JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1999, p. 3608–3611 Vol. 37, No. 11
0095-1137/99/$04.00+0

Prevalence of *Shigella* Enterotoxins 1 and 2 among *Shigella* Strains Isolated from Patients with Traveler's Diarrhea

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Received 30 December 1998/Returned for modification 23 June 1999/Accepted 9 August 1999

***Shigella* spp. are known primarily as a cause of bacillary dysentery. However, in an initial phase, numerous patients exhibit watery diarrhea that may or may not be followed by dysentery. New virulence factors associated with the species of *Shigella* have recently been described. These are enterotoxins 1 and 2 of *Shigella* (ShET-1 and ShET-2, respectively). The aim of the present study was to determine the prevalence of ShET-1 and ShET-2 in species of *Shigella* isolated from patients with traveler's diarrhea. During the period from 1993 to 1998, stool samples from 500 travelers with diarrhea were cultured for the isolation of *Shigella* spp. and other enteropathogens. The detection of ShET-1 and ShET-2 was performed by a PCR technique with specific primers. Among a total of 51 strains of *Shigella* isolated during this period (22 *S. flexneri*, 26 *S. sonnei*, and 3 *S. dysenteriae* strains), at least one enterotoxin was detected in 31 (60.78%) strains; 2 (9.09%; both of which were *S. flexneri* strains) produced only ShET-1, while 21 (41.17%; 3 *S. flexneri*, 15 *S. sonnei*, and 3 *S. dysenteriae* strains) produced ShET-2. Furthermore, 8 (15.69%) of 22 *S. flexneri* strains presented both enterotoxins. Our results show that the prevalence of ShET-2 was high in all the *Shigella* species studied and confirm that ShET-1 is detected only in *S. flexneri*.**

Shigella species are an important cause of diarrheal disease in developing countries (2) and in travelers to tropical countries (16, 18). About 50% of Spanish travelers who visit these countries develop diarrhea, and *Shigella* species are among the main etiological agents (6–8). Among the *Shigella* species, *Shigella dysenteriae* is especially known as a cause of bacillary dysentery; this has also been shown with *S. flexneri* (4). However, in a considerable number of patients, watery diarrhea is shown in the first phase of the infection, which may or may not be followed by dysentery, similar to what is seen for the species *S. flexneri*, *S. sonnei*, and *S. boydii*, although the last two species generally produce a self-limited, watery diarrhea (4, 9).

Several virulence factors have been associated with *Shigella* spp., the most common being the ability to colonize and invade the intestinal cells. This phenomenon is mediated, in part, by the invasion-associated locus (*ial*), which is carried on a plasmid of 120 to 140 MDa (5), and the invasion plasmid antigen H (*ipaH*) gene, which is present in multiple copies in both the plasmids and the chromosomes of these organisms (17).

Another virulence factor related to *S. dysenteriae* is its capacity to produce an exotoxin called Shiga toxin (Stx), which is not excreted by the bacteria but is released only during cell lysis (1). Despite its clear toxigenicity, the role of Stx in shigellosis is not clear, since it is known that Stx is not essential for invasion or cellular lysis.

Two new enterotoxins have recently been described in *S. flexneri* 2a. The first toxin is called *Shigella* enterotoxin 1 (ShET-1), which is encoded in the *set1* chromosomal gene. It has been suggested that the active toxin of ShET-1 has a configuration of one A subunit and several B subunits (A_1-B_n) (13).

The second enterotoxin, called *Shigella* enterotoxin 2 (ShET-2), is encoded in the *sen* gene. This gene is located on a plasmid of 140 MDa which is associated with invasion in these pathogens (12).

The main aim of this study was to determine the prevalence of ShET-1 and ShET-2 in *Shigella* species isolated from patients with traveler's diarrhea.

MATERIALS AND METHODS

Patients. During the period from 1993 to 1998, stool specimens from 500 patients with traveler's diarrhea (TD) were analyzed. TD was defined as the occurrence of three or more episodes of watery diarrhea within a 24-h period, with or without other symptoms, between 12 h after arrival in and 5 days after departure from the country visited or as the occurrence of unformed stools accompanied by one of the following: cramps, tenesmus, vomiting, nausea, fever, chills, and prostration.

Microbiological tests. To isolate *Shigella* species, stool samples were inoculated onto MacConkey agar and Salmonella-Shigella agar (Becton Dickinson, Heidelberg, Germany), and the resulting colonies which exhibited characteristics of *Shigella* spp. were identified by conventional biochemical methods (11). Subsequently, the species were identified with specific polyvalent antisera against *S. flexneri*, *S. sonnei*, and *S. dysenteriae* (Diagnostics Pasteurs, Marnes-la-Coquette, France) and *S. boydii* (Difco Laboratories, Detroit, Mich.).

PCR assay. The detection of ShET-1 (A and B subunits) was performed by amplifying both *set1A* and *set1B* genes by PCR. A PCR technique was also used to detect the *sen*, *ipaH*, *ial*, and *stx* genes. The isolated *Shigella* species were grown on MacConkey agar overnight. One colony of each isolate was suspended in 25 μl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM KCl, 3 mM $MgCl_2$, 0.1% gelatin, 400 μM (each) deoxynucleoside triphosphate, and 1 μM primer together with 2.5 U of *Taq* polymerase (GIBCO-BRL). The reaction mixture was overlaid with mineral oil and was subjected to the following program: 30 cycles at 95°C for 50 s, 55°C for 1.5 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Seven sets of primers, obtained from Boehringer Mannheim (Mannheim, Germany), were used to amplify the *set1A*, *set1B*, *sen*, *ipaH*, *ial*, and *stx* genes, as indicated in Table 1.

PCR assays were performed in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Emeryville, Calif.). A reagent blank, which contained all components of the reaction mixture with the exception of the bacteria, was included in every PCR procedure.

Amplification products were subjected to gel electrophoresis in 2% agarose and were detected by staining with ethidium bromide.

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TABLE 1. Primers used for identification of virulence factors of *Shigella* spp.

Gene encoding virulence factor	Primer	Oligonucleotide sequence (5′ to 3′)	Location within gene	Size of amplified product (bp)	Reference(s)
set1A	ShET-1A upper	TCACGCTACCATCAAAGA	460–477		
	ShET-1A lower	TATCCCCCTTTGGTGGTA	751–768	309	3
set1B	ShET-1B upper	GTGAACCTGCTGCCGATATC	70–89		
	ShET-1B lower	ATTTGTGGATAAAAATGACG	197–216	147	3
sen	ShET-2 upper	ATGTGCCTGCTATTATTTAT	380–399		
	ShET-2 lower	CATAATAATAAGCGGTCAGC	1158–1178	799	12
ipaH	Shig-1	TGGAAAAACTCAGTGCCTCT	1063–1083		
	Shig-2	CCAGTCCGTAAATTCATTCT	1466–1485	423	10
ial	ial upper	CTGGATGGTATGGTGAGG	5340–5357		
	ial lower	GGAGGCCAACAATTATTTCC	5640–5659	320	5
Stx	stx upper	CAGTTAATGTGGTTGCGAAG	50–69		
	stx lower	CTGCTAATAGTTCTGCGCATC	924–944	895	14, 15

RESULTS

The products from amplification DNA by the different PCRs are shown in Fig. 1. In the amplification reaction with the *set1A* and *set1B* genes, which encode the ShET-1 A and B subunits, respectively, bands of 309 bp (A subunit) and 147 bp (B subunit), respectively, were observed. The product amplified from the *sen* gene, which encodes ShET-2, was 799 bp. The PCR product of the *ipaH* gene was 423 bp, and that of the *ial* gene was 320 bp. Amplification of the *stx* gene produced a PCR product of 895 bp. All strains that were ShET-1A positive were also found to be ShET-1B positive. The DNA sequences of the PCR products were determined to confirm amplification of the correct gene. The geographic distributions of the *Shigella* spp. studied are provided in Table 2.

Among the 51 strains of *Shigella* isolated, 31 (60.78%) were found to produce ShET-1 and/or ShET-2 (Table 3). Among the toxin producers, 2 *S. flexneri* strains (3.92%) produced only ShET-1 (A and B subunit positive), 21 (41.17%; 3 *S. flexneri*, 15 *S. sonnei*, and 3 *S. dysenteriae* strains) produced only ShET-2, and 8 (15.69%) *S. flexneri* strains produced both enterotoxins. Furthermore, all strains of *Shigella* were also *ipaH* positive, whereas only 29 (57%) were *ial* positive. Only the three *S. dysenteriae* isolates were positive for the *stx* gene. If the detection of ShET-2 is considered in correlation with the detection of *ial*, 3 of 25 *Shigella* strains (2 *S. flexneri* strains and 1 *S. sonnei* strain) isolated during the period from 1993 to 1995 were positive for both the *sen* and the *ial* genes, whereas 26 of 26 *Shigella* strains (9 *S. flexneri*, 14 *S. sonnei*, and 3 *S. dysenteria* strains) isolated during the period from 1996 to 1998 were positive for both genes.

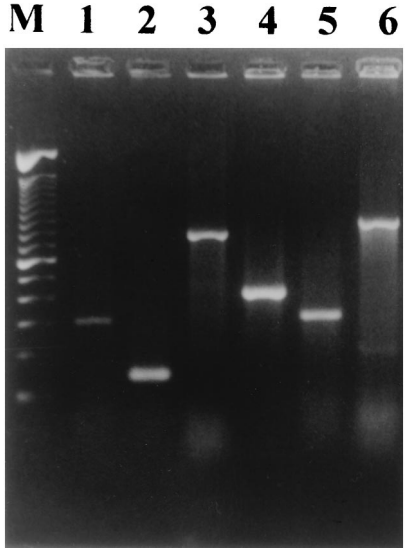

FIG. 1. Agarose gel electrophoresis showing PCR amplification products. Lane M, DNA molecular size markers (100-bp DNA ladder from Gibco-BRL). The products for detection of the *set1A* gene (lane 1), *set1B* gene (lane 2), *sen* gene (lane 3), *ipaH* gene (lane 4), *ial* gene (lane 5), and *stx* gene (lane 6) are shown.

DISCUSSION

Acute dysentery is a common disease in many developing countries, whereas in developed countries diarrhea is often acquired during travel abroad and hence is TD. In our study, different virulence factors associated with the pathogenicity of *Shigella* spp. were investigated. In the colon, the bacteria invade the mucosal cells. Some of the genes involved in the invasion have been identified and have been designated *ipa*, for invasion plasmid antigen. However, one of these genes, the *ipaH* gene, which encodes a 60-kDa antigen, is found in multiple copies on both the invasion-related plasmid and the chromosome. All *Shigella* species that we studied were positive for this gene. Another virulence factor gene associated with the invasion of the cell by *Shigella* species is the *ial* gene, which is plasmid encoded. In our study, 57% of the *Shigella* strains were positive for *ial*. This fact is discussed below. The geographic distribution (by continent) of the different species of *Shigella*, as well as those of the ShET-1 and/or ShET-2-producing strains, was homogeneous.

S. dysenteriae, as is already known, was the only species of the genus *Shigella* that produces Stx.

In a considerable number of patients, watery diarrhea is observed prior to the onset of dysentery. This is likely explained by the synthesis of two recently described enterotoxins, ShET-1 and ShET-2. However, in our study a significant difference was not observed when clinical symptoms and the isolation of ShET-1- and/or ShET-2-producing *Shigella* strains were compared (data not shown). Noriega et al. (13) found ShET-1 almost exclusively in *S. flexneri* 2a. In our study, although no serotyping was performed, ShET-1 was found in

TABLE 2. Geographic distribution of *Shigella* isolates from patients with TD

Geographic area	Total no. of isolates tested[a]	No. of isolates that produced the indicated toxin:									
		S. flexneri (n = 22)				*S. sonnei* (n = 26)				*S. dysenteriae* (n = 3)			
		ShET-1	ShET-2	ShET-1 + ShET-2	None	ShET-1	ShET-2	ShET-1 + ShET-2	None	ShET-1	ShET-2	ShET-1 + ShET-2	None
America (17)[b]													
Central America	15	1	1	1	2		5		5				
South America	2		1		1								
Africa (16)													
West Africa	9		1	4	2		1		1				
East Africa	4			1							3		
North Africa	3						3						
Asia (18)													
India	14	1		1	4		5		3				
Middle East	2						1		1				
Southeast Asia	2			1					1				
Total	51	2	3	8	9		15		11		3		

[a] Total number of strains in each geographic area.
[b] Values in parentheses are total number of strains from each continent.

TABLE 3. Prevalence of ShET-1 and ShET-2 in *Shigella* spp. isolated from patients with TD

Strain	Total no. of strains tested	No. (%) of strains that produced the following:		
		ShET-1	ShET-2	ShET-1 and ShET-2
S. flexneri	22	2 (9.09)	3 (13.64)	8 (36.36)
S. sonnei	26	0 (0)	15 (57.69)	0 (0)
S. dysenteriae	3	0 (0)	3 (100)	0 (0)
Total	51	2 (3.92)	21 (41.17)	8 (15.69)

45% of *S. flexneri* strains but was not found in either *S. sonnei* or *S. dysenteriae*, confirming the previous results. In 36% of *S. flexneri* strains, ShET-1 was found together with ShET-2. ShET-2 was also found alone in 14% of *S. flexneri* strains, 57% of *S. sonnei* strains, and 100% of *S. dysenteriae* strains. The ShET-1 toxin shows an A-B structure. The PCRs with primers designed to amplify either the *set1A* gene (which encodes the A subunit) or the *set1B* (which encodes the B subunit) showed complete correlation, although the intensity of the PCR product was stronger for the *set1B* gene than for the *set1A* gene. Although the strains carry the genes for the toxins, they may not express the toxin.

Nataro et al. (12) found the *sen* gene (which encodes ShET-2), using a DNA probe, in 73% of the *S. flexneri* strains that they studied, whereas we found this gene in 50% of the *S. flexneri* strains that we studied. However, our results showed bias because some of these strains have been stored since 1993, and as this gene is plasmid encoded, these strains could have lost the plasmid, which is known to occur frequently upon repeated subculturing and prolonged storage (10, 17). This is demonstrated by the fact that only 3 (12%) of 25 *Shigella* strains isolated from 1993 to 1995 were positive for both the *sen* and the *ial* plasmid-encoded genes, whereas 100% of *Shigella* strains isolated from 1996 to 1998 were positive for both genes.

In conclusion, this study shows the high prevalence of ShET-1 and ShET-2 in *S. flexneri* strains and the high prevalence of ShET-2 in *S. sonnei* and *S. dysenteriae* strains isolated from patients with TD.

ACKNOWLEDGMENT

This work was supported in part by grant FIS 94/0980 from Fondo de Investigaciones Sanitarias de la Seguridad Social of Spain.

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