Original Article

A Comparison between Culture and Multiplex PCR for Detection and Identification of *Shigella* Species in Patients with Shigellosis from Isfahan Province in 2014-2015

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Introduction: Awareness of the species of *Shigella* has particular importance in the way of dealing with an outbreak, controlling it, and treating patients. The purpose of this study was to use the Multiplex PCR method and comparison with the culture method to detect *Shigella* species, as well as analysing the antibiotic resistance pattern in its different species. **Methods:** Simultaneously, the fecal samples of the patients with diarrhea and a sample from each patient in Cary-Blair medium were sent to the laboratory. Cultivation, serotyping, and antibiogram analysis were performed using the samples inoculated in the Cary-Blair medium. DNA extraction of the fecal samples and amplifying of *invC*, *rfc*, *wbgZ*, and *rfpB* genes were performed. **Results**: Totally, 300 samples from the patients were analyzed, out of which 240 *Shigella* isolated (80%) were detected using the culture method and serotyping, and 260 *Shigella* isolated (86.6%) using the PCR method (*Shigella flexneri* (77%), *Shigella sonnei* (19.2%), and *Shigella dysenteriae* (3.8%)). 20 samples suspicious of *Shigella* were observed in the culture method but were not identified by serotyping. However, in the PCR assay, they were identified as *S. flexneri* (n=16) and *S. sonnei* (n=4). The resistance of *Shigella* isolated to Co-trimoxazole was observed to be (78.3%), Ceftriaxone (42.5%), Cefixime (42%), Azithromycin (40.7%), Ofloxacin (34.5%), Nalidixic acid (25 %), and Ciprofloxacin (16%). **Conclusion:** Due to annual outbreaks of various *Shigella* species in the country, it is recommended that the Multiplex PCR method, be used along with culture method in laboratories to identify *Shigella* isolated. The antibiogram results showed increasing resistance of *Shigella* to the available antibiotics. *J Med Microbiol Infec Dis, 2015, 3 (1-2): 6 pages.*

Keywords: *Shigella*, Diarrhea, Antibiotics, Multiplex PCR.

INTRODUCTION

Acute infectious diarrhea (AID) can cause severe diarrhea which is called shigellosis. *Shigella* spp. are a group of pathogenic bacteria transmitted through contaminated food [1, 2]. This group of bacteria causes about 165 million cases of diarrhea each year, out of which 163 million occur in developing countries, and 1.5 million in industrialized nations [3]. *Shigella* species are mainly transmitted fecal-orally from person to person. It may also spread indirectly through the fecal contamination of water or food [4]. The genus *Shigella* includes the species *Shigella sonnei, Shigella boydii, Shigella dysenteriae,* and *Shigella flexneri* [5]. Three species of *Shigella*, namely *S. flexneri*, *S. sonnei,* and *S. dysenteriae* are responsible for most cases of shigellosis. *S. flexneri* is seen in developing countries and *S. dysenteriae* only in cases of epidemics and pandemics [5-7].

The incidence of *Shigella* in the past was quite different from the current situation. *S. dysenteriae* was one of the most common species, which caused severe diseases. Now, *S. flexneri* has been replaced it in many countries [8].Over the past decade, *Shigella* species have gradually become resistant to broad-spectrum antibiotics [9]. Increased levels of antimicrobial resistance of various *Shigella* species have complicated the treatment of shigellosis. Changes in antimicrobial resistance patterns of *Shigella* species, depending on the geographical area as well as in one place over time, have led to therapeutic problems [10]. Multidrug resistance of pathogenic bacteria to antimicrobial is of great importance in the clinical cases and is an important issue in public health [11].

Therefore, it is necessary to be aware of the types of resistance which can be changed in time. Due to the changes in drug resistance patterns in different strains of *Shigella,* antibiotics should be prescribed after determining the antibiotic sensitivity of these strains [12]. Shigellosis is a significant infection in Isfahan province, and correct diagnosis of this disease faces several problems. These include improper sampling, delay in sending the samples, non-compliance with the cold chain, the sensitivity of *Shigella* to the ambient temperature, limited facilities in the laboratories, and time-consuming culture method (at least 48 h are needed to achieve a result). To solve these problems, it is necessary to use high repeatable, fast, and easy methods for the identification of *Shigella* isolates [13, 14].

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Hence, the purpose of the present study is to employ the Multiplex PCR method to identify the genus and species of *Shigella* and compare it with the culture method in the reference laboratory of Isfahan Provincial Health Deputy. At the same time, by using an antibiogram analysis, the antibiotic resistance pattern of *Shigella* species was identified.

MATERIAL AND METHODS

Clinical strains. This study was conducted from the beginning of 2014 through the end of 2015. According to the country-wide guideline of the care system for food-borne diseases, dysentery samples were gathered from all the patients visiting healthcare centers and hospitals in Isfahan Province. At the same time, a direct fecal sample and a sample inoculated in the Cary-Blair transport medium were obtained from each patient and sent to the reference laboratory of Health Deputy in cold boxes along with filled out personal information forms.

Inclusion criteria. Fecal samples obtained from patients suffering from dysentery or acute diarrhea associated with fever, abdominal cramps, nausea and vomiting in health care centers and hospitals across the province.

Exclusion criteria. Consumption of antibiotics before sampling, samples without a label and questionnaire, transferring the samples without compliance with the cold chain, sampling after the initial 24 h of the onset of symptoms, and receiving the sample more than 72 h after collection.

Identification of the bacterial strains. The swab samples in Cary-Blair transport medium were cultured on MacConkey's agar medium and in more specialized media such as xylose-lysine-deoxycholate (XLD), and deoxycholate citrate agar. The isolated *Shigella* were identified by their biochemical properties and by cultivating them in differential media according to the standard methods of microbiology including Kliger's Iron Agar (KIA), Urea Agar, SIM, lysine iron agar, and MRVP (all the media from Merck Company in Germany) [15]. Then, the isolated *Shigella* were serotyped using specific groups of polyvalent and monovalent antisera (Mast, UK).

Antimicrobial susceptibility test. The disk diffusion method was used on the Muller Hinton Agar medium (Merck, Germany) to determine the antibiotic resistance pattern. The drug susceptibility results were reported after 18 h storage at 37°C and based on the recommendation of NCCLSI [16].The prepared and dehydrated antibiotic discs (Padtan Teb, Iran) used in this study were co-trimoxazole (trimethoprim-sulfamethoxazole) (1.25-23.75 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftriaxone (30 µg), cefixime (5 µg), and azithromycin (15µg). The standard strains *Escherichia coli* (ATCC 25922), and *S. flexneri* (ATCC 12022) were used as controls.

DNA Extraction and PCR. Simultaneous with cultivation, DNA was extracted from fecal samples using DNA Stool Kit (Qiagen Mini Kit) according to the instructions provided by the manufacturer. The DNA samples were kept in a -70°C until used.

Specific primers pairs for amplification of *invC* gene from the genus *Shigella* was used. For isolated identification, the *rfc* fragment from *S. flexneri*, *wbgZ* from *S. sonnei*, and *rfpB* from *S. dysenteriae* were amplified in a Multiplex PCR assay using the primers (Table 1) designed by Sowash Chandra *et al.* [17].

Table 1. Primers used in this study.

Primer	Primer sequence (5'-3')	Gene target	Reference
SgenDF1	TGC CCA GTT TCT TCA TAC GC	*InvC*	Sowash Chandra *et al.* [17]
SgenDR1	GAA AGT AGC TCC CGA AAT GC		
SflexDF1	TTT ATG GCT TCT TTG TCG GC	*Rfc*	Sowash Chandra *et al.* [17]
SflexDR1	CTG CGT GAT CCG ACC ATG		
SsonDF1	TCT GAA TAT GCC CTC TAC GCT	*Wbg*	Sowash Chandra *et al.* [17]
SsonDR1	GAC AGA GCC CGA AGA ACC G		
SdysDF1	TCT CAA TAA TAG GGA ACA CAG C	*rfpB*	Sowash Chandra *et al.* [17]
SdysDR1	CAT AAA TCA CCA GCA AGG TT		

The 25 µL reactions contained 1X PCR buffer, 2.5 mM $MgCl_2$, 200μM dNTPs, 0.4 pmol primers for *rfc* and *rfpB*, 0.3 pmol primers for *invC*; 0.2 pmol primers for *wbgZ*, 0.05 U/µl Taq DNA polymerase, and 10 pg of extracted DNA. The standard strains of *Shigella* available at Pasteur Institute of Iran including *S. flexneri* (ATCC 12022), *S. sonnei* (ATCC 9290), *S. boydii* (ATCC 9207), and *S. dysenteriae* were used as positive controls, and *Salmonella typhi* (ATCC 14028), *Salmonella paratyphi B*, *E. coli* (ATCC25922), and deionized distilled water as negative controls. The PCR was performed in a thermocycler (TC- 320) with one cycle of initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing for 45 s at 60°C, and extension at 72°C for 45 s and a final extension at 72°C for 3 min. Finally, the PCR products were electrophoresed on a 2% agarose gel. The expected 875 bp product was observed in all *Shigella* strains, and the 211 bp, 430 bp and 537 bp fragments indicative of the species *S. dysenteriae*, *S. sonnei*, and *S. flexneri*, respectively.

RESULTS

In this study, 300 samples were simultaneously analyzed by using PCR and culture methods; 240 *Shigella* isolates (80%) were detected using the culture method followed by serotyping, and a total of 260 *Shigella* isolates (86.7%) using the PCR method (Figure 1). Using the culture method, 184 species belonging to *S. flexneri* (76.6%), 46 to *S. sonnei*

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(19.1%), and 10 to *S. dysenteriae* (4%) were isolated. Twenty samples suspicious of *Shigella* were observed in the culture method but were not identified by serotyping. However, in the PCR assay, they were identified as S. *flexneri* (n=16) and *S. sonnei* (n=4). PCR assay identified 200 isolates belonging to *S. flexneri* (% 77), 50 to *S. sonnei* (% 19.2), and 10 to *S. dysenteriae* (% 3.8) (Table 2). Also, 40 other Gram-negative isolates that were obtained by the culture method were 27 *E. coli* strains, 8 *Vibrio cholera inaba* strains, 2 *Morganelal morganii* strains, 2 *Pseudomonas aeruginosa* strains and 1 *S. paratyphi* B strains.

The samples were collected from at least 25 urban and rural centers in Isfahan Province. The patients were ranging in age from 2 to 86 years. About 15% of patients were under 5 years old, 35% between 5 and 20 years old, and 50% over 20 years old (Table 3).The age of the patients had no relationships with the type of the isolated bacteria (P= 0.124).

The standard strains of *Shigella* and non-*Shigella* strains obtained from the Pasteur Institute of Iran were used as positive and negative controls, and the PCR results of all the non-*Shigella* strains were negative, and they did not interfere with the reaction at all (Table 4).

The PCR was also performed for two samples of *S. boydii* that only reacted with the primer *invC* which determines the genus of *Shigella*, but the results were negative for the other primers, which is a sign of the specificity and accuracy of the Multiplex PCR method being used (Figure 2).

Given that *Shigella* are genetically very similar to *E. coli* (especially to the *E. coli* in EIEC subgroups) and are sometimes even called pathogenic *E. coli* [18], in this study, from among 27 samples of *E. coli*, 6 samples were EIEC on which Multiplex PCR was performed, and the results were negative.

The results of the antibiotic susceptibility test are summarized in Table 5. In this study, the drug resistance of *S. flexneri* was observed to be less than that of *S. sonnei*. Except for one case, where the drug resistance to Co-trimoxazole was higher in *S. flexneri* (90%) than in *S. sonnei* (70%) and *S. dysenteriae* (75%). Generally, the mean drug resistances in the 3 *Shigella* species were as follows: The moderate resistance was observed to Ceftriaxone (42.5), Cefixime (42), Azithromycin (40.7), and Ofloxacin (34.5%). The lowest resistance was observed to Ciprofloxacin (16%) and Nalidixic acid (25%). And multidrug resistance was observed to Co-trimoxazole (70%), Cefixime (71%), and Ceftriaxone (60%) in *S. sonnei* (Table 5).

Statistical analysis. The results obtained by descriptive and analytical statistical methods were evaluated, and the sensitivity and specificity of the Multiplex PCR method were determined relative to those of the culture method. And the Chi-square test was used to compare the frequency distributions.

Table 2. Comparison of the results between culture and PCR Method

Bacterial strains	No. of specimen tested (%) PCR Method	No. of specimen tested (%) Culture Method
Shigella genus	260 (86.7%)	240 (80%)
S. flexneri	200 (77%)	184 (76.6%)
S. sonnei	50 (19.2%)	46 (19.1%)
S. dysenteriae	10 (3.8%)	10 (4%)
Unidentified	40 (13.3%)	20 (6.6%)
Other Gram-negative	-	40 (13.4%)
Total No.	300	300

Table 3. Distribution of gender and the age groups of the patients

Variable	Number of patients	(%)
Gender		
Male	**140**	**46.6**
Female	**160**	**53.4**
Age group (years)		
≤5	**45**	**15**
5-20	**105**	**35**
>20	**150**	**50**
Total	**300**	**100**

Table 4. Bacterial species used in this study and results of multiplex PCR.

Bacterial strains	*InvC*	*Rfc*	*wbgZ*	*rfpB*
S. flexneri (ATCC 12022)	+	+	-	-
S. sonnei (9290)	+	-	+	-
S. boydii (ATCC 9207)	+	-	-	-
S. dysenteriae	+	-	-	+
Salmonella Typhi (ATCC 14028)	-	-	-	-
Salmonella Paratyphi B	-	-	-	-
E. coli (ATCC25922)	-	-	-	-

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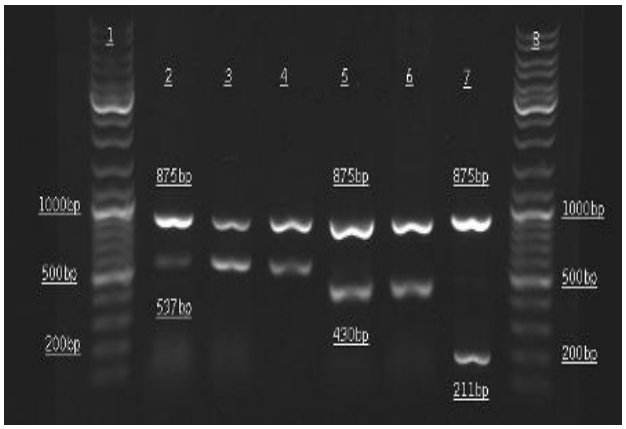

Fig. 1. Multiplex PCR assay profile. Lane 1, Ladder Mix (Thermo Scientific); lanes 2, 3, 4, strain (*rfc - S. flexneri*, *inv C-Shigella* genus); lanes 5, 6, strain (*wbgZS. Sonnei*, *invC-Shigella* genus); lane 7, strain (*rfpB S. dysenteriae*, *invC- Shigella* genus); lane 8, Ladder Mix (Thermo Scientific).

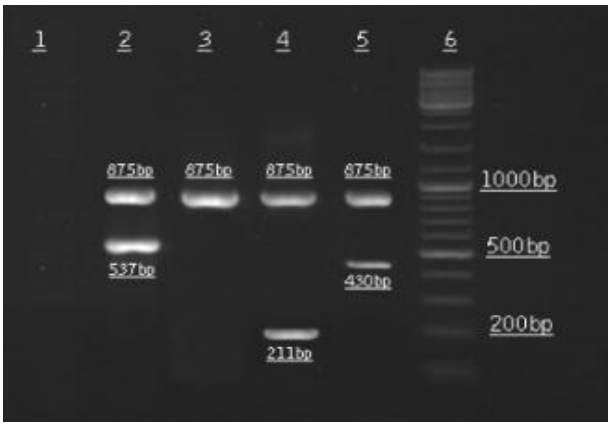

Fig. 2. Multiplex PCR assay profile with reference strains. Lane 1, *S. typhi* (ATCC 14028); lane 2, *S. flexneri* (ATCC 12022) (*rfcS. flexneri*, *invC -Shigella* genus); lane 3, *S. boydii* (ATCC 9207) (*invC - Shigella* genus); lane 4, *S. dysenteriae*375 strain (*rfpBS. Dysenteriae*, *invC-Shigella* genus); lane 5, *S. sonnei* (*wbgZ S. sonnei*, *invC –Shigella* genus); lane 6, Ladder Mix (Thermo Scientific).

Table 5. Antibiotic susceptibility profile of *Shigella* Spp. isolated in Isfahan province during 2014-2015

Antibiotic		Type of bacteria			Total
		S. sonnei	*S. flexneri*	*S. dysentry*	
Co-trimoxazole	R	70%	90%	75%	**78.3%**
	S	30%	10%	25%	22%
Cefixime	R	71%	30%	25%	**42%**
	I	0%	15%	12.5%	9.2%
	S	29%	55%	62.5%	48.8%
Ciprofloxacin	R	15%	8%	25%	**16%**
	I	25%	20%	12.5%	19.2%
	S	60%	72%	62.5%	64.8%
Ofloxacin	R	32%	9%	62.5%	**34.5%**
	I	0%	21%	0	7%
	S	68%	70%	37.5%	58.5%
Ceftriaxone	R	60%	30%	37.5%	**42.5%**
	I	10%	8%	12.5%	10.2%
	S	30%	62%	50%	47.3%
Nalidixic acid	R	35%	15%	25%	**25%**
	I	25%	45%	25%	31.6%
	S	40%	40%	50%	43.4%
Azithromycin	R	45%	27%	50%	**40.7%**
	I	5%	30%	12.5%	15.8%
	S	50%	43%	37.5%	43.5%

R: resistant, I: Intermediate susceptibility, S: sensitive

DISCUSSION

Controlling shigellosis or the dysentery due to *Shigella* species is of particular importance around the world. Because the agent of this disease in a very small amount (only 10 of them) can cause dysentery, which is easily transmitted fecal-orally from one person to another, and can cause big outbreaks by polluting water and food sources [19, 20]. In *Shigella* outbreaks, mortality is significant, especially in children, the elderly, and people with immune deficiency and malnutrition [21, 22].

The usual method to examine the agent of dysentery is culture and performing specific biochemical tests to identify the genus and species of bacteria, which is a time consuming and laborious method. Nowadays, DNA-dependent molecular methods, especially the PCR method, are widely used in scientific and research centers for the detection of shigellosis. Various PCR assays have been developed for the rapid detection of *Shigella*. But most of these methods cannot identify *shigella* species [23, 24].

The multiplex PCR method is an appropriate method which makes it possible to rapidly and simultaneously detect and identify several target genes in a single reaction [25, 27]. Ranjbar and colleagues employed a Multiplex PCR method to detect *S. boydii*, *S. sonnei*, and *S. flexneri* [28]. But, it did not simultaneously detect *Shigella* genus and species.

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We simultaneously compared the culture and Multiplex PCR methods in the patient samples. For the PCR method, we used the primers designed by Sowash Chandra *et al.* which is a sensitive, fast, and simple method, and which is capable of detecting the genus and the three species: *S. flexneri*, *S. sonnei*, and *S. dysenteriae* [17].

The results of this study verified the accuracy and precision of the PCR method compared with the culture method. And the Gram-negative strains had no longer any interference in the reaction.

Being aware of the species of the bacterium is effective in the way of dealing with an outbreak, controlling it, and also in treating patients. What has increased the importance of shigellosis in the world in recent years, is the incidence of progressive antibiotic resistance, particularly in developing countries [29]. Recent studies showed the high prevalence of antibiotic resistance in all *Shigella* isolates, especially the prevalence of multidrug resistance *S. sonnei*, in Iran [13, 30, 31]. In an outbreak of *S. sonnei* in Isfahan in summer 2013 1,086 individuals were reported with dysentery, and a high resistance to available antibiotics was detected [32].

Our results showed that the resistance of *Shigella* isolates to commonly prescribed antibiotics was still high and has increased, so that the resistance to ciprofloxacin (16%), and ofloxacin (34.5%) has increased compared with the previous study (14.8% and 25.9%). The multidrug resistance was also observed in *S. sonnei*, but in *S. flexneri* was less than in *S. sonnei*.

During the widespread outbreak of *S. flexneri* in Isfahan in 2015, 9,869 cases of dysentery were reported. The data showed the growing trend of shigellosis in Isfahan and Iran, which needs more care, rapid detection of outbreaks, and necessary measures to treat patients. Hence, before antibiotic treatment which causes higher drug resistance, it is recommended that, for the prompt and timely diagnosis of outbreaks, the Multiplex PCR method, as an exact and rapid method, be used with culture method in reference laboratories to classify *Shigella*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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