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Enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis as a reliable evidence for suspected *Shigella* spp. outbreaks


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ABSTRACT

Background: Shigellosis remains a serious public health problem and an important cause of morbidity and mortality worldwide. The aim of this study was to characterize *fliC* and the genetic relatedness of *Shigella* spp. isolated during a one-year period from children in a suspected outbreak in Tehran, Iran.

Methods and results: Fifty *Shigella* spp. were isolated from 3779 stool samples of children with diarrhea (prevalence rate: 1.32%). Among the isolates, 92% were characterized as *Shigella sonnei*, while 6% and 2% were identified as *S. flexneri* and *S. boydii*, respectively. *S. dysenteriae* was not recovered from the patients. All isolates were negative for *fliC* except for *Shigella* standard strains. The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) profiles allowed differentiating the 50 isolates into 5 ERIC types, which were grouped into five clusters (ET1–ET5). Computer-assisted clustering of the strains showed a high degree of similarity among the isolates.

Conclusion: In conclusion, given the clonal correlation of the *Shigella* strains isolated in this study and the lack of *fliC* among them, we propose that probably a single or limited *fliC*-defected *Shigella* clone spread and caused the outbreak.

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Introduction

Shigellosis is an infectious disease and a health problem in both developed and developing countries. It is also a major cause of diarrheal diseases among children in Iran.¹ *Shigella* spp. are highly specific human pathogens with variable epidemiological and pathological features.² Based on their biochemical characteristics and antigenic properties, *Shigella* spp. are classified to four species or serogroups: *Shigella dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C), and *S. sonnei* (group D).

Although the genomic information of *Shigella* has described this species as non-flagellated organisms, controversial published data have shown cryptic flagellin genes for *S. flexneri* and *S. sonnei* strains.^{3,4} In addition, a study using electron microscopy proved that prototypic strains of all four *Shigella* species possess flagella.⁵

The organism has a well-documented ability to cause sustained outbreaks from a wide variety of sources and high rates of infection in populations, making accurate typing of patient isolates mandatory for understanding the transmission dynamics and control of further spread.⁶

A wide variety of typing systems has been developed to examine and compare isolates of *Shigella* spp. for outbreak inspection and taxonomic characterization. Bacterial genomes are generally considered to be continually reorganizing with numerous families of short interspersed repetitive sequences. Enterobacterial repetitive intergenic consensus (ERIC) sequences are described as intergenic repetitive units, different from most other bacterial repeats in being distributed or present among a wider range of species. ERIC sequences are imperfect palindromes of 127 bp⁷ and were first described in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, other *Enterobacteriaceae* members, and *Vibrio cholerae*.

The success of ERIC-PCR as a simplified typing method for a wide and progressively expanding number of organisms makes this method suitable for hospital-based epidemiology or localized epidemiology, where a highly recreated typing method that requires minimal disease-specific reagents is an expedient characteristic.⁷

The aim of this study was to characterize *fliC* and genetic relatedness of *Shigella* spp. isolated during a 1-year period from children in a suspected outbreak in Tehran, Iran.

Materials and methods

Ethical approval was obtained from the Faculty of Medical Sciences, Tarbiat Modares University.

Bacterial strains

A total of 3779 stool specimens were collected from outpatient children, younger than 5 years, with diarrhea characterized by ≥ 3 episodes of loose or watery stools with or without blood, mucus, and stomach cramps referred to two major hospital laboratories from November 2012 to October 2013. For the preliminary isolation of *Shigella* spp., the specimens were cultured on selective agar plates including *Salmonella*-

Shigella agar and XLD agar (Merck, Hamburg, Germany).⁸ Putative *Shigella* organisms were selected and preliminarily characterized by biochemical tests including TSI, SIM, ODC, LIA, Simmons Citrate and Urea agar and confirmed by an API-20E strip kit according to manufacturer instructions (API-bioMérieux, Inc., La Balme les Grottes, France).⁹ The phenotypic biochemical profiles of the isolates were compared with the World Health Organization (WHO) criteria for biochemical identification of *Shigella* spp.¹⁰ *S. sonnei* ATCC 9290, *S. flexneri* ATCC 12022, *S. boydii*, and *S. dysenteriae* control strains were kindly provided by the WHO and used as controls in each assay.

DNA preparation, molecular confirmation of *Shigella* spp., and serotyping

The isolates were identified by biochemical tests and confirmed by PCR for *ipaH* with a specific set of primers (Table 1). Additional primer pairs that amplify *wbgZ* and *rfc* specifically for *S. sonnei* and *S. flexneri*, respectively, were used for identifying the two most commonly found *Shigella* species. All PCR reactions were performed with DNA preparations obtained by the boiling method.

Freshly cultured plates of *Shigella* isolates confirmed with molecular identification of *ipaH* were prepared for agglutination on glass slides by commercially available polyclonal antisera (Baharafshan Institute of Research & Development, Tehran, Iran) using the standard slide agglutination method.¹¹

Investigation of *fliC*

Two sets of primer pairs were used for amplifying *fliC* with regard to the complete and truncated flagellin sequences in GenBank. Primer names, their sequences, and amplification conditions are presented in Table 1.

CLC software (<http://www.clcbio.com>) was used for designing specific primer pairs for truncated *fliC* according to the genome sequence of *S. sonnei* ATCC 9290 (GenBank accession number HE616528).

ERIC PCR performance and analysis

ERIC-PCR was performed on *Shigella* strains according to Versalovic et al. (1991). Each PCR reaction (25 μ L) contained 10 $\times$ buffer 2.5 μ L, dNTPs (2.5 mmol/L) 2.5 μ L, Taq DNA polymerase (5 U/ μ L) 0.5 μ L, MgCl₂ (25 mmol/L) 1.5 μ L, primers (100 mol/L) each 1 μ L, template DNA 1 μ L, and addition of distilled water to 25 μ L. All PCR reactions were performed in the following program: initial denaturation of 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 5 min followed by a final extension of 72 °C for 10 min. Primer sequences are presented in Table 1.

The amplicon was electrophoresed on 2% agarose gel with DNA safe stain along with a 1-kb DNA ladder. An image was captured for further analysis with Gel compare II software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). The clustering of the isolates was performed on the basis of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis and Dice similarity coefficient.

Table 1 – Oligonucleotide primers used in this study.

Primer name (target)	Sequence (5' to 3')	Product size (bp)	Annealing temperature	Reference
<i>Shigella</i> (<i>ipaH</i>)	GTTCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	619	55 °C	24
<i>Shigella sonnei</i> (<i>wbgZ</i>)	TCTGAATATGCCCTCTACGCT GACAGAGCCCGAAGAACCG	430	56 °C	25
<i>Shigella flexneri</i> (<i>rfe</i>)	TTTATG GCTTCTTTGTCTG GC CTG CGTGATCCGACCATG	537	56 °C	25
ERIC	ATGTAAGCTCCTGGGGATTAC AAGTAAGTGAAGGGGTGAGCG	–	54 °C	12
<i>fliC</i> -truncate	AACTCCCTCATTGACACCTTT ATCCATTACACCTTCAGCG	1500	58 °C	This study
<i>fliC</i>	GGGTGGAACCCCAATACG GCGCATCAGGCAATTTGG	1714	58 °C	26

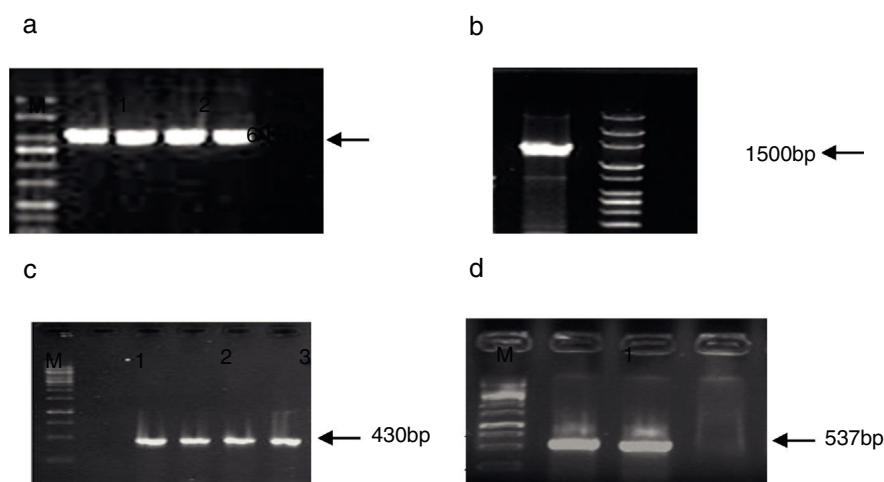


Fig. 1 – PCR amplification of *ipaH* gene. Lane 1: positive control; Lane 2–4: positive isolates; Lane 5: negative control; M: mid-range DNA ladder (100 bp to 3 kb). PCR amplification of *fliC* gene. Lane 1: *Shigella flexneri* ATCC1202 and M: mid range DNA ladder (100 bp to 3 kb). PCR amplification of *wbgZ* gene in *Shigella* spp. L. Lane 1: negative control; Lane 2: positive control; Lane 3–5: positive isolates; M: 1 kb DNA ladder. PCR amplification of *rfe* gene in *Shigella* spp. Lane 1: positive control; Lane 2: positive isolate; Lane 3: negative control; M: 1 kb DNA ladder.

Results

Molecular confirmation of *Shigella* spp. isolates and detection of flagellin genes

Based on the biochemical and molecular analyses, 50 *Shigella* spp. were isolated from 3779 stool samples of children with diarrhea (prevalence rate: 1.32%). All suspected *Shigella* spp. (by biochemical tests) were positive for *ipaH* and showed a fragment of 619 bp, which was further confirmed by sequencing (Fig. 1). Among the isolates, 46 (92%) were characterized as *S. sonnei* and 3 (6%) as *S. flexneri* by serogrouping, which was confirmed by amplifying 430-bp and 537-bp bands related to *wbgZ* and *rfe*, respectively. Among the isolates, *S. boydii* accounted for 2%, while *S. dysenteriae* was not recovered from the patients. All isolates were negative for *fliC* except for standard *Shigella* strains (Fig. 1).

ERIC PCR analysis

The genotyping profiles of 50 *Shigella* strains according to ERIC-PCR fingerprinting are shown in Fig. 2. The ERIC-PCR profiles

allowed differentiating 50 isolates into 5 ERIC types, which were grouped into 5 clusters (ET1–ET5) (Fig. 2). All 50 *Shigella* spp. under analysis produced 7–9 amplicons ranging from 500 to 3200 bp. Computer-assisted clustering of the strains showed a high degree of similarity among the isolates, with similarity indices ranging from 92.4% for the most diverse isolates to 100% for identical isolates.

Discussion

The number of intestinal infections caused by *Shigella* spp. is increasing continuously and is turning into a prominent public health concern worldwide. The prevalence rate of *Shigella* spp. in this study was 1.32% with *S. sonnei* constituting the most prevalent species (92% of all isolates). The data from other studies have shown *S. flexneri* as the most frequent *Shigella* spp. in developing countries and in Iran.^{13–15} In the last decades, most of incidences of shigellosis in Iran were caused by *S. flexneri*; however, recent studies have revealed a growing tendency of shigellosis caused by *S. sonnei*.^{16,17} The present results upon comparison with those from the studies in developed countries where *S. sonnei* is the most prevalent

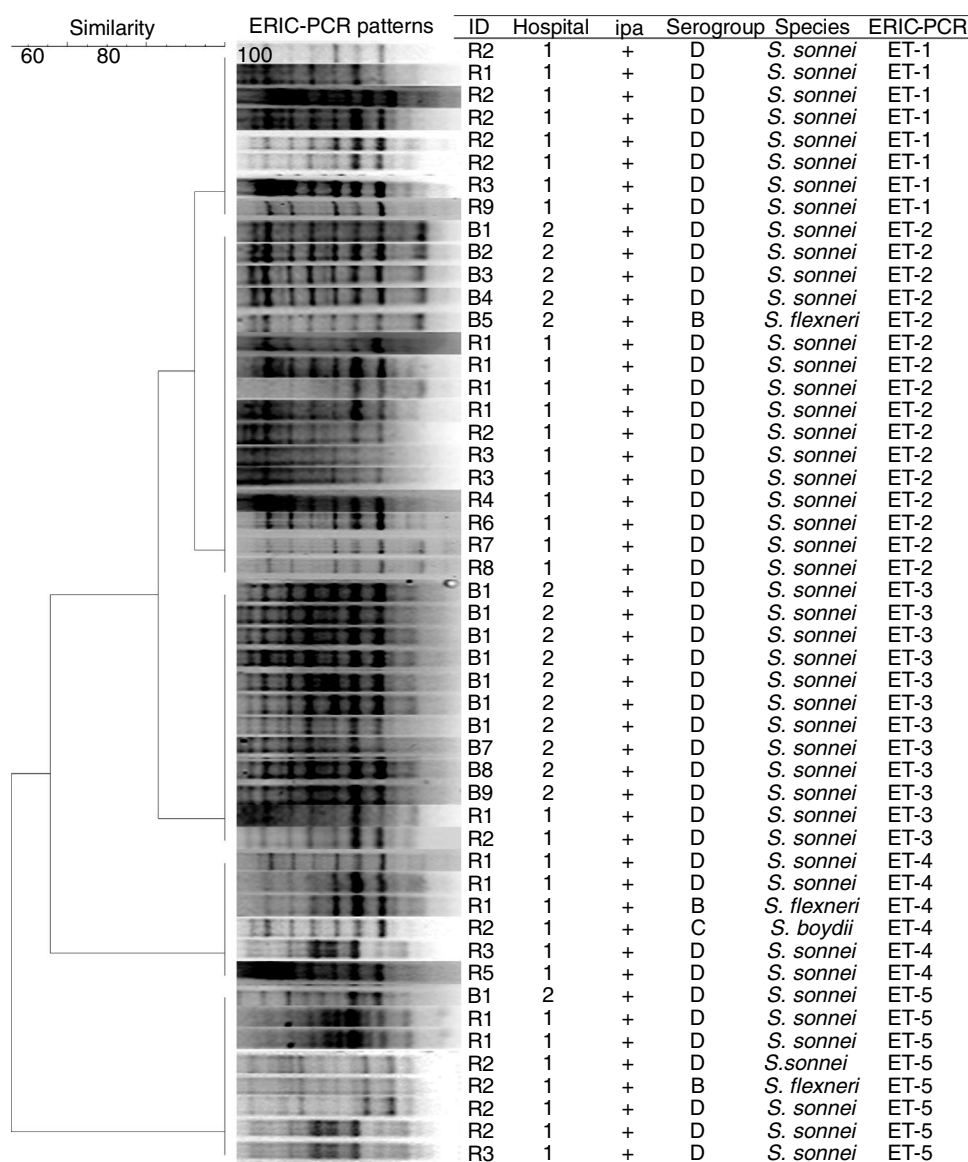


Fig. 2 – Dendrogram generated from enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) banding pattern of 50 *Shigella* spp. isolated from children with diarrhea. The similarity analysis was performed with Dice coefficient and UPGMA method.

causative agent of shigellosis reflect the industrialization of the capital city, Tehran, and the improvement of hygiene levels in Iran.¹⁷

Although the standard *Shigella* standard strains carried *fliC* gene, in this study, *fliC* was not detected in the clinical isolates despite several attempts using two sets of primers. The absence of *fliC*, even in the truncated form,¹⁸ among all isolates under study may be due to mutational or recombinational events within the coding sequence of this gene. These changes may have caused nucleotide changes in the primer-annealing sequence or deletion of the entire gene from the *Shigella* genome. In a study by Coimbra et al. (2001), all *Shigella* serotypes amplified the *fliC* region except for the reference strain *S. boydii* serotype 12 NCDC266-59, which failed to give a PCR product.¹⁸ The data from the present study along with limited observations on the existence and expression of *fliC*

in *Shigella* spp. make further studies necessary to track the changes in *fliC* in the genome of *Shigella* spp.

Several methods exist for investigating the molecular diversity of bacteria in the community and hospitals. ERIC-PCR has a proven discriminatory power and is a quick and relatively easy technique, making it useful for routine epidemiological investigations. Using the ERIC-PCR technique, genetic relatedness among *Shigella* strains isolated from pediatric patients in Iran was investigated and the isolates were differentiated into five clusters (ET1–ET5) in this study. The isolates showed less than 6-band differences in their profile, which could be considered to be a confirmation for the incidence of a *Shigella* outbreak. Ranjbar et al. (2013) showed the presence of limited ERIC-PCR profiles among *S. sonnei* isolates recovered during 2008–2010, with most isolates showing an identical banding pattern, confirming the epidemic nature of the *S. sonnei* strain

leading to several outbreaks annually in Iran.¹⁹ ERIC-PCR has been also used for typing the epidemic isolates of *Shigella* spp. and other enteric bacteria for outbreak detection in many parts of the world, including Iran.^{20–23}

Interestingly, *S. flexneri* isolates in this study showed an identical banding pattern with the pattern presented by *S. sonnei* isolates, which may suggest that inter-species differences do not necessarily affect the ERIC-PCR banding profiles of *Shigella* strains.

Conclusion

In conclusion, given the clonal correlation of the *Shigella* strains isolated in this study and the lack of *fliC* among them, we propose that probably a single or limited *fliC*-defected *Shigella* clone spread and caused the outbreak.

Authors' contribution

BB designed the study and drafted the manuscript. NA participated in the molecular tests. FF helped in designing the study and participated in statistical analysis.

Conflicts of interest

Authors declare no conflicts of interest.

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