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Drug resistance and plasmid profile of shigellae in Taiwan

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SUMMARY

One hundred and twenty-eight shigella strains isolated from newborn and infant human faecal specimens at Kaohsiung Medical College Hospital in Taiwan were serogrouped, serotyped and examined for drug-resistance patterns and for the presence of plasmids. Forty-seven per cent of the isolates were found to belong to the *Shigella sonnei* serogroup, 41% to the *S. flexneri* group, 9% to the *S. boydii* group and 3% to the *S. dysenteriae* group. The serotype with the greatest number of strains was *S. sonnei* I. (29%) followed by *S. flexneri* 1 (27%). Each strain was tested for resistance to 11 antimicrobial agents. Eighty-eight per cent of the strains were resistant to tetracycline, 87% to chloramphenicol, 84% to streptomycin, 52% to ampicillin, 25% to nalidixic acid, 29% to kanamycin, 11% to cephalothin, 11% to neomycin, 10% to cotrimoxazole, 1% to amikacin and none to gentamicin. The most prevalent resistance pattern was ApCmSmTc (28%). Clinical isolates demonstrating multiple resistance were found to harbour a large transmissible plasmid of 45–75 MDa while isolates without multiple resistance did not. Two large virulence plasmids of 123 and 110 MDa were found in 12 strains of *S. flexneri* and 4 strains of *S. sonnei* phase I. Small plasmids of 4·5, 4·2, 3·5, 2·8, 2·5. 2·0 and 1·5 MDa were also present in all strains. These small plasmids were species specific and can be used as marker plasmids to identify species.

INTRODUCTION

Shigellosis is one of the most prevalent and important diseases in the developing countries [1–3], and is the major cause of dysentery in Taiwan [4]. Resistance of *Shigella* spp. to antibacterial drugs has been reported from different parts of the world and is increasing [5]. First observed in *S. flexneri* in Japan [6], the development of transferable multi-drug resistance was soon reported elsewhere, including Taiwan [4], and was also observed in other shigellae [7]. In spite of the prevalence of shigellosis in Taiwan, there have been few epidemiological studies, and resistance patterns in this area have not as yet been investigated. The present study was undertaken to determine the relative prevalence of different species, serotypes and patterns of drug resistance among shigella isolated from clinically diagnosed cases of acute dysentery, gastroenteritis or colitis.

Plasmid analysis has revealed that bacteria usually harbour a heterogeneous population of plasmids which can confer resistance to different antibiotics, and

can contribute to pathogenicity in different ways, notably by encoding proteins essential for virulence. In this study, the plasmid profiles of the different shigella serotypes were surveyed and compare with their drug resistance patterns in an attempt to reveal any possible correlations.

MATERIALS AND METHODS

From August 1982 to August 1987, 140 shigella strains isolated from newborn and infant faecal specimens were collected from patients diagnosed at the Kaohsiung Medical College Hospital, Kaohsiung, Taiwan, as having acute dysentery, gastroenteritis, or colitis.

Isolation and identification

For the isolation of shigella from stool specimens, GN broth (Difco, Laboratories, Detroit, Michigan, USA) was used for enrichment. After 8–12 h of incubation at 35 °C, inoculum from GN broth was streaked on XLD (xylose lysine desoxycholate) agar (Difco) plates. Translucent colonies were picked after incubation for 24 h at 35 °C. Shigella serotypes were identified by biochemical assay and serological testing [4]. Once identified, strains were then preserved in tryptic soy broth (Difco) containing 15% glycerol and stored at −70 °C for further study.

Serological typing

Strains of shigella were serogrouped and serotyped by slide agglutination (antisera from Denka Seiken Co, Japan). Strains showing strong agglutination with the polyvalent antiserum were subsequently tested with specific monovalent antisera and placed in one of the four serogroups.

In vitro *sensitivity testing*

The standardized disk–agar diffusion method was used to determine *in vitro* susceptibility [8]. The antimicrobial disks were obtained from BBL (Baltimore Biological Laboratory, Cockeysville, MD, USA). The minimal inhibition concentration (MIC) were determined by the agar plate dilution method [9]. A bacterial suspension of $4{\cdot}5 \times 10^8$ (CFU/ml) was diluted 1:20 and inoculated on Mueller–Hinton medium (Difco) containing a series of concentrations of antimicrobial agents. The agents used were ampicillin (Ap), kanamycin (Ka), cephalothin (Cr), chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), neomycin (Nm), gentamicin (Gm), nalidixic acid (Nx), trimethoprim-sulphamethoxazole (TMP-SMZ) and amikacin (An) (Sigma, USA). These agents were originally obtained in powder form and dissolved in an appropriate diluent, and stored at −70 °C. The value of the geometric mean of the MIC was calculated for all of the strains isolated.

Isolation of plasmid DNA

The rapid procedure for the detection and isolation of shigella plasmid was used as previously described [4]. Plasmid DNA was extracted from 0·5 ml of the overnight culture with phenol-chloroform. A 10 μl sample was mixed with 3 μl of

tracking dye (25% sucrose, 0·05% bromphenol blue, 0·1% sodium dodecyl sulphate, 5 mM sodium acetate) and loaded onto a 0·7% horizontal agarase gel (BRL cat. no. 5510VA, USA). A Tris-acetate buffer system (pH 8·0) was used for electrophoresis.

Conjugation experiments

Nutrient broth cultures of the donor shigella strain and the recipient strain (*Escherichia coli* K-12 14R525, Lac^+, F^-, Nx^r) were incubated with agitation at 37 °C to mid-exponential phase (4 h). Donor and recipient cells were mixed in equal volumes (1 ml) and the incubated at 37 and 28 °C for 18–20 h without agitation. After incubation, the mating mixture was serially diluted in phosphate buffered saline and various dilutions were plated on EMB (Eosin Methylene Blue) agar (Difco) containing 30 mg/l nalidixic acid and one other appropriate antibiotic.

RESULTS

Of the 140 strains isolated, 128 were serotypable. Eighty-eight per cent of the serogrouped isolates were either *S. sonnei* (60 strains, 47%) or *S. flexneri* (53 strains, 41·4%); Eleven (8·6%) were *S. boydii* and (3·1%) *S. dysenteriae*. The distribution of serotypes amongst the isolates are presented in Table 1.

Over all, 88% of strains were resistant to Tc, 87% to Cm, 84% to Sm, 52% to Ap, 25% to Nx, 20% to Ka, 11% to Cr, 11% to Nm, 10% to TMP-SMZ, 1% to An and 0% to Gm (Table 2). Higher MIC values were found for the isolates of *S. boydii* than for other isolates. Table 3 shows the predominant resistance patterns identified. Resistance patterns Sm Cm Tc and Sm Cm Tc Ap were the two most frequently found amongst the strains of all four serogroups. Resistance to Ka, TMP-SMZ, Nm, Nx and Cr were very variable amongst the strains of *S. flexneri* and *S. sonnei* serogroups (data not shown). A variety of resistance patterns were found amongst the *S. boyddi* strains that were not found in other groups. Most involved three or more drugs.

Agarose gel electrophoresis showed more than one plasmid in every strain. All strains with multiple resistance were found to harbour a plasmid of 45–75 MDa. This plasmid was not found in any of the strains without multiple resistance. In conjugation experiments, this plasmid was the only transferred, and with it, multiple resistance to all the antibiotics except Sm.

No precise correlations were found between plasmid profiles and specific drug resistance. As shown in Table 4, several prominent patterns were observed among the plasmid profiles of *S. sonnei* and *S. flexneri*. Most of the 35 strains of *S. flexneri* 1a and 1b contained 4 other plasmids besides the 45–75 MDa: 3·5 MDa, 80% (28/35); 2·8 MDa, 71·4% (25/35); 2·5 MDa, 88·6% (31/35); 2·0 MDa, 88·6% (31/35), representing a total of 43% of all 53 *S. flexneri* strains isolated. All 8 strains of serotypes 2a and 2b were found to contain 2 plasmids of 3·5 and 2·8 MDa, and 5 strains harboured a smaller plasmid of 1·5 MDa. Three of the four strains of serotype 3b contained 4 plasmids of 4·2, 3·5, 2·8 and 2·5 MDa. In addition to the 45–75 MDa plasmid, 48 of the 60 *S. sonnei* strains were found to have a second plasmid of 4·5 MDa. No specific patterns were observed in the plasmid profiles of the 11 *S. boydii* strains isolated or in the profiles of the 4 strains of *S.*

Table 1. *The serotypes distribution of* Shigella *species*

Species	%	Serotype	No. of strains
S. dysenteriae	3·1	1	1
		8	2
		2	1
S. flexneri	41·4	1a	20
		1b	15
		2a	6
		2b	2
		3a	1
		3b	4
		4a	1
		5b	1
		6	2
		VX*	1
S. boydii	8·6	1	1
		2	3
		3	3
		13	1
		14	3
S. sonnei	47·0	I	38
		II	22

* VX, Variant strain of *Shigella flexneri*.

dysenteriae. Lastly, 2 large virulence-conferring plasmids of 123 and 110 MDa described in a previous study [10] were found in 12 strains of *S. flexneri* and in 4 strains of *S. sonnei* phase I. Representative profiles of plasmids of each of the four serogroups are presented in Figures 1–4.

DISCUSSION

The shigellae continue to be important aetiological agents of dysentery gastroenteritis in India [1, 11, 12], the United States [13], Nigeria [2], Australia [3], Vellore [14] and Bangladesh [15]. This is also true for Taiwan [4, 10]. In a previous study [4], of 249 shigella strains isolated, *S. flexneri* was the species most commonly found (73·1%), followed by *S. sonnei* (22·2%), *S. boydii* (3·2%) and *S. dysenteriae* (1·6%). In the 128 shigella strains isolated in the present study, the proportion of *S. sonnei* isolates increased to 47%, while that of *S. flexneri* fell to 41·1%. In other reports [1–3, 14, 16, 17] from different parts of India, *S. flexneri* and then *S. boydii* or *S. dysenteriae* were the most prevalent species. Changes in the relative frequencies of *Shigella* species isolated in a given area may be caused by improvements in the levels of environmental and personal hygiene. *S. sonnei* is generally transmitted by personal contact [1] and is thus most common in more developed areas. In contrast, in many developing areas, infections with *S. boydii* and *S. dysenteriae* are the more prevalent and *S. flexneri* is more common than *S. sonnei*. This decrease in the proportion of *S. flexneri* strains isolated in Taiwan as well as in the overall incidence of shigella infection may reflect just such hygienic improvements in the country over the past 10 years.

Table 2. *Percentage of strains showing drug resistance and geometric mean of minimal inhibitory concentration (MIC) in shigella*

Strain (no. isolated)	Percentage showing drug resistance (geometric mean of MIC, μg/ml)										
	Ap	Ka	Cr	Cm	Tc	Sm	Nm	Gm	Nx	TMP | SMZ	An
S. dysenteriae (4)	25	0	0	75	75	75	0	0	0	0	0
	(11·3)	(4·0)	(4·8)	(128·0)	(64·0)	(107·6)	(8·0)	(5·7)	(4·0)	(2·8)	(8·0)
S. flexneri (53)	81	23	11	89	92	92	9	0	17	9	0
	(48·6)	(5·6)	(4·3)	(85·3)	(66·6)	(68·3)	(5·9)	(1·5)	(7·3)	(7·9)	(5·0)
S. boydii (11)	64	45	27	100	100	82	36	0	27	0	0
	(128·0)	(53·0)	(14·1)	(225·7)	(106·0)	(64·0)	(38·7)	(2·7)	(14·1)	(2·5)	(5·5)
S. sonnei (60)	27	13	8	84	84	78	8	0	33	13	2
	(13·9)	(5·5)	(4·1)	(170·9)	(85·4)	(57·0)	(5·2)	(2·1)	(9·0)	(16·6)	(4·3)
Average	52	20	11	87	88	84	11	0	25	10	1
	(37·4)	(9·6)	(5·1)	(117·6)	(78·7)	(63·9)	(8·4)	(2·1)	(8·6)	(11·6)	(4·8)

Ap, ampicillin; Tc, tetracycline; Nx, nalidixic acid; An, amikacin; Ka, kanamycin; Sm, streptomycin; Cr, cephalothin; Nm, neomycin; Cm, chloramphenicol; Gm, gentamicin; TMP-SMZ: sulphamethoxazole/trimethoprim.

Table 3. *Drug resistance pattern and plasmids detected in shigella isolates*

Species (no. of isolates)	Drug resistance pattern	No. of drug resistant strains	Molecular weight (MDa)										
			123	110	45–75	17	8·5	4·5	4·2	3·5	2·8	2·5	2·0	1·5
S. dysenteriae (4)	Sm Cm Tc	2	·	·	2	·	1	1	·	·	·	·	·	·
	Sm Cm Tc Ap	1	·	·	1	1	·	1	·	·	·	·	·	·
	a11*S	1	·	·	·	1	·	·	·	·	·	·	·	·
S. flexneri (53)	Sm Cm Tc Ap	29	10	·	29	·	·	4	5	25	24	23	26	6
	Sm Cm Tc	5	1	·	5	·	·	·	·	4	4	4	5	·
	Sm Cm Tc Ap Ka TMP SMZ	5	1	·	5	·	·	1	1	5	4	5	4	·
	No multiple drug resistance	5	·	·	·	·	·	·	1	2	4	·	1	·
	Others	9	·	·	9	·	·	·	1	6	6	5	1	·
S. boydii (11)	Sm Cm Tc Ap Ka Nx Nm	3	·	·	3	·	·	·	·	·	·	·	·	·
	Sm Cm Tc	2	·	·	2	·	·	1	1	·	·	2	1	·
	Sm Cm Tc Ap Ka Nm Cr	1	·	·	1	·	·	·	·	·	·	1	1	·
	Sm Cm Tc Ap Ka Nm	1	·	·	1	·	·	·	·	·	·	·	1	·
	Sm Cm Tc Ap Cr	1	·	·	1	·	·	1	·	·	·	·	·	·
	Sm Cm Tc Ap	1	·	·	1	·	·	1	·	·	·	·	·	·
	Cm Tc Nx	1	·	·	1	·	·	·	1	1	·	·	·	·
	Cm Tc	1	·	·	1	·	·	·	·	·	·	·	·	·
S. sonnei (60)	Sm Cm Tc	16	·	3	16	·	·	13	·	2	3	·	·	3
	Sm Cm Tc Nx	12	·	·	12	·	·	8	·	·	2	·	·	1
	Sm Cm Tc Ap	5	·	1	5	·	·	4	·	1	·	·	·	·
	Cm Tc	5	·	·	5	1	·	2	·	·	1	·	·	1
	No multiple drug resistance	6	·	·	·	·	·	6	2	·	·	1	·	1
	Others	16	·	·	16	·	1	15	·	·	·	2	·	1

S, Susceptible to drugs; Ap, ampicillin; Tc, tetracycline; Nx, nalidixic acid; An, amikacin; Ka, kanamycin; Sm, streptomycin; Cr, cephalothin; Nm, neomycin; Cm, chloramphenicol; Gm, gentamicin; TMP-SMZ: sulphamethoxazole/trimethoprim.

Table 4. *The plasmids found among shigella serotypes*

Serotype (no of strains)	Plasmid MW (MDa) Number of strains											
	123	110	45–75	17	8·5	4·5	4·2	3·5	2·8	2·5	2·0	1·5
S. flexneri 1a (20)	·	·	17	·	·	3	2	16	14	18	18	·
S. flexneri 1b (15)	9	·	14	·	·	1	1	12	11	13	13	·
S. flexneri 2a (6)	1	·	6	·	·	·	·	6	6	·	1	4
S. flexneri 2b (2)	·	·	2	·	·	·	·	2	2	·	·	1
S. flexneri 3a (1)	·	·	1	·	·	·	·	·	·	1	1	·
S. flexneri 3b (4)	1	·	3	·	·	1	3	4	4	3	·	·
S. flexneri 4a (1)	·	·	1	·	·	·	·	·	1	·	1	·
S. flexneri 5b (1)	·	·	1	·	·	·	·	1	1	1	1	·
S. flexneri 6 (2)	1	·	2	·	·	·	2	·	2	·	1	1
S. flexneri VX (1)	·	·	1	·	·	·	·	1	1	1	1	·
S. sonnei I (38)	·	4	35	·	1	32	·	1	4	3	·	6
S. sonnei II (22)	·	·	19	1	·	16	2	2	2	·	·	1

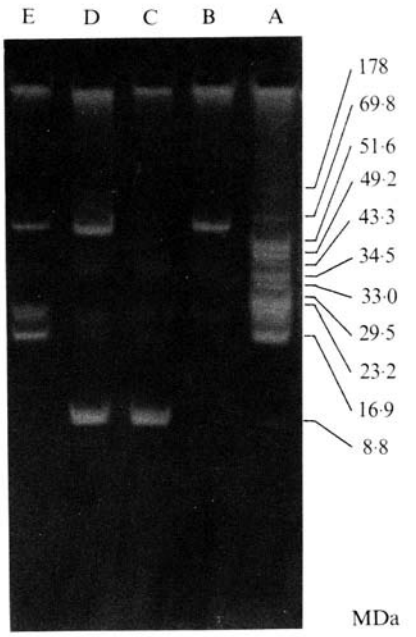

Fig. 1. Agarose gel electrophoresis profiles of plasmid DNA obtained from *Shigella dysenteriae* (A), *Erwinia stewartii* SW2 (MW marker); (B–E), *Shigella dysenteriae* strains.

S. dysenteriae serotypes 1, 8 and 2, were isolated in this study, whereas in 1976 serotype 2 was the most frequently found [4]. Thirty-five of the 128 strains isolated were *S. flexneri* 1a and 1b (27·3%) or 2a and 2b (6·25%). This compares with the 61% of *S. flexneri* 2s isolated in 1976. Changes in the relative incidence of *S. flexneri* serotypes have been reported elsewhere. In Yugoslavia *S. flexneri* 6 was the most prevalent in 1969, in contrast to previous years when it was relatively uncommon [18], and in Kenya *S. flexneri* 6 was the most prevalent

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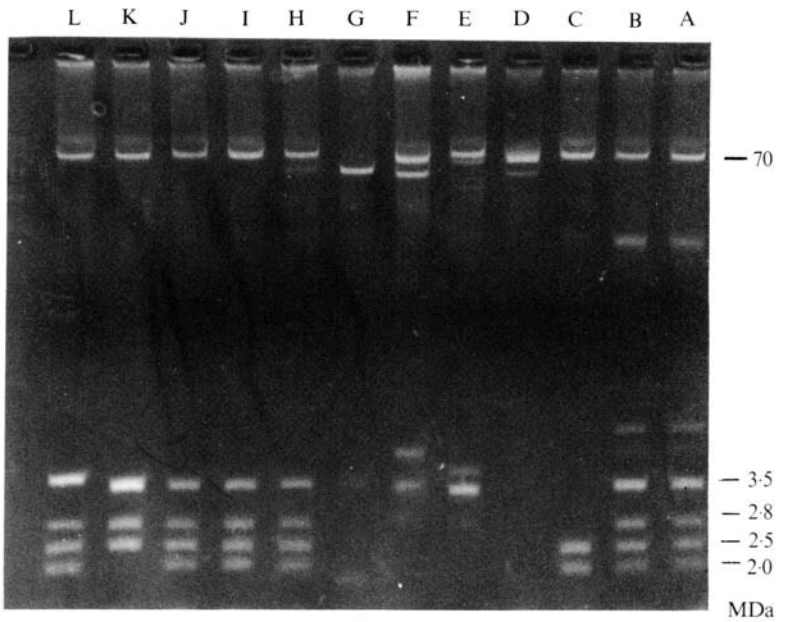

Fig. 2. Agarose gel electrophoresis profiles of plasmid DNA obtained from *Shigella flexneri* (A–L).

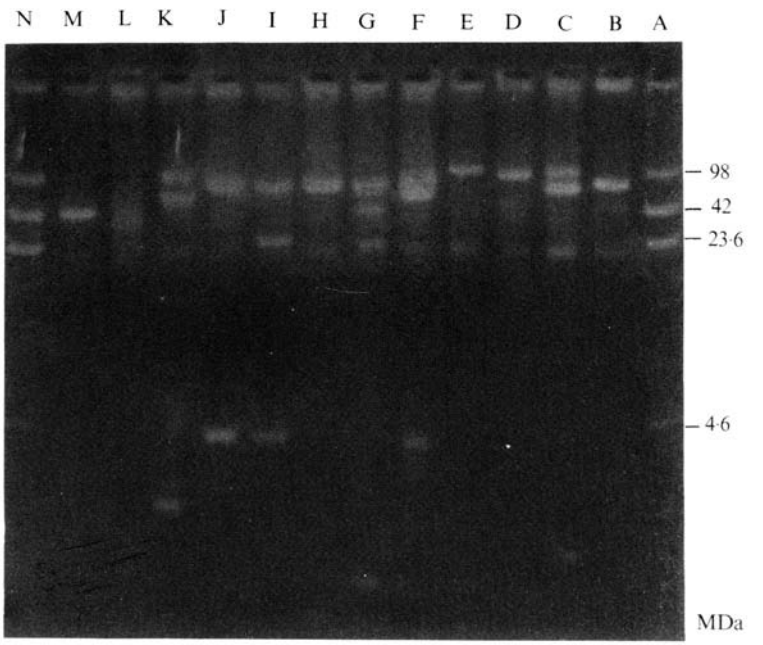

Fig. 3. Agarose gel electrophoresis profiles of plasmid DNA obtained from *Shigella boydii* (A, N), *Escherichia coli* strain 39R861 (MW marker); (B-M), *Shigella boydii* strains.

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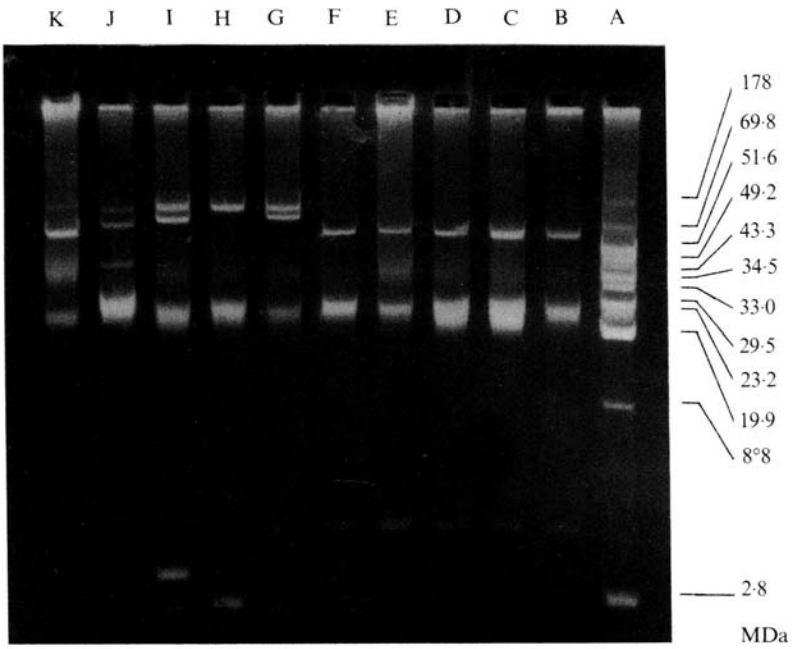

Fig. 4. Agarose gel electrophoresis profiles of plasmid DNA from *Shigella sonnei*. (A), *Er. stewartii* SW2 (MW marker); (B-K), *Shigella sonnei* strains.

serotype isolated during 1977/78 [19], replacing type 2 which had been dominant in the 1960s. Factors leading to these changes are not known. *S. boydii* was found infrequently in both these studies, and *S. sonnei* phase I was found more often than phase II strains.

The two most common patterns of antibiotic drug resistance were Ap Cm Sm Tc (28%) and Cm Sm Tc (20%). Eighty-eight per cent of strains were resistant to tetracycline. This finding was similar to those reported from India and Bangladesh [1, 11, 20] and may reflect the widespread overuse of tetracycline. There had been a particularly significant rise in the proportion of strains resistant to tetracycline and ampicillin since the last survey in 1975 [4].

Plasmid profiles revealed a large 110–123 MDa plasmid in 16 of the isolated strains. This plasmid was previously studied and found to contain a gene conferring virulence [10]. As the form I antigen which is requisite for virulence [21] was only present in the *S. sonnei* phase I, the virulence plasmid of 110 MDa in our isolates was solely found in these strains. This result is compatible with the findings of Sansonette and colleagues [22]. Because the large plasmid was very unstable and easily lost on serial subculture [21], only a small number of strains was found to have the large virulence plasmid. Several small plasmids of unknown function were also observed in all shigella. Some differences were found among the molecular weights of these small plasmids, and the results are closely consistent with the reports of Haider and colleagues [23] and Sakaguchi and colleagues [24].

Shigella strains with plasmid-mediated multiple drug resistance have been well documented in Central America [25] and South-east Asia [26], although these plasmids have not been further studies. In this study, a medium-sized transferrable

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plasmid of 45–75 MDa was contained in all 117 shigella demonstrating multiple drug resistance, while none of the other 11 strains harboured this plasmid. It would seem therefore that a close relationship exists between this plasmid and drug resistance. In our preliminary experiments, we found that 26 strains of *S. flexneri* demonstrating ApCmSmTc harboured a common plasmid of 75 MDa. Incompatibility testing revealed they belonged to HI_2 incompatibility group. Another plasmid of 70 MDa belonging to HII incompatibility group was found in 21 strains of *S. sonnei* with CmSmTc. The characterization of these plasmids will be reported in the future.

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