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SHORT PAPER
Prevalence of the genes for shigella enterotoxins 1 and 2 among clinical isolates of shigella in Israel

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SUMMARY

Two enterotoxins, shigella enterotoxin 1 (SHET1) and shigella enterotoxin 2 (SHET2) have been recently characterized and are believed to play a role in the clinical manifestation of shigellosis. One hundred and twenty-one isolates of *Shigella* spp. of 13 different serotypes and variants and 10 isolates of enteroinvasive *Escherichia coli* (EIEC) isolated in Israel, were examined by polymerase chain reaction for the presence of SHET1 and SHET2 genes. SHET1 was only prevalent among isolates of *S. flexneri* 2a while SHET2 was found in all the serotypes that were tested except for several isolates of *S. flexneri* 1b that lost their virulence plasmid during storage. In addition, we found that the *S. flexneri* 2a vaccine strain T-32 Istrati contains the gene encoding for SHET1 but not that encoding for SHET2, suggesting that the latter is located within a large deletion occurring in the 140 Mda plasmid of this *S. flexneri* 2a non-invasive vaccine strain.

Shigella species are important pathogens responsible for diarrhoeal diseases and dysentery occurring all over the world. The morbidity and mortality due to shigellosis are especially high among children in developing countries. Travellers from developed to developing regions and soldiers serving under field conditions are also at an increased risk to develop shigellosis. Two enterotoxins have recently been recognized as virulence factors: (a) chromosome encoded shigella enterotoxin 1 (SHET1) which is present in all *Shigella flexneri* 2a [1] but rarely found in other shigella serotypes [2], and (b) shigella enterotoxin 2 (SHET2) which is located on a large plasmid associated with virulence of shigella [3]. SHET2 was found in many, but not all, shigella of different serotypes and also in enteroinvasive *Escherichia coli* (EIEC) [3, 4]. The soluble toxins, SHET1 and SHET2, show significant enterotoxic activity *in vitro* when tested in rabbit ileal loops and Ussing chambers. Furthermore, inactivation of these enterotoxins through genetic engineering is used for attenuation of new shigella vaccine candidates [5].

There is limited information on the presence of SHET1 and SHET2 among clinical isolates of different shigella serotypes. In the present work we examined the prevalence of the two enterotoxins among various shigella strains of different serotypes isolated from patients with dysentery in Israel.

One hundred and twenty-one isolates of shigella and 10 of EIEC were examined. Two sets of primers were designed from the published sequence data of SHET1 [6], (gene bank accession no. Z47381) and of SHET2 [3], (gene bank accession no. Z54211). The primers for SHET1 amplified a 722 fragment encoding the complete ORF including SHET1A and SHET1B (1-5′ATGGTTCAGCGTAATATTCCCTTCATACTGG; 2-5′TTACTGGATCTTAAGGCTCAGGATACAAATG). The primers for SHET2 amplified a

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Table 1. *Prevalence of SHET1 and SHET2 among* shigella *and EIEC isolates*

Serotype	SHET1 positive (isolates tested)	SHET2 positive (isolates tested)	No. of different strains*
S. flexneri 1b	0 (26)	24 (26)	3
S. flexneri 1b mannitol negative	0 (11)	1 (10)†	1
S. flexneri 2a	19 (19)	18 (18)	9
S. flexneri 6	0 (6)	7 (7)	6
S. flexneri 6 mannitol negative	0 (3)	3 (3)	3
S. sonnei	0 (20)	20 (20)	7
S. boydii (2 serotypes‡)	0 (14)	13 (13)	4
S. dysenteriae (4 serotypes§)	0 (18)	20 (20)	7
T-32 istrati(8)	1 (1)	0 (1)**	
EIEC††	0 (10)	10 (10)	10

* Isolates that were involved in a single outbreak were counted as a single strain.
† All SHET2 negative strains were also *virF* and *ial* negative.
‡ *S. b.* 2; *S. b.* 10.
§ *S. d.* 1; *S. d.* 2; *S. d.* 3; *S. d.* E112707 (provisional type).
** *VirF* positive, *ial* negative.
†† O144 serotype (10).

440 bp fragment located within the ORF of SHET2 (1-5′CGTTAGAATTACTTTTGGCAGC; 2-5′GG-CCAGCAAATTTACAATATCC). Bacteria were subcultured from a frozen stock onto McConkey agar plates and grown overnight at 37 °C. Five to ten colonies of each strain were suspended in water, boiled for 10 min and 5 μl of the boiled suspension was used in the PCR. Each reaction was performed in a 50 μl mixture consisting of 10 mM Tris–HCL, pH 8·8, 1·5 mM $MgCl_2$, 150 mM KCl, 0·1 % Triton X-100, 0·25 mM dNTP's mix, 0·8 unit DyNAzyme II DNA polymerase (Finnzymes, Finland), 0·4 μM of each primer, and 5 μl template. PCR was performed in a PTC-200 Thermal Cycler (MJ Research, USA). The amplification procedure included pre-incubation at 95 °C for 30 sec, 30 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 60 sec at 72 °C, and final incubation at 72 °C for 5 min. The products were separated by electrophoresis on a 0·8 % agarose gel and visualized by staining with 0·5 μg/ml ethidium bromide. The specificity of the assay was verified by testing salmonella (8 variants), klebsiella, enterobacter, proteus (2 variants), *E. coli* O157 and enterotoxigenic *E. coli*. All of the above were negative. Assays to detect the presence of two other plasmid encoded virulence factors of shigella, *virF* and *ial*, were carried out as described by Yavzori et al. [7].

The main findings of the study are summarized in Table 1. The chromosome encoded enterotoxin, SHET1 was found in all the isolates of *S. flexneri* 2a that were tested, including the Romanian attenuated vaccine strain T-32 Istrati [8]. In contrast to the report of Noriega et al. [2], we did not detect SHET1 in any shigella serotypes isolated in Israel except for *S. flexneri* 2a.

The plasmid encoded enterotoxin SHET2 was identified in all the strains that were tested except for 9 out of 10 mannitol-negative *S. flexneri* 1b and the T-32 Istrati vaccine strain. Vankatesan et al. [1] showed that the attenuation of the Istrati strain is the result of a large deletion in the virulence plasmid, covering several virulence genes, *virG*, *invA*, and *ipaBCDA*. In agreement with the previous results, we found that the Istrati strain was negative for *ial* that is associated with the *ipa* genes [7] but was positive for *virF*, showing that the plasmid was not lost. Therefore, our findings suggest that the locus encoding for SHET2 is probably part of the deleted region in the large 140 MDa plasmid of the Istrati strain and that the absence of SHET2 may contribute to the high degree of attenuation of this vaccine strain.

Previous studies have showed SHET2 in only 83 % [3] and 41 % [4] of shigella strains. In our study however, only a single serotype was negative for SHET2 and the isolates that were tested originated from a single outbreak and were stored for 12 years.

They were also negative for all three plasmid-encoded genes SHET2, *virF* and *ial*, indicating that the virulence plasmid was lost in these specific isolates perhaps as a result of storage. According to our records, the original strain was fully virulent and was associated with a typical clinical picture of dysentery including symptoms such as fever, bloody diarrhoea, stomach-ache, headache, nausea and vomiting. However, none of the other shigella isolates that were stored under the same conditions showed a significant plasmid loss as evidenced by the presence of the SHET2 gene. We further attempted to test the rate of loss of the virulence plasmid by different shigella serotypes *in vitro* during growth in a rich medium. The marker for the presence of the virulence plasmid was positive Congo-red morphology [9]. Bacteria were plated on Congo-red agar plates and incubated overnight at 37 °C. Several positive colonies were passaged three times in BHI medium and suspensions were again plated on Congo-red agar plates. The relative number of negative colonies estimated the rate of plasmid loss. No significant plasmid loss *in vitro* was found in *S. flexneri* 2a, *S. flexneri* 6 (mannitol negative), *S. flexneri* 6 (mannitol positive) and *S. boydii*. However, a significant plasmid loss rate ranging between 7% and 95%, depending on the isolate, was found for *S. flexneri* 1b (both mannitol-negative and mannitol-positive strains). The relatively high rate of plasmid loss *in vitro* by *S. flexneri* 1b as compared to other shigella serotypes remains unexplained but may account for the plasmid loss during storage among the *S. flexneri* 1b isolates in our collection.

In summary, among the 13 shigella serotypes and variants that were tested, only *S. flexneri* 2a carried the SHET1 gene. SHET2 gene is probably present in all shigella serotypes isolated from clinical samples. We provided evidence that some serotypes may lose the virulence plasmid more rapidly then others during processing or storage and this may account for the lower prevalence of SHET2 reported in previous studies [3, 4]. Further, we suggest that the absence of SHET2 may contribute to the high safety profile of the T32 Istrati vaccine strain.

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