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Use of AFLP, plasmid typing and phenotyping in a comparative study to assess genetic diversity of *Shigella flexneri* strains

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SUMMARY

Shigella flexneri infections are one of the main causes of acute diarrhoea in Cuba. Twenty strains isolated from sporadic cases in nine different Cuban provinces were characterized. Serotyping, antibiotic-resistance typing, plasmid-typing and AFLP-typing were used to determine their suitability for use in epidemiological studies of *S. flexneri*. The predominant serotypes were serotype 6 (35%) and serotype 2 (35%). Eleven different plasmid profiles were detected (Diversity Index = 0·92). AFLP-typing discriminated 12 different patterns (DI = 0·95), these patterns were not coincident with plasmid-typing patterns. Both techniques combined distinguished 14 patterns among the 20 studied strains (DI = 0·99). There was no consistent relationship between plasmid-typing and AFLP-typing patterns or antibiotic-resistance typing patterns. Ninety-five percent of *S. flexneri* strains were multiresistant.

INTRODUCTION

Shigella species infections are an important cause of acute diarrhoea disease in both developing and developed countries. In Cuba, *S. flexneri* and *S. sonnei* are the two commonest species involved [1, 2].

Most of recent studies of typing methods have focused on *S. sonnei*, because of its high prevalence and the absence of subserotypes. Phenotyping techniques such as bio-typing, antibiotic-resistance typing and phage-typing have been complemented with genotyping methods such as ribotyping, plasmid-typing, PFGE-typing and ERIC-PCR typing [3, 4]. Several studies, comparing different epidemiological methods with *S. flexneri* strains have been published. Phenotypic (serotyping and antibiotic-typing) and genotypic (plasmid-typing and PFGE-typing) have been tried [5–7].

AFLP-typing is a PCR based technique that studies the size of polymorphic restriction fragments. This technique has proved useful to characterize different microorganisms [8–10].

The goal of this study was to test sporadic Cuban *S. flexneri* strains by AFLP-typing, antibiotic-typing, serotyping and plasmid-typing and to compare the results of each.

MATERIALS AND METHODS

Bacterial strains

During a 6-month time period, stool specimens from children under 5 years with diarrhoea were collected

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in nine different Cuban provinces. The isolates were sent for species confirmation to the Laboratorio Nacional de Referencia de Enfermedades Diarreicas Agudas, Instituto Pedro Kourí, Cuba. Further antibiotic susceptibility and molecular characterization of 20 independent selected isolates were performed at the Spanish Reference Laboratory for *Salmonella* and *Shigella*.

Serotyping

S. flexneri strains were grown on Trypticase Soy Agar plates and serological reactions were determined by the slide agglutination technique. Commercial group and type-specific antisera (Seyken, Oxoid, Unipath, Spain) were used.

Antibiotic susceptibility testing

Antibiotic susceptibility was assessed by the disk diffusion method [11]. Susceptibility patterns were assessed using the NCCLS criteria [12]. Antibiotics utilized were supplied by Oxoid (Unipath, Spain). The following 12 antibiotics were tested: ampicillin (A, 10 μg); chloramphenicol (C, 30 μg); sulphonamides (Su, 300 μg); streptomycin (S, 10 μg); tetracycline (T, 30 μg); gentamicin (G, 10 μg); kanamycin (K, 30 μg); nalidixic acid (Nx, 30 μg); ciprofloxacin (Cip, 5 μg); cefotaxime (Ctx, 30 μg); cephalotin (Kf, 30 μg); trimethoprim-sulphamethoxazole (SxT, 25 μg). Strains resistant to four or more antibiotics were considered as multiresistant.

Plasmid-typing

S. flexneri plasmid profiles were determined using the Kado and Liu method [14]. Plasmid extraction was run on a horizontal electrophoresis 0·8% agarose gel, ethidium bromide stained and visualized under UV light. Plasmid size was determined by comparison to a supercoiled DNA marker (Gibco–BRL, Invitrogen, Spain). Regression analysis was performed to determine the linear relationship between the molecular size base 10 logarithm and the plasmid mobility. Plasmid profiles were elaborated by considering the number and size of plasmid present. Only bright and stable bands were considered for analysis. Fine and non-consistent bands were considered as relaxed DNA forms of supercoiled plasmids. Only plasmid bands below the chromosomal DNA band were taken into account for profile assignment because of instability of the large plasmids [7].

Amplified fragment length polymorphism (AFLP)

Whole-cell DNA was extracted by the CTAB method [15]. The restriction-ligation reaction was performed with *Pst*I, *Pst*I adapter pairs, and T4 ligase (Roche, Madrid, Spain) as previously described [8]. The tagged DNA fragments were then precipitated by the addition of ammonium acetate to a final concentration of 2·5 M, and absolute ethanol. After a washing step in 70% ethanol, DNA was resuspended in 100 μl of TE pH 8·0. Resuspended DNA was diluted 1:10 with distilled water and 5 μl were used for PCR amplification. Four reactions were performed with different primers: AFLPPstIT (5′-GACTGCGTACATGCAGT-3′); AFLPPstIA (5′-GACTGCGTACATGCAGA-3′); AFLPPstIC (5′-GACTGCGTACATGCAGC-3′) or AFLPPstIG (5′-GACTGCGTACATGCAGG-3′). PCR amplification was performed using a Ready-to-Go system (Amersham Pharmacia Biotech Inc.). PCR amplification was as follows: denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2.5 min; and one final extension cycle at 72 °C for 7 min. Amplified fragments were analysed by electrophoresis in a 1·5% agarose gel (Agarose MS8, Hispanalab, Madrid, Spain) in 1 × TAE buffer and were visualized under UV light by ethidium bromide staining.

Statistical methods

The discriminatory index (DI), i.e. the probability that two unrelated strains sampled from the population would be placed into different typing groups, was calculated by Simpson's Index of Diversity [16]. Clustering analysis and dendrograms were achieved by using the Dice coefficient using the MVSP software (Kovach Computing Services, Wales, UK).

RESULTS

Shigella flexneri serotyping

Serotypes 6 and 2 were the two commonest *S. flexneri* serotypes with seven strains for each serotype (Table 1). Subserotypes were not determined.

Antimicrobial resistance patterns

All strains were resistant to tetracycline, and 95% were resistant to sulphonamides, trimethoprim-sulphamethoxazole and streptomycin. All strains were susceptible to gentamicin, ciprofloxacin, kanamycin,

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Table 1. *Characteristics of* S. flexneri *strains tested*

Strain	Serotype	Plasmid profile	Antibiogram patterns	AFLP		Cuban Province
				Pattern	Clusters	
6785	1	A	Su SxT S T	P5	II	Las Tunas
6786	1	A	Su SxT S T	P5	II	Las Tunas
6792	1	A	Su SxT S T	P8	IV	Granma
6787	1	B	Su SxT S T	P3	II	Las Tunas
6790	1	C	Su SxT S T	P6	II	Santiago de Cuba
6781	2	D	T	P4	II	Matanzas
6788	2	D	A Su SxT S T	P6	II	Isla de la Juventud
6791	2	E	A C Su SxT S T	P7	III	Granma
6793	2	F	A C Su SxT S T	P7	III	Camagüey
6794	2	G	A Su SxT S T	P9	III	Camagüey
6795	2	G	A Su SxT S T	P9	III	Camagüey
6798	2	F	A C Su SxT S T	P11	II	Pinar del Rio
6778	3	H	Su SxT S T	P2	I	Cienfuegos
6779	6	I	Su SxT S T	P3	II	Cienfuegos
6780	6	I	Su SxT S T	P1	I	Cienfuegos
6799	6	I	Su SxT S T	P12	I	Pinar del Rio
6797	6	I	Su SxT S T	P10	I	Pinar del Rio
6801	6	I	Su SxT S T	P1	I	Ciudad Habana
6796	6	J	Su SxT S T	P10	I	Camagüey
6800	6	K	Su SxT S T	P1	I	Pinar del Rio

nalidixic acid and cefotaxime. Four strains were intermediate to cephalotin (Table 2). Multiresistance percentage was very high (95%). Multiresistant patterns present were: Su SxT S T (65%); A Su SxT S T (15%); and A C Su SxT S T (15%) (Table 1). All ampicillin and chloramphenicol resistant strains belong to serotype 2 (Table 1).

Plasmid profiles

Eleven different profiles were detected, DI = 0·92 (Fig. 1). All strains harboured 3–5 bands, between 1·4 kb and 6·2 kb. One 3·9 kb band was present in all strains (Fig. 1). Twenty-five percent of strains were pattern I. Thirty percent of strains had a unique plasmid pattern. All strains with the same plasmid profile were the same serotype (Table 1).

AFLP profiles

AFLP-*Pst*I-A primer was selected because of its power resolution and high band variability. Twelve different patterns were obtained, DI = 0·95 (Fig. 2). Twenty-five percent of strains had unique and specific patterns (Table 1). Four different clusters, considering a similarity index of 0·80 or higher, were detected (Table 1, Fig. 3). Strains similarity index ranged between 0·52 and 0·93. The two commonest clusters were I and II and were distributed all over Cuba (Table 1).

DISCUSSION

S. flexneri and *S. sonnei* are the two commonest species involved in acute diarrhoea diseases in Cuba [1, 2]. Epidemiological studies of *Shigella* spp. have been based on phenotypic characteristics such as serotyping, antibiotic-resistance typing and genotypic characteristics such as plasmid-typing. These methods have had limited usefulness in the epidemiological study of related strains coexisting in a geographic region during a certain period of time [6, 17]. PFGE has become as a powerful epidemiological tool, improving discriminating power. AFLP has been reported to be a very discriminative technique to differentiate bacteria, which were highly related or identical by other typing methods [10]. In addition, AFLP is less laborious, time consuming and technically demanding than PFGE. Thus, we compared serotyping, antibiotic-resistantyping, plasmid-typing and AFLP-typing on a selection of *S. flexneri* strains from Cuba.

Antibiotic treatment has been recommended in shigellosis, because it reduces severity of illness, prevents

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Table 2. *Percentage of antimicrobial susceptibility among strains of* S. flexneri

	Susceptible		Intermediate		Resistant	
Drug	*n*	%	*n*	%	*n*	%
Ampicillin	14	70	0	0	6	30
Chloramphenicol	17	85	0	0	3	15
Sulphonamides	1	5	0	0	19	95
Gentamicin	20	100	0	0	0	0
Ciprofloxacin	20	100	0	0	0	0
Kanamycin	20	100	0	0	0	0
Trimethoprim-Sulphamethoxazole	1	5	0	0	19	95
Streptomycin	0	0	1	5	19	95
Cephalotin	16	80	4	20	0	0
Nalidixic acid	20	100	0	0	0	0
Cefotaxime	20	100	0	0	0	0
Tetracycline	0	0	0	0	20	100

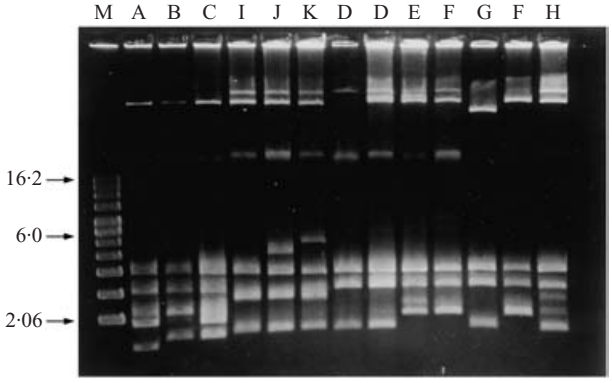

Fig. 1. *S. flexneri* plasmid profiles (0·8% agarose gel). Lane M, molecular weight supercoiled DNA marker (Gibco–BRL, Invitrogen, Spain). Lanes A–H plasmid profiles A to K.

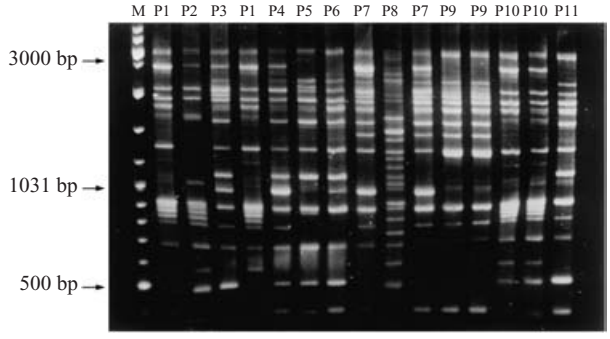

Fig. 2. AFLP profiles of *S. flexneri* strains performed with selective primer AFLPPstI-A. Lane M, molecular weight marker GeneRuler DNA ladder mix (MBI Fermentas Inc., Quimigranel, Spain). Lanes P1–P11 different AFLP profiles.

complications and shortens the excreting period, thereby reducing the risk of disease dissemination [18]. Several publications highlight the emergence of multiresistant *Shigella* spp. strains in several countries [6, 19, 20]. We have found that 95% of studied strains were multiresistant, with Su SxT S T the commonest and widely distributed multiresistant pattern (Table 1).

Because of past high resistance to ampicillin, nalidixic acid is currently the antibiotic of choice in shigellosis treatment in Cuba [21]. All strains tested were susceptible to this antibiotic (Table 2). It is important to note that there have been isolated *S. sonnei* strains resistant to nalidixic acid in Cuba (unpublished observations). In some countries where shigellosis treatment changed from ampicillin to nalidixic acid, *S. flexneri* strains resistant to nalidixic acid have appeared [18, 20]. Thus, periodic surveillance for resistance and evaluation of treatment are ongoing needs.

Plasmid typing appears to provide a useful epidemiological marker (DI = 0·92). It is also a technique that is cheap, rapid and easy to perform. One limitation for epidemiologic studies is that plasmids may be unstable and consequently can be lost or acquired. Our study did not show a consistent relationship between plasmid profiles and antibiotic-resistance patterns or serotypes. *Shigella* spp. have a heterogeneous plasmid population, between two and ten, some of them are related with virulence and/or antibiotic resistance and some other are cryptic plasmids. About 45–73% of them are transferable. These circumstances together with the existence of stable chromosomal locus, carrier of antibiotic resistance [5], and the endemicity of this disease, suggest the circulation of resistant strains with a high degree of genetic variability in Cuba.

Some studies have found a correlation between plasmid profiles and serotype [5, 22]. In this study

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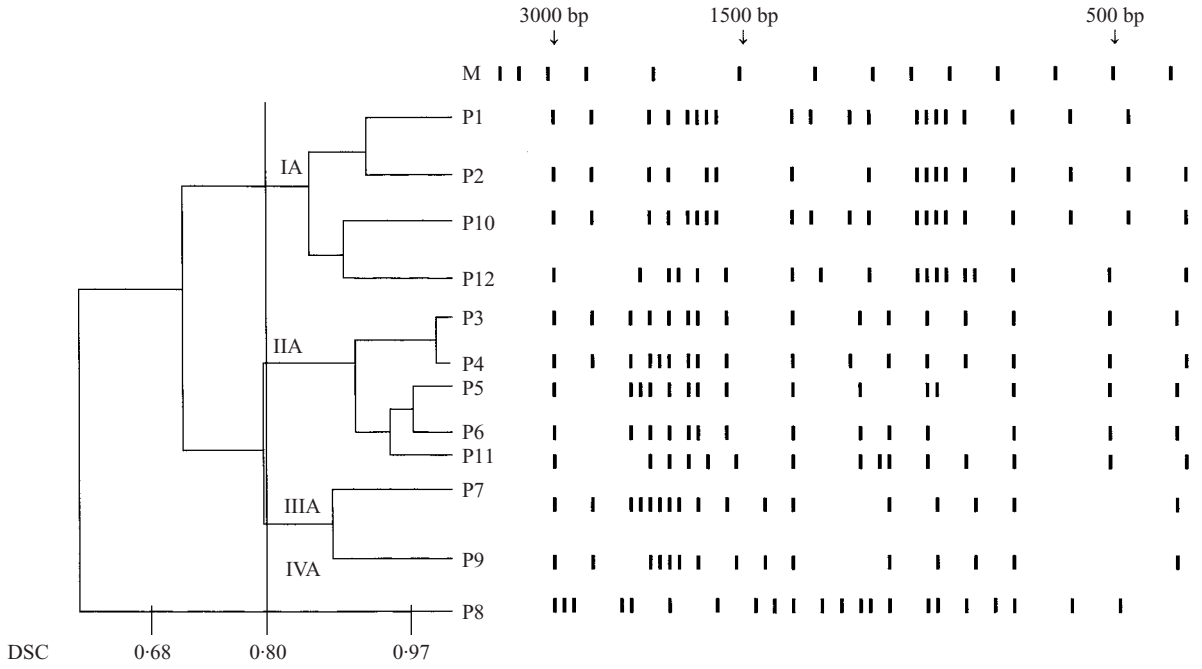

Fig. 3. Dendrogram and schematic representation showing AFLP profiles for *S. flexneri* strains obtained using the selective primer AFLPPstI-A. Lane M, GeneRuler DNA ladder mix (MBI Fermentas Inc., Quimigranel, Spain). Lanes P1–P12 different AFLP profiles. DSC: Dice similarity coefficient.

there was variability among plasmid profiles both within and between serotypes. Plasmid pattern A was predominant in serotype 1 strains. Observed variations were due to the acquisition or loss of one or two plasmids. Plasmid pattern I was predominant in serotype 6 strains. Observed variations were exclusively due to the acquisition of one plasmid (Table 1, Fig. 1).

AFLP has been established as a broadly applicable genotyping method with a high degree of reproducibility and discriminatory power. However, its use with *Shigella* sp. has not been reported. The results obtained in the present study show that AFLP, using *Pst*I as restriction enzyme, better discriminated between non related *S. flexneri* strains (DI = 0·95) than plasmid typing (DI = 0·92). The reproducibility of the technique was examined by performing duplicate AFLPs runs with two separate DNA extractions. Also, DNAs from several of these *S. flexneri* strains were amplified in two different thermal cyclers. Under all these different conditions, the fragments for each AFLP profile were identical. However, variations in the intensities of some of the bands were observed with different PCR runs. The combination of plasmid and AFLP profiles allowed discrimination between identical AFLP patterns and vice versa. The AFLP dendrogram considering a 0·80 or over genetic distance, identified four clusters (Table 1, Fig. 3). Serotype 1 strains were mainly cluster II.

Serotype 2 strains presented the highest variability with AFLP and plasmid techniques. Six out of seven serotype 2 strains had different plasmid patterns. Five different AFLP patterns that grouped in two clusters (II and III) were obtained. Cluster II strains were isolated in the oriental zone of the island, while cluster III strains were isolated in the occidental zone.

Serotype 3 was only represented by one strain, that also has unique plasmid and AFLP pattern, although it is included within cluster I.

Serotype 6 strains were widely geographically distributed, and were very homogeneous, four different AFLP patterns were obtained but most of them pertained to cluster I except for strain no. 6779 that pertained to cluster II.

AFLP-plasmid-typing combination allowed to differentiation of 14 different patterns, increasing the discriminatory power of both separated techniques (DI = 0·98). *S. flexneri* has been typed mostly using plasmid-typing and not until recently using PFGE-typing, particularly in regions with a high rate of *S. flexneri* incidence. AFLP as herein described could also be used as complementary typing technique, because of its high discriminatory power, easy performance,

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speediness and high reproducibility. However, more additional studies are necessary to show if strains belonging to an outbreak with a common source fall into the same AFLP subtype.

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