 2a strains yielded the 1676-bp fragment specific for this serotype, according to sequence analysis. Similarly for all 129 strains of *S. sonnei*, the 1097-bp fragment was detected. Interestingly, the 401-bp fragment was amplified in 58/72 (81%) *S. flexneri* non-2a strains. Of the 14 *S. flexneri* non-2a strains lacking the 401-bp fragment, two were *S. flexneri* serotype Y and amplified a band similar to the 1676-bp fragment specific for *S. flexneri* 2a; the other 12 strains corresponded to *S. flexneri* serotype 6. No amplicons were detected in Chilean *S. boydii* isolates.

To determine the universal applicability of the mPCR, 79 *Shigella* strains isolated from other geographical regions were tested (Table 1). Table 3 shows that in all (3/3) *S. sonnei* strains and all (4/4) *S. flexneri* 2a strains the expected 1097-bp and 1676-bp fragments, respectively, were detected. However, the 1676-bp fragment was also found in 10 strains of *S. flexneri* non- 2a used as a template (6 strains corresponding to serotype 2b, 1 to serotype 3a, 1 to serotype 4a and 2 to serotype Y). From the remainder of the *S. flexneri* non-2a strains (28/38), the 401-bp fragment was detected in 22 strains, and no amplification products were found in any of six *S. flexneri* serotype 6 tested. No amplicons were detected using *S. boydii* and *S. dysenteriae*.

Detection of *Shigella* virulence determinants

To enhance the utility of this mPCR as a tool for detection of *Shigella* associated-virulence determinants, specific primers were incorporated to detect *set*, *sen* and *virF* genes. The *set* gene (ShET-1 enterotoxin) is encoded chromosomally within the *she* PAI [13]; *sen* (ShET-2 enterotoxin) and *virF* (*Shigella* virulence

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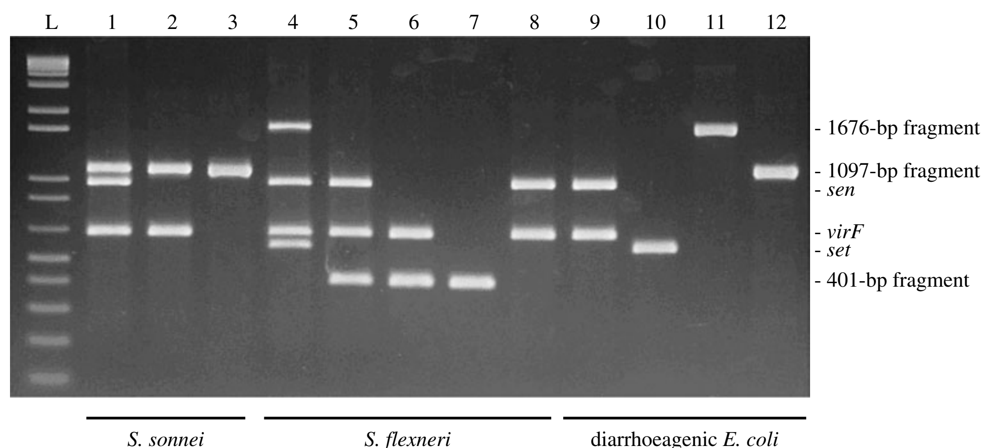

Fig. 2. Agarose gel electrophoresis showing the amplification patterns to discriminate *Shigella* spp. using the ten primers in the mPCR reaction. L, Molecular size markers (1 kb plus ladder from Invitrogen); lanes 1–3, *S. sonnei*; lane 4, *S. flexneri* harbouring *she* pathogenicity island (PAI); lanes 5–8, *S. flexneri she* PAI-negative strains; lanes 9–12, diarrhoeagenic *E. coli* (lane 9, EIEC; lane 10, EAEC; lane 11, STEC; lane 12, EHEC/EPEC).

regulator) genes are encoded in the 220-kb virulence plasmid [21, 22].

The different amplification patterns using the ten primers simultaneously are shown in Fig. 2. For *S. sonnei* three amplification patterns were found (Fig. 2, lanes 1–3); in contrast, all *S. flexneri* strains that harboured the *she* PAI displayed a unique amplification pattern (Fig. 2, lane 4). Four patterns were observed for *S. flexneri she* PAI-negative strains (Fig. 2, lanes 5–8); three of these strains were characterized by amplification of the 401-bp fragment, indicating the absence of *she* PAI in the *pheV* boundary (Fig. 2, lanes 5–7); the fourth pattern, distinguished by the sole amplification of *virF* and *sen* markers was exclusive to *S. flexneri* serotype 6 (Fig. 2, lane 8). All *S. boydii* and *S. dysenteriae* strains also presented this amplification pattern (*virF*$^+$ and *sen*$^+$), indicating the presence of the virulence plasmid. Interestingly, for *S. sonnei* and *S. flexneri* non-2a a potential loss of the 220-kb virulence plasmid was detected, since no amplification of *virF* and *sen* markers was observed (Fig. 2, lanes 3 and 7).

Analysis of the 383 *Shigella* strains showed that the *sen* gene marker was present in 308 (80%) of them whereas the *set* gene marker was found only in *Shigella* strains that harboured the *she* PAI (115/383) (Table 4).

Specificity and sensitivity of the mPCR

The mPCR proved to be specific for *Shigella* and was negative with all other species tested with the exception of diarrhoeagenic *E. coli* strains which displayed some amplified products which are probably related to the PAI present in these pathogens (Fig. 2, lanes 9–12). For the EIEC strain, which is known to harbour the *Shigella* 220-kb virulence plasmid, amplicons compatible with the *sen* and *virF* genes were detected, displaying the same pattern observed for *S. flexneri* 6, *S. boydii* and *S. dysenteriae* (Fig. 2, lanes 8, 9). For EAEC strains, the *set* marker was amplified (Fig. 2, lane 10), as previously described [23]. In both of these *E. coli* pathotypes no amplification for *S. flexneri* 2a or *S. sonnei* integrase was obtained, which allows for the differentiation of these pathogens from *S. flexneri* (with the exception of *S. flexneri* 6) or *S. sonnei* strains. For STEC, a band similar to the 1676-bp fragment specific for *S. flexneri she* PAI-positive *int* gene was detected, but not the *set* gene (Fig. 2, lane 11). Finally, for EHEC and EPEC, a band similar to the 1097-bp fragment specific for *S. sonnei int* gene was amplified, displaying the amplification pattern observed for *S. sonnei* lacking the virulence plasmid (Fig. 2, lane 12). Sensitivity tests with *S. flexneri* 2a, *S. sonnei* and *S. flexneri* non-2a cultures revealed the expected amplicons in 100 c.f.u. of organisms, and repeated tests with freshly prepared bacterial template gave the expected amplification products for all strains.

DISCUSSION

Classical methods for determining the presence of *Shigella* are time-consuming and labour-intensive; therefore, the detection of several virulence markers in a single PCR reaction represents an important advance in the identification of this microorganism

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Table 4. *Frequency of the virulence-determinant markers in 383* Shigella *isolates analysed*

Serotype	Total strains	Number of strains positive for the markers: *set*	*sen*	*virF*	No markers
S. flexneri 2a	103	103	103	103	0
S. flexneri non-2a	110	12	86	90	20
S. sonnei	132	0	81	89	43
S. boydii	21	0	21	21	0
S. dysenterieae	17	0	17	17	0
Total (%)	383 (100)	115 (30)	308 (80)	320 (84)	63 (16)

[24, 25]. Most of the mPCR assays to identify *Shigella* so far described are confined to the detection of the *ipaH* gene, a marker present in all *Shigella* isolates as well as in EIEC strains [7, 9, 26–28]. The lack of specific markers to recognize a particular species or serotype has limited the design of new mPCRs. In this study, we designed a mPCR based on the detection of markers present on the *she* PAI. This PAI correspond to a 46-kb segment mostly found in *S. flexneri* 2a [13] and is partially present in the *S. sonnei* genome (Fig. 1*a*). Using bioinformatic analyses the integrase gene located in the 5′-end of this PAI can be differentiated from its homologous gene present in *S. flexneri* 2a with specific primers (Fig. 1*a*). Using this approach, four primers were designed to differentiate *S. flexneri* harbouring the *she* PAI and *S. sonnei* by the specific amplification of a 1676-bp and a 1097-bp fragment, respectively. Moreover, a 401-bp fragment was found that indicated the absence of the insertion of the *she* PAI in the *pheV* boundary in most (73%) of *S. flexneri* non-2a obtained from different regions worldwide.

Also included in the mPCR reaction were specific primers for ShET-1 and ShET-2 enterotoxin genes and the 220-kb *Shigella* virulence plasmid marker *virF* gene. Recently, vaccine trials using live attenuated *Shigella* strains suggested that ShET-1 or ShET-2 or both contribute to human disease, indicating their importance as virulence factors [16, 17]. Thus ten primers were incorporated in the same PCR reaction and used to test a large collection of *Shigella* strains. A high prevalence of the ShET-2 marker was found, in agreement with previous reports [29–31]. Considering that 16% of the strains were *virF* negative (63/383 strains), which suggests the lack of the 220-kb virulence plasmid might probably be due to long storage [32], the prevalence of the ShET-2 marker could be even higher. On the other hand, the presence of the *she* PAI, indicated by the amplification of the 1676-bp fragment and the *set* gene marker, was found in all *S. flexneri* 2a strains as well as in a minority of *S. flexneri* non-2a strains, a result that is in accord with previous studies [13, 29, 33]. Interestingly, the absence of the *set* gene in all *S. flexneri* strains not harbouring the *she* PAI was correlated with the amplification of the 401-bp fragment, with the exception of all *S. flexneri* 6 strains assayed. The lack of this fragment in *S. flexneri* 6 isolates might be explained by insertion of a long DNA sequence or changes in the *pheV* boundary that affect the amplification with primers She1 and She16. For *S. boydii* and *S. dysenteriae* no amplification of *she* PAI markers was evident, but these serotypes exhibited an amplification pattern similar to *S. flexneri* 6 where the virulence plasmid markers *sen* and *virF* genes were detected.

A DNA microarray targeting O-serotype-specific genes to detect all 34 distinct O-antigen forms of *Shigella* was recently developed [34, 35]. Even though this technique proved to be specific, sensitive and reproducible, its application as a diagnostic or epidemiological tool is difficult, even in industrialized countries, in view of the elevated cost, instruments and qualified personnel necessary to perform this technique [36]. The mPCR described here might offer a more practical approach for rapid, easy and affordable identification of *Shigella*, particularly in developing countries where *Shigella* incidence is high and resources are limited. Although our mPCR was tested on pure cultures rather than food or clinical samples, the application of this technique using a single colony grown on selective media from food or stool samples might reduce the time of the

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identification of *Shigella* compared to conventional methods.

The mPCR described here represents a new approach based on the identification of several serotypes of clinical or epidemiological importance. As the assay is not based exclusively on the detection of genes present in the 220-kb virulence plasmid, this assay might allow the differentiation between *S. sonnei* and most of *S. flexneri* serotypes from EIEC strains. In addition, as *Shigella* vaccine development is mostly focused on *S. flexneri* 2a and *S. sonnei* [3, 4], the mPCR described here may prove to be a valuable tool in epidemiological studies to identify specifically the most frequent *Shigella* isolates and contribute to the surveillance of the virulence determinants ShET-1 and ShET-2 enterotoxins.

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