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Molecular epidemiology of shigella infections in Israel

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SUMMARY

The DNAs of *Shigella sonnei* or *Shigella dysenteriae* type 1 strains isolated in outbreaks of shigellosis or in sporadic cases were analysed by restriction fragment length polymorphism (RFLP). Southern blots of the DNAs of 36 *S. sonnei* isolates digested by 8 restriction enzymes were hybridized with an *Escherichia coli* rRNA probe. The *S. sonnei* strains were unexpectedly diverse in their RFLP. Antibiotypes of the same isolates showed clusters of strains corresponding to the various outbreaks. On the other hand, RFLP analysis suggested concomitant multiple sources of infection rather than a common source and thereby introduced a new insight in the epidemiology of shigellosis. RFLP was also used to trace *S. dysenteriae* type 1 transmission in a recent cluster of clinical cases. Although antibiotic resistance patterns indicated the presence of more than one strain, RFLP analysis showed that the six isolates were identical clones and suggested the loss of an R episome after one person-to-person passage.

INTRODUCTION

Epidemiological investigations of shigella infections can be refined by serotyping [1]. However further refinement would be useful, particularly for *Shigella sonnei* which is represented by a single serotype that accounts for at least 50% of the shigella infections occurring in Israel [2] and is frequently isolated worldwide. Better differentiation of other prevalent serotypes of *S. flexneri* such as *S. flexneri* 2a or *S. flexneri* 6 would also be helpful. Strains of *S. dysenteriae* which produce Shiga toxin and cause the most severe disease belong to a single serotype, *S. dysenteriae* type 1 and although cases of dysentery caused by these organisms rarely occur in Israel, it is of public health interest to trace their origin and mode of transmission.

Epidemiological markers used in addition to serotyping such as phage and colicin typing lack adequate standardization [3, 4]. Plasmid profile analysis has been used in the differentiation of isolates belonging to *S. sonnei* and *S. dysenteriae* type 1 isolates, mostly during the investigation of outbreaks [5, 6, 7]. However the loss of plasmids due to long-term storage or numerous passages represents a serious drawback in this method [3, 8, 9]. Restriction fragment length poly-

morphism (RFLP) analysis of bacterial chromosomal DNA has recently been employed as a tool in the molecular epidemiology of various infections and has proven to be very discriminating [10, 11].

The objective of the present study was to examine the usefulness of RFLP analysis as an epidemiological tool to differentiate shigella strains within the same serotype. This evaluation was carried out on strains of *S. sonnei* and *S. dysenteriae* type 1 isolated in clusters of shigellosis. It was of interest to clarify if a single clone of *S. sonnei* was responsible for all the cases of *S. sonnei* shigellosis in outbreaks caused by this organism. It was also important to verify if a single or more strains infect very closely located sites one year after the other indicating the existence of a long term reservoir of these organisms in that region.

Because of the rarity of *S. dysenteriae* in Israel [7], it was of interest to verify if the cluster of cases of Shiga dysentery occurring by the end of 1989 was infected by an organism identical to that involved in an outbreak of dysentery among Ethiopian immigrants in 1985.

MATERIALS AND METHODS

Bacterial isolates and epidemiological background

Twenty-five *S. sonnei* strains isolated from three outbreaks occurring in three military bases, between 1986–9, were studied. Two outbreaks of *S. sonnei* shigellosis occurred in Unit S in 1986 and 1989, and a third one, in 1988, occurred in Unit C situated two kilometres away from Unit S. We compared the RFLP and the antimicrobial susceptibility (AS) patterns of these epidemic isolates with 11 *S. sonnei* isolates from sporadic cases of shigellosis occurring during September–October 1989 among the civilian population (a gift from the Shigella National Reference Center).

Six strains of *S. dysenteriae* type 1 isolated from an unusual cluster of severe shigellosis occurring in Israel towards the end of 1989 and two strains of *S. dysenteriae* type 1 isolated in Israel during an outbreak of dysentery among Ethiopian immigrants in 1985 were also studied.

All the isolates were characterized by biochemical and serological tests [12]. The identified strains were maintained as stocks in brain heart infusion broth (BHI) with 20% glycerol at −70 °C. For DNA preparation, strains were grown overnight in Luria Broth (LB).

DNA preparation and labelling

Bacteria grown overnight in 100 ml LB were centrifuged for 10 min at 7000 g. The resulting pellet was washed once in TES (NaCl 75 mM, Tris 25 mM, EDTA 50 mM) and bacterial DNA was extracted after lysis as described by Brenner and colleagues [13].

Plasmid pKK_{3535} was a gift of Dr Glaser of the Hebrew University Medical School. The DNA of this plasmid, carrying the *E. coli* rrnB operon [14], was extracted by alkaline lysis and purified by caesium-chloride ethidium bromide equilibrium density gradient centrifugation as previously described [15]. The 2·5 Kb *Eco* RI-*Hind* III restriction fragment encoding for the 16S rRNA and part of 23S rRNA [14], used as a probe, was separated by low melting agarose gel

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electrophoresis. The same procedure was employed to extract DNA from the pDT_6 plasmid containing the gene encoding for Na^+/H^+ antiport. The fragment 1·8 kb obtained from this plasmid after cleavage with Dra I and Ava I was used as a control probe [16]. DNA in the slice of the gel containing the relevant restriction fragment was radiolabelled in the agarose by random oligo priming with ($\alpha - {}^{32}P$) dCTP (25 ci/mmol) according to the instructions of the manufacturer (Amersham International plc, Buckinghamshire, UK). Plasmid DNA of *S. dysenteriae* type 1 was extracted by a rapid alkaline lysis procedure [17].

Southern blot analysis

About 2 μg of purified chromosomal DNA was digested with restriction endonucleases (*Eco*R I, *Bam*H I, *Sac* I, *Sma* I, *Kpn* I, *Bgl* II, *Pvu* I, *Pvu* II) (International Biotechnologies Inc, New Haven, US). The restriction fragments were separated by electrophoresis in 0·8% agarose gel and transferred to Genescreen plus membranes (DuPont, New England Nuclear, US) according to the supplier's instructions. Lambda DNA digested with *Hind* III was used as molecular mass marker on each gel. The membranes were incubated in a prehybridization solution for 4 h and then hybridized at 65 °C for 16–18 h with the boiled denaturated ^{32}P labelled probe as previously described [17]. Membranes were washed and exposed to X-ray film as described [17]. The hybridization banding patterns of *S. sonnei* and *S. dysenteriae* type 1 were analysed.

Antimicrobial susceptibility (AS)

Isolates were tested for susceptibility to ampicillin (Amp), cotrimoxazole (trimethoprim-sulfamethoxazole) (SxT), tetracycline (Te), nalidixic acid (Na), norfloxacin (Nor), chloramphenicol (C), clindamycin (CC), ciprofloxacin (Cip) and ampicillin-sublactam (SAM) by the Kirby-Bauer disk diffusion method [18] (Difco, Detroit, Michigan. US antibiotic discs).

RESULTS

Analysis of AS and RFLP patterns of S. sonnei

Seven different AS patterns were found among the 36 strains of *S. sonnei* studied (Table 1). All the strains were susceptible to nalidixic acid, norfloxacin, ciprofloxacin and ampicillin-sublactam and resistant to clindamycin. 17, 42, 33 and 3% of the strains were resistant to ampicillin, trimethoprim-sulfamethoxazole, tetracycline and chloramphenicol, respectively. There were no differences in the AS pattern of the *S. sonnei* strains belonging to the same cluster. AS patterns in strains isolated from unit S were different from that of strains isolated in unit C and the largest variety of AS patterns occurred in isolates of *S. sonnei* obtained from sporadic cases of shigellosis.

A single pattern of RFLP was obtained after digestion of the *S. sonnei* DNAs with *Bgl* II, *Sac* I and *Pvu* I and hybridization with the rRNA probe (data not shown). Two patterns which were different by one or two fragments were obtained after cleavage with *Eco*R I, *Sma* I and *Pvu* II (Table 2). *Bam*H I provided two entirely different patterns while *Kpn* I generated the highest degree of

Table 1. *Antimicrobial susceptibility patterns**

Pattern	Antibiotics†								
	Amp	Sxt	NA	Nor	C	TE	CC	Cip	SAM
A	S	S	S	S	S	S	R	S	S
B	S	R	S	S	S	R	R	S	S
C	R	R	S	S	R	R	R	S	S
D	S	R	S	S	S	S	R	S	S
E	R	R	S	S	S	R	R	S	S
F	R	R	S	S	S	S	R	S	S
G	R	S	S	S	S	S	R	S	S

* According to the Kirby–Bauer disk diffusion method.

† Ampicillin (Amp), cotrimoxazole (SxT), nalidixic acid (NA), norfloxacin (Nor), chloramphenicol (C), tetracycline (TE), clindamycin (CC), ciprofloxacine (Cip), ampicillin-sublactam (SAM).

Table 2. *RFLP patterns of* S. sonnei *chromosomal DNA after digestion with various restriction enzymes and hybridization with* ^{32}P *labelled probe encoding for* E. coli *rRNA*

Enzyme	RFLP pattern	Size in kbp of detected fragment
*Eco*R I	I	15·1; 11·56; 10·39; 7·9; 5·47; 3·76; 3·04; 1·6
	II	15·1; 11·56; 10·39; 5·47; 3·76; 3·04; 1·6
*Bam*H I	I	15·48; 13·67; 10·66; 8·31; 6·48
	II	7·81; 5·05; 4·2; 1·99; 0·94
Sma I	I	10·6; 9·7; 7·3; 6·32; 6·02
	II	9·7; 9·28; 7·3; 6·32; 6·02; 0·47
Kpn I	I	15·46; 14·4; 12·9; 9·78; 7·64; 6·8; 2·56; 1·18
	II	15·46; 14·4; 12·9; 10·5; 7·3; 2·85
	III	15·46; 14·4; 12·9; 10·05; 10·13; 7·12; 5·01; 2·5; 1·18
Pvu II	I	22·03; 11·04; 10·36; 5·98; 3·67; 3·24; 2·15; 1·84; 1·3
	II	22·03; 11·04; 10·36; 9·43; 5·98; 3·24; 2·15; 1·84; 1·3

polymorphism (three different RFLP patterns) among the 36 *S. sonnei* strains studied (Table 2). In order to verify that DNAs were completely digested by the various enzymes used in this study, an additional probe pDT_6 (a gift from S. Schuldiner, The Hebrew University), encoding the *ant* gene that is present in a single copy on the *E. coli* chromosomal DNA, was hybridized to the membranes before using the rRNA probe. This approach was developed to assure that the RFLPs obtained with the rRNA probe were not artifacts due to incomplete digestion of the DNA by the restriction enzymes. Fig. 1 shows that two similar bands appeared after digestion of the *S. sonnei* DNAs and hybridization with pDT_6, validating this internal control whereas 5–10 bands were revealed when using pKK_{3535} as a probe. Among the *S. sonnei* isolates obtained from sporadic cases of shigellosis, both AS and RFLP revealed a relative high number of different patterns (Table 3).

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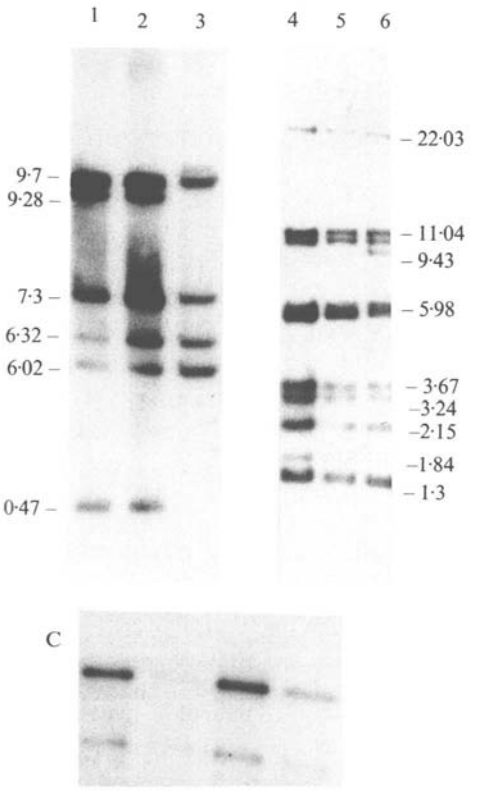

Fig. 1. RFLP patterns of DNA of *S. sonnei* strains after hybridization with *E. coli* rRNA gene. (A) DNA of three *S. sonnei* strains digested with *Sma* I: Pattern II in lanes 1–2 and pattern I in lane 3 (presence of an additional band at 10·6 kb and absence of the 0·47 kb band). (B) DNA of the same strains digested with *Pvu* II: Pattern II in lanes 4–5 and pattern I in lane 6 (an additional band in 9·43 kb). The band in 1·84 kb appears in all the patterns although less clearly displayed by the picture. (C) DNA from the same isolates plus an additional strain of *S. sonnei* digested with *Pvu* II after hybridization with the $pIDT_6$ probe all showing the same pattern.

AS and RFLP analysis of S. dysenteriae *type I* (*Shiga*)

During a 2-month period 6 isolates of *S. dysenteriae* type 1 were reported from 5 different laboratories all over the country (Table 4). Detailed investigation of the cases revealed a clear epidemiologic association among them. Cases 2–6 (see Table 4) served in military unit B at the time of or prior to the onset of the disease. Case 1 served at base A which is situated about two kilometres away from base B. During the acute phase of his diarrhoeal disease, this subject was isolated and treated in the dispensary of base B. Cases 4, 5, and 6 were in close contact sharing food and water among themselves and case 3 was their instructor during their pre-military training in base B. No contact other than service in the same base and consumption of food prepared by the same kitchen, was noticed between case 2 and the other cases of Shiga.

The AS patterns of the strains isolated in cases 2–6 were identical but different

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Table 3. *RFLP and antiobiotic susceptibility (AS) patterns of* 36 S. sonnei *isolates*

Source	No. of strains	AS patterns	RFLP patterns after digestion with: *Bam* HI	*Eco* RI	*Kpn* I	*Pvu* II	*Sma* I
Unit S 5–7/86	5	A	I (5)	II (5)	I (3) II (1) III (1)	I (4) II (1)	I (3) II (2)
Unit C 5–7/88	6	B	I (4) II (2)	I (4) II (2)	I (2) II (1) III (3)	I (6)	I (6)
Unit S 4–6/89	14	A	I (14)	I (14)	I (5) II (1) III (8)	I (1) II (13)	I (10) II (4)
Sporadic cases	11	A (1), E (2) B (3), C (2) D (1), F (1) G (1)	I (10) II (1)	I (11)	I (5) II (2) III (4)	I (6) II (5)	I (5) II (6)

Table 4. *Cases of dysentery caused by* S. dysenteriae *type 1 in Israel, October–December 1989, their epidemiological data, AS, plasmid profile and RFLP patterns*

Case no.	Date of disease (1989)	Permanent residence of onset	Military unit patient	AS* pattern	Plasmid profile	RFLP pattern
1	7 Oct.	Tel-Aviv	A†	R	I‡	I
2	25 Nov.	Petah Tikva	B	S	II	I
3	2 Dec.	Jerusalem	B§	S	II	I
4	5 Dec.	Haifa region	B	S	II	I
5	5 Dec.	Haifa region	B	S	II	I
6	5 Dec.	Haifa region	B	S	II	I

* R, Resistant to ampicillin, chloramphenicol and tetracycline. S, Susceptible to ampicillin, cotrimoxazole, tetracycline, nalidixic acid, norfloxacin chloramphenicol, clindamicin, ciprofloxacin, ampicillin-sublactam.

† Isolation and medical treatment due to dysentery in base B.

‡ Plasmid profile 1: presence of a small plasmid (Fig. 3) which disappears in profile II.

§ Very close connection (direct contact, sharing of food and drinking water among cases 3, 4, 5 and 6).

from that of case 1. The isolates of cases 2–6 were sensitive to all the following antibiotics: ampicillin, cotrimoxazole, nalidixic acid, norfloxacin, chloramphenicol and tetracycline. The strain isolated in case 1 was susceptible to cotrimoxazole, nalidixic acid and norfloxacin but resistant to ampicillin, chloramphenicol and tetracycline. DNAs of the 6 isolates digested with 8 different restriction enzymes (the same 8 restriction enzymes used to study the *S. sonnei* isolates) and hybridized with the rRNA probe showed an identical RFLP pattern (Table 4 and Fig. 2) but different from the Ethiopian immigrant's strain (Fig. 2). The plasmid profile was identical for the last five strains of *S. dysenteriae* type 1 while the first strain presented an additional DNA band (Fig. 3) which probably corresponds to an additional plasmid.

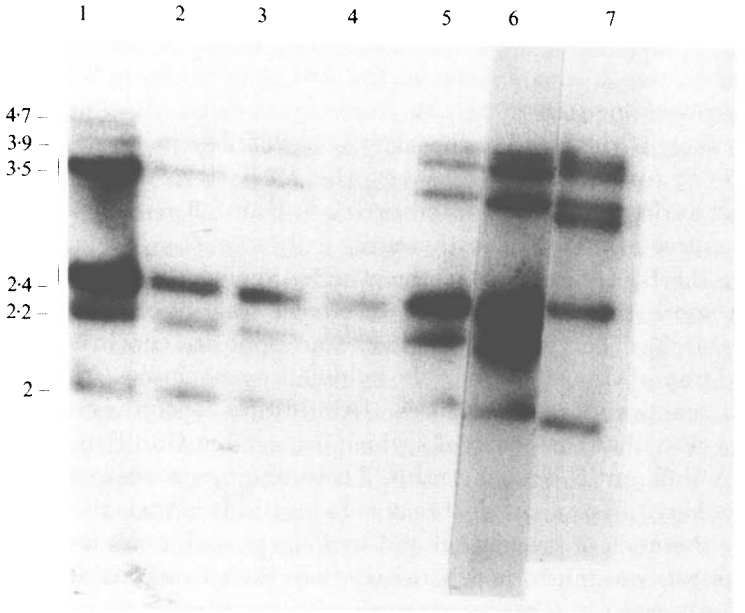

Fig. 2. RFLP pattern of the six isolates of *S. dysenteriae* type 1 after digestion with *Sma* I and hybridization with *E. coli* rRNA genes. Lanes 1–6, *S. dysenteriae* type 1 strains from the 1989 cluster with an identical RFLP; Lane 7, one strain isolated from an Ethiopian new immigrant in 1985 with a different RFLP pattern from the six other isolates (absence of the 2·2 kb band and presence of an additional band around 3·3 kb).

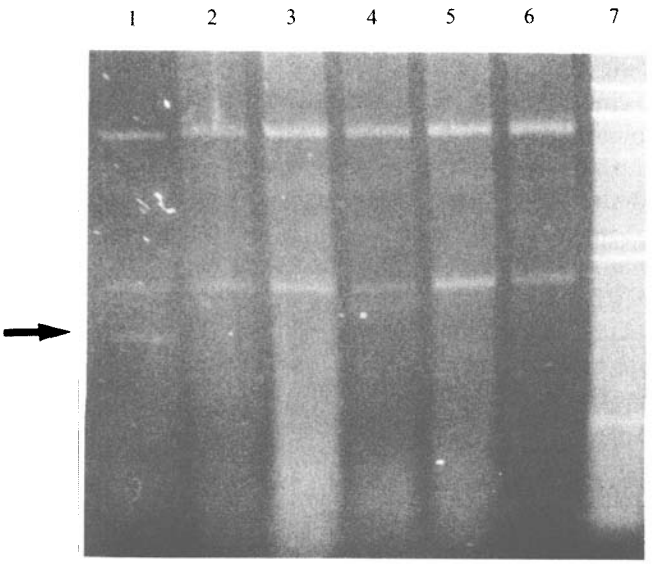

Fig. 3. Plasmid profile of six *S. dysenteriae* type 1 strains. Lanes 2–6, strains isolated in cases 2–6 with identical profile. Lane 1, the strain which was isolated in the first case and was resistant to ampicillin, tetracycline and chloramphenicol has an additional band around 1 kb (arrow). Lane 7, size markers (kb).

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DISCUSSION

With regard to the *S. sonnei* isolates, the AS patterns indicated that a single strain was involved in each of the three outbreaks and therefore it could be assumed that each of the three outbreaks were probably point source epidemics due to a single *S. sonnei* strain. Moreover, the AS pattern was identical for the strains isolated during the outbreaks occurring in Unit S 3 years apart, suggesting a permanent source of infection in this unit.

In contrast, the RFLP analysis of the strains involved in the three outbreaks revealed that more than one strain of *S. sonnei* was involved in each of the outbreaks (Table 3). Therefore RFLP led to an opposite conclusion concerning the source and way of transmission of the epidemic agent, indicating the existence of more than one simultaneous source. In addition, according to the RFLP analysis it was clear that the outbreaks which occurred in Unit S in 1986 and 1989 were caused by different *S. sonnei* strains. These findings increase the complexity of the epidemiology of apparent outbreaks of shigellosis in endemic countries and as a result the methods of prevention and hygiene in such cases must be revised. The RFLP analysis was much more sensitive than the antimicrobial susceptibility analysis when applied on *S. sonnei* strains isolated during outbreaks.

The RFLP analysis of *S. dysenteriae* serotype 1, a serotype rarely found in Israel, suggested that all the clinical cases in this cluster of Shiga dysentery were infected by the same clone, although the AS pattern of the first isolate was different from that of the five following isolates. We can assume that the organism that infected the first individual was resistant to ampicillin, tetracycline and chloramphenicol and probably lost an R plasmid after one person to person passage (see Fig. 3) and became susceptible to these antibiotics while keeping intact its chromosomal structure. A single autotransferring plasmid, conferring resistance to ampicillin, chloramphenicol, and tetracycline, the same resistance pattern found in the first isolate of *S. dysenteriae* type 1 in our study, has previously been identified [19]. It has also been shown that bacterial plasmids and particularly R factors are subject to frequent gain, loss and rearrangement of their DNA sequence as a result of environmental conditions [3, 8, 20].

In this study, RFLP analysis of bacterial chromosomal DNA was shown to be an important epidemiological tool to differentiate between shigella strains of a single serotype such as *S. sonnei* and *S. dysenteriae* type 1 (Shiga). The DNA probe used in the present study comprised genes with highly conserved sequences (rRNA genes) which are present in seven copies in Enterobacteriaceae [10]. Using a similar DNA probe Hinojosa-Ahumada and colleagues studied the RFLP patterns of sporadic and epidemic *S. sonnei* organisms isolated in US [21]. The degree of diversity of *S. sonnei* isolates according to the RFLP analysis was lower in this recently reported study as compared to ours. This finding may be a result of a much higher incidence of shigellosis and of a more dynamic transmission of *S. sonnei* in Israel (mainly under field conditions) as compared to the United States. Most of the RFLP diversity which could be revealed with the probe we used is probably related to the polymorphism of the flanking regions of these genes. These characteristics ensured a high specificity of the RFLP as an epidemiologic tool and allowed to discriminate among *S. sonnei* or *S. dysenteriae* type 1 strains. The

specificity of the RFLP technique was also documented by the uniqueness of the RFLP pattern found in the six strains of *S. dysenteriae* type 1 (Shiga) examined which contrasted with a relative high polymorphism found in strains of *S. sonnei* isolated in outbreaks or among sporadic cases (Table 3 and Fig. 1) using the same restriction enzymes and the same rRNA probe. Using a similar RFLP protocol with a ribosomal RNA probe, Strockbine and colleagues [22] identified three different RFLP patterns of chromosomal DNA in 83 isolates of *S. dysenteriae* type 1 (Shiga) after digestion with a single restriction enzyme (*Pvu* II). On that occasion, the RFLP analysis led to the conclusion that isolates of *S. dysenteriae* type 1 (Shiga) in Mexico were different from Shiga strains responsible for outbreaks in Africa and Asia, indicating no significant exchange of organisms between continents in recent decades [22]. The strains we examined were also different in their RFLP from those described above. In conclusion, our findings and those of Strockbine and colleagues indicate that the RFLP analysis of shigella strains appears to be a powerful epidemiologic tool in the investigation of outbreaks due to this pathogen. This tool is particularly important for shigella belonging to a single serotype. Out of eight restriction enzymes studied, *Kpn* I, *Pvu* II and *Sma* I generated the highest degree of polymorphism and their use should be suitable for the construction of a regional or international 'RFLP scheme' of *Shigella* sp. strains.

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