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Molecular Diagnosis of *Salmonella enterica* and *Shigella* spp. in Stool Sample of Children With Diarrhea in Tehran

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Background: Diarrheal illnesses caused by *Salmonella* and *Shigella* spp remain a serious public health problem in industrializing countries, and are still an important cause of morbidity and mortality in developed as well as undeveloped countries. Rapid detection of agents that cause diarrhea may help prevent occurrence of outbreaks.
Objectives: The aim of this study was to compare conventional biochemical tests, namely API 20E strip system and Polymerase Chain Reaction (PCR) for identification of *Salmonella enterica* and *Shigella* spp. isolated from stool samples of patients with diarrhea.
Materials and Methods: Stool samples were collected from patients with diarrhea during a three-year period (2009-2012). Conventional biochemical tests were used for identification of *Shigella* spp. and *Salmonella enterica* isolates. API 20E strips and PCR methods with oligonucleotide primers specific for *ipaH* of *Shigella* genus and *hilA* of *Salmonella enterica* were used to confirm the identity of the isolates.
Results: Of the 81 suspected *S. enterica* and 112 *Shigella* spp. identified by conventional biochemical tests, 77 and 105 were identified as *S. enterica* and *Shigella* spp. by API 20E, respectively. All of the isolates were assigned to bacterial species with 99.9% probability value. All of the 81 suspected *Salmonella enterica* isolates produced 784 bp amplification bands of *hilA* gene. Among the 112 *Shigella* isolates confirmed by PCR, 90 (80.35%) were positive for *wbgZ* and 14 (12.5%) were positive for *rfc* genes indicative of *S. sonnei* and *S. flexneri*, respectively.
Conclusions: In conclusion, the results of this study suggest that the PCR amplification of *hilA* and *ipaH* is a promising method for identification of *Salmonella enterica* and *Shigella* spp. The outcomes of this study can help towards more accurate and easy screening of large population of patients with *Salmonella enterica* and *Shigella* spp infections.

Keywords: Salmonella enterica; *Shigella*; API 20E; Polymerase Chain Reaction

1. Background

Diarrheal illnesses are still amongst serious health problems causing morbidity and mortality among children in both industrialized and developing countries. It is estimated that 17% of the annual incidents of diarrhea in children under five years old leads to death in developing countries (1). *Salmonella enterica* and *Shigella* spp. are still among the major causes of bacterial diarrhea in these countries, especially within rural areas (2).

Due to the importance of the disease caused by these bacteria, appropriate and rapid detection of microorganisms has a crucial role in directing the disease and the results especially among children of younger age. Different detection methods have been devised as a diagnostic tool to detect *Salmonella* and *Shigella* spp. Traditional culture methods require five to seven days for presumptive results and additional biochemical and serologic tests are required to identify specific serotypes. These conventional methods are generally time-consuming and labor intensive.

Therefore a rapid, specific and sensitive method for identification of microorganisms in different clinical samples is needed among which, by far Polymerase Chain Reaction (PCR) using genus-specific oligonucleotide primers is the most well-known and successfully implemented technique for detection nucleic acid (3). The API 20E diagnostic strip is a conventional method used to identify members of Enterobacteriaceae based on their biochemical properties. This test strip can detect 20 biochemical reactions (4). The *ipaH* gene is a multi-copy element that is involved in *Shigella* invasion. It is encoded on both the chromosome and the invasion plasmid. Thus, amplification of the *ipaH* genetic marker would make it possible to detect *Shigella* spp. through PCR amplification of this gene (5). The hyper-invasive locus A (*hilA*) gene is one of the important features of *Salmonella* pathogenesis, which is necessary for colonization of the extracel-

Implication for health policy/practice/research/medical education:
PCR amplification of *hilA* and *ipaH* is a reliable method for identification of *Salmonella enterica* and *Shigella* spp. The outcomes of this study can help towards more accurate and easy screening of large population of patients with *Salmonella enterica* and *Shigella* spp infections.

lular compartment of the host intestine and is present in all invasive strains of *Salmonell*a and absent from closely related genera such as *Escherichia* (6, 7).

2. Objectives

The aim of this study was to compare biochemical tests, namely API 20E strip system and Polymerase Chain Reaction (PCR) using specific primers for identification of *Salmonella* and *Shigella* spp. isolated from stool samples of patients with diarrhea.

3. Materials and Methods

3.1. Bacterial Isolates

Stool samples were collected from patients with diarrhea during a three-year period (2009-2012) from three major hospitals in Tehran, Iran. After a four-hour enrichment in GN broth, each sample was streaked onto XLD agar (Merck, Hamburg, Germany) and incubated at 37 °C for 24 hours. The suspicious gram negative and oxidase negative bacterial colonies were selected for further analysis.

3.2. Biochemical Tests

Preliminarily detection of the isolates was performed by conventional tests including TSI, SIM, ODC, LIA, Simmons Citrate and Urea. The phenotypic biochemical profiles of the isolates were compared with WHO criteria for biochemical identification of *Salmonella* and *Shigella* (4).

3.3. API 20E Strips

API 20 E strips (API-bioMérieux, Inc., La Balme les Grottes, France) consisting of 20 mini-test tubes of essential biochemical tests for identification of Enterobacteriaceae were used according to the manufacturer's instructions.

A saline suspension of the fresh cultures was inoculated into the tubes. The dehydrated substrates in the plastic tubes became rehydrated after adding a suspension of the bacteria. A few tubes were fully filled (CIT, VP and GEL), and some tubes were overlaid with mineral oil so that anaerobic reactions could be carried out (ADH, LDC, ODC, H_2S, URE). After inoculation, each strip was placed in the incubation box (tray and lid) provided by the manufacturer with 5 mL of distilled water in the tray of the incubation box to provide a humid atmosphere. The records from each tube were documented on the API 20 E analytical profile index. The software supplied by the manufacturer was used for analysis and data reporting.

3.4. Molecular Confirmation of Shigella spp. and Salmonella Enterica Isolates

Molecular confirmation of suspicious *Salmonella* or *Shigella* isolates was carried out via Polymerase Chain Reaction. Primer oligonucleotides specific for *Shigella* spp. (*ipaH*) and *Salmonella* spp. (*hilA*) were used for detection of each bacterial species. Subsequently, additional primer pairs specific for *S. sonnei* and *S. flexneri* were used for differentiation the two most commonly found *Shigella* species (Table 1).

DNA was extracted from logarithmic phase cultures by the boiling method. PCR was performed in a reaction mixture with total volume of 25 μL, containing 2.5 μL 10x Taq polymerase buffer, 0.3 μL dNTPs (10 $mmol.L^{-1}$), 1 U Taq DNA polymerase, 0.6 μL $MgCl_2$ (50 $mmol.L^{-1}$) and 0.3 $mol.L^{-1}$ of each primer. The PCR procedure was as follows: initial denaturation step at 94°C for 5 minutes followed by 30 cycles consisting of denaturation (94°C for 1 minute), annealing (58°C *ipaH*, 65°C *hilA*, 60°C *rfc* and *wbgZ*) for 1 minute, was set separately for each primer pair, and extension (72°C for 1 minute), followed by a final extension step at 72°C for 5 minutes. The specificity of the oligonucleotide primers were evaluated by using a series of enteric bacteria including *E. coli* ATCC25922, *Vibrio cholerae* ATCC14035, *Entrobacter faecalis* ATCC19433, *Aeromonas hydrophila* ATCC 7965 as negative controls and standard reference strains including *S. sonnei* ATCC 1202 and *S. flexneri* ATCC 9290, *S. dysenteriae* and *S. Boydii* kindly provided by the World Health Organization (WHO) as positive controls.

Table 1. Oligonucleotide Primers Used in This Study

Primer target	Sequence (5' to 3')	Amplicon size (bp)	Reference
***ipaH*-F**	GTTCCTTGACCGCCTTTCCGATACCGTC	619	(8)
***ipaH*-R**	GCCGGTCAGCCACCCTCTGAGAGTAC		
***hilA*-F**	CGGAACGTTATTTGCGCCATGCTGAGGTAG	784	(9)
***hilA*-R**	GCATGGATCCCCGCCGGCGAGATTGTG		
***Sflex*-F**	TTTATGGCTTCTTTGTCGGC	537	(10)
***Sflex*-R**	CTGCGTGATCCGACCATG		
***Sson*-F**	TCT GAATATGCCCTCTACGCT	430	(10)
***Sson*-R**	GACAGAGCCCGAAGAACCG		

4. Results

4.1. Biochemical Identification of Isolates

All of the isolates with Alk/A feature of TSI with H_2S production, LDC$^+$, Indole$^-$, motility$^+$ in SIM tube, ODC$^+$, Urease$^-$ and Simmons Citrate$^+$ were assigned to *Salmonella* spp. Furthermore, *Shigella* spp. were characterized by Alk/A without hydrogen sulphide production in TSI agar, LDC$^-$, Indole$^-$, motility$^-$ in SIM tube, Urease$^-$ and Simmons Citrate$^-$ and Ornithine decarboxylation activity (negative in *S. dysenteriae*, *S. flexneri*, *S. boydii* and positive in *S. sonnei*) in LDC tube, negative urease activity and inability of citrate utilization in Simmons Citrate tube. A total of 112 *Shigella* spp. and 81 *Salmonella enterica* were isolated from patients with acute diarrhea from Tehran, Iran.

4.2. API 20E Analysis

Of the 81 suspected *S. enterica* isolates, 77 were identified as *S. enterica* by API 20E and 105 out of 112 biochemically identified *Shigella* spp. were confirmed by API 20E. All of the isolates were assigned to bacterial species with 99.9% probability value (Table 2).

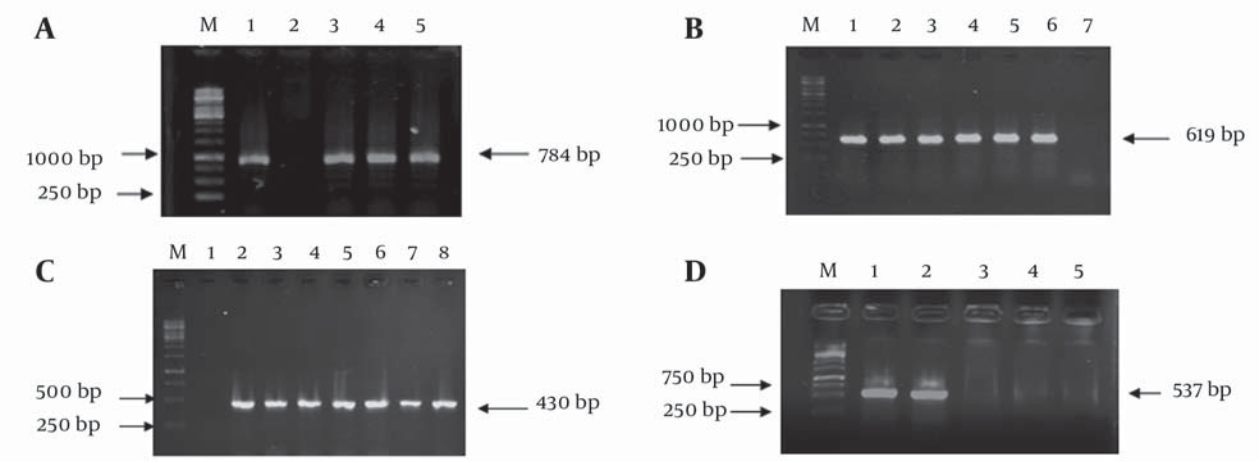
4.3. Molecular Detection of Salmonella and Shigella Isolates

PCR amplification of *ipaH* gene showed a single band of expected size (619bp) in all 112 suspected *Shigella* spp. under study. All of the 81 suspected *Salmonella enterica* isolates produced 784 bp amplification band of *hilA* gene. Among the total 112 *Shigella* isolates, 90 (80.35%) were positive for *wbgZ* and 14 (12.5%) were positive for *rfc* genes indicative of *S. sonnei* and *S. flexneri*, respectively. Four *ipaH* + isolates were negative for both rfc and *wbgZ* genes, which may be an indication of probable *S. boydii* or *S. dysenteriae* (Figure 1. A-D). The Comparison of biochemical, API 20E and PCR tests results of *Salmonella* and *Shigella* isolates are shown in Table 3.

Table 2. Comparison of Identification of Isolates by two Different Methods

Test	No. of Isolates With Positive Reactions	
	Shigella spp.	*Salmonella enterica*
API 20E	105	77
PCR	112	81
Total	112	81

Figure 1. Detection of *Salmonella enterica*



A) Detection of *Salmonella enterica* by *hilA* gene. Lane 1, positive control; lane 2, negative control; lane 3-5, isolates under study; M, 1kbp size marker. B) Detection of *Shigella* spp. by *ipaH* gene. Lane 1, positive control; lane 2-6, isolates under study; lane 7, negative control; M, 1 kbp size marker. C) Detection of *S. sonnei* by *wbgZ* gene. Lane 1, positive control; lane 2-7, isolates under study; lane 8, negative control; M, 1kbp size marker. D) Detection of *S. flexneri* isolates by *rfc* gene. Lane 1, positive control; lane 2-4 isolates under study; lane 5, negative control; M, 1kbp size marker.

Table 3. The Comparison of Biochemical, API 20E and PCR Tests Results for *Salmonella* and *Shigella* Isolates [a]

Isolates	Biochemical tests	API 20E	PCR			
			ipaH	*wbgZ*	*rfc*	*hilA*
S. sonnei	85	70	90	90	0.0	0.0
S. flexneri	0.0	0.0	14	0.0	14	0.0
***Shigella* spp**	26	34	4	0.0	0.0	0.0
***S. sonnei* ATCC 1202**	01	01	01	01	0.0	0.0
***S. flexneri* ATCC 9290**	0.0	0.0	01	0.0	01	0.0
***S.dysenteriae* WHO**	0.0	0.0	01	0.0	0.0	0.0
***S. boydii* WHO**	0.0	0.0	01	0.0	0.0	0.0
Salmonella enterica	81	77	0.0	0.0	0.0	81
V.cholerae	01	0.0	0.0	0.0	0.0	0.0
***E. coli* ATCC 25922**	01	01	0.0	0.0	0.0	0.0
***E. faecalis* ATCC 19433**	01	01	0.0	0.0	0.0	0.0
***A. hydrophila* ATCC 7965**	01	01	0.0	0.0	0.0	0.0
False Negative	0.0	11	0.0	0.0	0.0	0.0

[a] Abbreviation: PCR, polymerase chain reaction.

5. Discussion

Considering conventional biochemical tests as the gold standard for identification of these microorganisms, sensitivity of API strips was calculated as, [(number of isolates determined as positive by both API and conventional biochemical tests)/(total number of isolates determined as positive by conventional biochemical tests)] × 100], and sensitivity of PCR method was calculated using the following formula, [(number of isolates determined as positive by both PCR and conventional biochemical tests)/(total number of isolates determined as positive by conventional biochemical tests)] × 100]. Sensitivity of API strip was 95% and 93.7% for *Salmonella enterica* and *Shigella* spp., respectively; however, the sensitivity of PCR was 100% for both microorganisms. There are multiple studies that report controversial results on API, either as a reliable (11, 12) or imprecise diagnostic method (13, 14).

Commensurate to the findings of Nucera et al. which declared that PCR and API 20E (at 99.9% likelihood level) are accurate diagnostic tests and either of them can be used to give similar results (15); we believe that API is an accurate tool for detection of *Salmonella* and *Shigella* but should not be used as the sole diagnostic test and could only be used in screening large number of specimens in outbreaks or epidemics and confirmation should be through a promising molecular method including PCR. The most important deficiency of the API 20E test is the false negative results of this technique which may be due to the fact that it does not present all the necessary tests for identification of some species of bacteria. However, the need for equipment like a thermocycler and gel electrophoresis apparatus in the PCR test (16), makes API 20E test an economical option in low income conditions. Our study provided strong evidence indicating that a number of strains may be misidentified through false negative results of the API 20E test, which may lead to crucial strain losses. Moreover, considering the costs and time of analysis, the PCR method is an advisable, faster and a more specific method for identification of *Salmonella enterica* and *Shigella* spp.

In an international research project for the standardization and validation of the PCR assay for detection of five major food borne pathogens including *Salmonella*, the most selective primer pair was found to be a region that targets the *hilA* gene. Further studies have reported that PCR with primers for *hilA* which are specific for *Salmonella* spp. are sensitive and specific for detection of *Salmonella* in many clinical samples (17-19) as well as carriers (9).

One of the important properties of *Shigella* pathogenesis is its invasiveness which can be traced by detecting the virulence gene, *ipaH*, located on the large invasive plasmid. The multi copies of *ipaH* gene and its presence in all *Shigella* spp. makes it possible to detect all suspected *Shigella* isolates through gene detection. All of the *Shigella* spp. isolates in this study harbored the *ipaH* gene, a feature, which has also been reported previously (20). The majority of shigellosis cases are caused by three species of *Shigella: S. flexneri*, *S. sonnei*, and *S. dysenteriae,* among which *S. flexneri* is mainly encountered in developing and *S. sonnei* in industrialized countries (21, 22). The dominance of *S. sonnei* isolates among the Iranian population provides additional documents on the improvement of hygienic and public health standards in Iran.

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Authors' Contribution

All author participated equally in the present study.

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There is no conflict of interest.

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