Plasmid profiles of *Shigella dysenteriae* type 1 isolates from Ethiopia with special reference to R-plasmids

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Summary. Plasmid profiles of 80 *Shigella dysenteriae* type 1 (Shiga's bacillus) strains, collected between 1974 and 1985 in Ethiopia, were examined. Strains with the dominant antibiotic-resistance (R-) type—resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphadiazine (Su) and tetracycline (T)—showed two distinct plasmid profiles (PP). Six plasmid bands were demonstrated in "Ethiopian strains" with PP-1A isolated between 1974 and 1982. In mating experiments with these strains, *Escherichia coli* K12 recipients showed plasmids pYH10a (72 Mda, atypical Inc F_{I_{me}}, coding for ACSSuT resistance). Eight plasmid bands were demonstrated in strains with PP-2A. These strains were first isolated in 1980 and carried plasmid pYH11a (40 Mda, Inc X, coding for ACT resistance). Strains with PP-2A were identical with a "Zairian strain" described elsewhere. Strains with R-type ACT were "Zairian strains" lacking the 5.1- and 4.2-Mda plasmids. Those with R-type CSSuT were temporally clustered in 1978–1980 and carried plasmid pYH12 (58 Mda, Inc B, coding for the same R-type). A trimethoprim-resistant strain (Gimira strain) had a pattern of small plasmids similar to those of the "Zairian strain" and is probably a subclone of the latter. The fact that a limited number of plasmid profiles have remained constant over many years shows the limitation of plasmid profile analysis as an epidemiological tool. However, when the usual profile is known for a given area, identification of a distinctly different pattern becomes easy and epidemiologically useful.

Introduction

Shigellosis is a major health problem in developing countries where conditions still favour endemic disease. In most of these countries there is continuous transmission with sporadic outbreaks or epidemics of serious proportions.¹ The emergence of plasmid-bearing antibiotic, multi-resistant *Shigella* strains has been widely recognised, and has become a serious problem for chemotherapy. However, considering the magnitude of the problem, laboratory investigations of the causative shigellae have been very scanty. In Ethiopia, there have been limited reports of the prevalence of various serotypes^{2,3} and their resistance patterns.^{4–6} Reports of R-factors have appeared only recently^{7,8} and studies of plasmid profiles or R-plasmid characterisation have not been done.

Within the last few years, some of the previously arcane methods of molecular biology have been

simplified. It is now possible to use relatively simple, quick and inexpensive methods to prepare plasmid DNA from bacterial isolates and to visualise it after agarose gel electrophoresis. Accordingly, the molecular analysis of plasmids has been applied to the investigation of epidemiological problems. The usefulness of plasmid analysis for typing *S. sonnei* isolates was evaluated by Prado *et al.*,⁹ but similar studies on the epidemiologically important *S. dysenteriae* type 1 (Shiga's bacillus) have not been reported. The purpose of this study was to analyse the plasmid patterns of *S. dysenteriae* type 1 isolates in Ethiopia, and to evaluate plasmid profiles as an epidemiological tool. An attempt was also made to characterise the R-plasmids found in these isolates.

Materials and methods

Shigella strains

A total of 133 strains of *S. dysenteriae* type 1 was isolated from 1974 to 1985 at the National Research

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Institute of Health (Addis Ababa, Ethiopia) from stools or rectal swabs of patients suffering from acute diarrhoea. The patients were from different parts of the city and had no recognised epidemiological link. Isolates were stored at -70°C in trypticase soy yeast broth with glycerol 25% v/v. From this collection, 76 randomly-selected strains were used for plasmid analysis. For comparative study, four strains of Shiga's bacillus isolated from rural areas were used.

Antibiotic susceptibility

Sensitivity tests were done as described previously.⁷ The agents tested were: ampicillin (A); chloramphenicol (C); kanamycin (K); streptomycin (S); sulphadiazine (Su); tetracycline (T); trimethoprim (Tp). The subscript 'i' with these abbreviations refers to partial (intermediate) resistance.

Plasmid extraction

Plasmid DNA from wild-type Shiga's bacillus and *E. coli* K12 recipients¹⁰ was extracted according to the method of Birnboim and Doly.¹¹ Eppendorf-type 1.5-ml polypropylene tubes and centrifugation at 10 000 *g* were used. All chemicals used were Analar grade or equivalent (BDH or Sigma). Plasmid designation was according to Novick *et al.*¹⁸

Agarose-gel electrophoresis

Agarose (A-6877, Sigma) was dissolved by heating in TE buffer (40mM Tris-acetate, 2mM disodium EDTA, pH 8.0). The agarose was allowed to solidify at room temperature in a horizontal gel apparatus (BRL, Model H4). About 35 μl of plasmid DNA from strains of Shiga's bacillus or their *E. coli* K12 counterparts was mixed with 6 μl of tracking dye,¹² bromocresol purple 0.1% w/v in glycerol 50%, and run at a constant voltage of 150 V for 3.5 h. Gels were then soaked in an aqueous solution of ethidium bromide 0.5 $\mu\text{g}/\text{ml}$ for 1 h. Plasmids were visualised on a Blak-Ray model 61 ultraviolet transilluminator (Ultraviolet Products, San Gabriel, USA) and photographed with an MP4 land camera (Polaroid Corporation, Cambridge, MA, USA) equipped with type 57 land film and an orange wratten filter (Eastman Kodak Co., Rochester, NY, USA).¹³

Mol.-wt determination

Mol. wt of plasmids in wild-type Shiga's bacillus and *E. coli* K12 recipients¹⁰ was determined in relation to the mobility of reference plasmids carried in *E. coli* 39R861 (NCTC 50192, harbouring plasmids of 98, 42, 23.9 and 4.6 Mda). *E. coli* V517¹⁴ with plasmids of 35.8, 4.8, 3.7, 3.4, 2.6, 2.0 and 1.8 Mda and *E. coli* with plasmids TP 116 (143.7 Mda) and TP 124 (120 Mda) (Plasmid Reference Centre, Stanford, CA, USA) were routinely included to check the suitability of the mini-preparation

of Birnboim and Doly¹¹ for the detection of large and small plasmids. Open circular (OC) forms of plasmid DNA were identified after selective extraction at acid pH and low ionic strength.¹⁵

R-plasmid incompatibility testing

E. coli K12 strains carrying single plasmids and derived by mating experiments¹⁰ with Shiga's bacillus were, in turn, mated with NCTC reference strains carrying reference plasmids of the following incompatibility groups: B, C, D, FI, FII, FIII, FIV, FV/OF, H₁, H₂, H₃, I₁, I₂, J, K, M, N, P, T, U, W, X, FI_{me} and MP10. Incompatibility testing was by the methods of Grindley *et al.*¹⁶ and Anderson and Threlfall.¹⁰

Restriction enzyme analysis

Purified plasmid DNA (c. 1–2 μg) was digested with an excess of *Eco*R1 enzyme (BRL, Cambridge). The restriction-enzyme mixture contained 50 mM NaCl, 10 mM MgCl₂ and 10 mM Tris-HCl, pH 7.5; the procedure was that of Maniatis *et al.*¹⁷

Results

Table I shows two main plasmid profiles (PPs) associated with the dominant R-type (ACSSuT). Six plasmid bands of 120, 72, 12.5 OC, 6.0, 5.2 and 2.4 Mda were demonstrated in strains with PP-1A. These strains were isolated between 1974 and 1982. Eight plasmid bands of 120, 40, 12.5 OC, 6.0, 5.2, 2.4 and 1.7 Mda were demonstrated in isolates of Shiga's bacillus with PP-2A. The strains were first isolated in 1980. Isolates of Shiga's bacillus with R-type ACT had plasmid profiles (PP-4) similar to that of PP-2A, but lacked the 5.1- and 4.2-Mda plasmids. Those isolates with R-type CSSuT and PP-3 were temporally clustered in 1978–1980. An epidemic-associated trimethoprim-resistant strain of Shiga's bacillus (Gimira strain) showed a plasmid profile (PP-9) very similar to that of strains with PP-2A.

The mol. wts and incompatibility groups of R-plasmids associated with the plasmid profiles described above are shown in table II. Plasmid pYH10a (72 Mda, Inc FI_{me}, coding for ACS_iSuT resistance) was characteristic of strains with PP-1A, whereas plasmid pYH11a (40 Mda, Inc X, coding for ACT resistance) characterised strains with PP-2A. Plasmid pYH12 (58 Mda, Inc B, coding for CSSuT resistance) was associated with the plasmid profile (PP-3) of strains with R-type CSSuT. A 40-Mda, Inc X plasmid coding for TTp resistance was demonstrated in the Gimira strain (PP-9). The overall distribution of incompatibility

grouping of R-plasmids showed that Inc FI_{me} and Inc X plasmids are common in isolates of Shiga's bacillus from Ethiopia.

An *Eco*R1 restriction enzyme digest of plasmid pYH11a showed fragments of 15, 5.4, 5.2, 3.4, 2.1, 1.7, 1.4, 1.3 and 0.9 Mda.

Discussion

In a recent paper,⁷ we demonstrated that there were two distinct patterns of R-plasmid transfer-frequencies in strains of Shiga's bacillus with R-type ACSSuT: the ACSSuT resistance determinant in strains isolated before 1980 transferred to *E. coli* K12 at low frequencies ($<10^{-4}$ resistant progeny per recipient cell), whereas 50% of conjugative plasmids in strains isolated after 1980 transferred at very high frequencies (10^0 – 10^{-2}) and expressed the ACT determinant only. The present study of plasmid profiles of strains of Shiga's bacillus has further clarified the difference between these two series of isolates. Strains with PP-1A carried plasmid pYH10a, which transferred at low frequencies. This strain, which we refer to as an "Ethiopian strain", was the only strain found in Ethiopia between 1974 and 1980. In an earlier report by Frost *et al.*,¹⁹ a single isolate of Shiga's bacillus from Somalia was found to harbour an Inc FI_{me} plasmid coding for ACSSuT resistance along with an Inc X plasmid coding for ACT resistance.

Isolates of Shiga's bacillus with PP-2A first appeared in Ethiopia in 1980, and carried plasmid pYH11a which transferred at high frequencies. This plasmid profile (PP-2A) was closely related to that of the "Zairian strain" E22106 described by Frost *et al.*²⁰ The Ethiopian isolate of the "Zairian strain" harboured a conjugative plasmid (pYH11a) of which the mol. wt, resistance-phenotype and Inc group were identical to that of the R-plasmid in strain E22106. The 40-Mda plasmid from strains isolated in Ethiopia also showed nine *Eco*R1-generated fragments identical to those in the "Zairian strain".²⁰

Our study has shown that the "Zairian strain" from Ethiopia was linked to severe epidemics in parts of three administrative regions of Ethiopia: Gondar (1981), Illubabor (1983), and Hararge (1983). We presume that this represents an extension of the Central African epidemic into Ethiopia. This becomes even more plausible when we consider the fact that the Central African focus started in 1979²⁰ and identical strains appeared in Ethiopia in 1980. The Tanzanian epidemic of 1982–1983²¹ is probably linked with the Central African epidemic. It would be useful to analyse representative strains of Shiga's bacillus from different African countries to see if they are genetically related.

Haider *et al.*²² analysed the plasmid profiles of strains of Shiga's bacillus from different geographical locations other than Africa. The major resist-

Table I. Plasmid profile analysis of *S. dysenteriae* type 1 isolates from Addis Ababa

Resistance type*	Number of isolates	Plasmid profile	Number of isolates	Year of isolation	Plasmids (size in Mda)											
ACSSuT	112	PP-1A	38	1974–82	120	72	...	...	...	12.5†	6	5.2	...	...	2.4	...
		PP-1B	1	1979	120	72	57	...	...	12.0	6	5.3	...	...	2.5	...
		PP-1C	1	1978	120	72	...	...	...	12.0	6	5.3	...	...	2.5	1.9
		PP-1D	1	1982	120	...	...	...	...	12.0	6	...	4.3	3.0	2.5	...
		PP-2A	15	1980–84	120	...	...	40	...	12.5	6	5.1	4.2	...	2.4	1.7
		PP-2B	1	1980	120	...	...	40	...	13.0	6	5.1	4.3	4.0	2.4	1.7
		PP-2C	1	1983	120	...	50	41.5	...	12.5	6	5.1	4.3	...	2.5	1.8
		PP-2D	1	1984	120	70	...	39	...	12.5	6	5.1	4.2	...	2.4	1.7
CSSuT	9	PP-3	9	1978–80	120	...	58	...	...	12.5	6	...	...	...	2.4	...
ACT	5	PP-4	5	1982–85	120	...	...	38.5	...	12.5	6	...	...	...	2.4	1.6
SSuT	3	PP-5A	2	1982	120	...	60	...	...	12.5	6	...	4.2	...	2.4	1.6
		PP-5B	1	1982	120	...	60	...	...	12.5	6	5.1	4.2	...	2.4	1.6
CSSu	1	PP-6	1	1983	120	...	56	...	...	12.8	6	...	...	...	2.4	...
Su	1	PP-7	1	1977	120	70	...	...	...	12.8	6	...	...	...	2.4	...
ACKSSuT	1	PP-8	1	1978	120	72	63	...	...	12.5	6	...	...	3.0	2.4	...
ACSSuTTP	1	PP-9§	1	1983	120	...	...	40	35	12.5	6	5.1	4.3	...	2.5	1.7
Total	133	PP:1–9	80	1974–85												

* See *Materials and methods*.

† The 12.5–13.0 Mda plasmid represented an open circular (OC) form.

‡ Three isolates were from epidemics in rural Ethiopia.

§ An epidemic strain from Gimira, Keffa, SW Ethiopia.

Table II. Molecular weight and incompatibility group of R-plasmids from *S. dysenteriae* type 1

Plasmid profile	Number of isolates	Recipient R-type*	Number of recipient strains	Plasmid designation	Plasmid size (Mda)	Incompatibility group†
PP-1A	38	ACS ₂ SuT	38	pYH10a	72	FI _{me}
PP-1B	1	ACS ₂ SuT	1	pYH10b	72	FI _{me}
PP-1C	1	ACS ₂ SuT	1	pYH10c	72	FI _{me}
PP-1D	1	ACS ₂ SuT	1	pYH10d	72	FI _{me}
PP-2A	15	ACT	15	pYH11a	40	X
PP-2B	1	ACT	1	pYH11b	40	X
PP-2C	1	ACT	1	pYH11c	41.5	X
PP-2D	1	ACT	1	pYH11d	39	X
PP-3	9	CSSuT	8	pYH12	57.5	B
PP-4	5	ACT	5	pYH11e	38.5	X
PP-5	3	T	3	pYH13	60	N
PP-6	1	CSSu	1	pYH14	56	FII
PP-7	1	Su	1	pYH15	70	B
PP-8	1	ACKSSuT	1	—	72, 63	—
		AK	1	pYH16	63	UNC‡
		ACS ₂ SuT	1	pYH10e	72	FI _{me}
PP-9	1	ACSSuTTp	1	—	40, 35, 5.1, 4.3	—
		AC	1	pYH17	35	X
		TTp	1	pYH18	40	X

* See *Materials and methods*: recipient was *E. coli* K12, F⁻, Lac⁺ (nalidixic acid resistant, prototrophic; Enteric Reference Laboratory no. 14R525).

† Inc FI_{me} plasmids were atypical.

‡ Unclassified; lack of a suitable marker.

ance phenotypes encountered were R-types CSSuT, CSSuTTp and ACSSuTTp. The authors concluded that three plasmids of 140, 6.0 and 2.0 Mda were common to all isolates of Shiga's bacillus. Our study showed that the large plasmid in Shiga's bacillus, as was reported by Frost *et al.*,²⁰ was typically about 120 Mda and all of our strains carried a cryptic plasmid of about 2.4 Mda. The 6.0-Mda plasmid, mentioned above, was a constant feature of all isolates of Shiga's bacillus in Ethiopia, and is known to code for one or more functions for "O" antigen production and bacterial virulence.²³ This plasmid was found earlier in African strains of Shiga's bacillus.²⁰ It seems to be strongly associated with isolates of Shiga's bacillus of African and Asian origin, and is probably serotype specific (unpublished observations).

Our data show that the plasmid profile characteristic of the "Ethiopian strain" was observed over a 9-year period. The "Zairian strain" isolated in Ethiopia also had a reproducible plasmid profile between 1980 and 1984. The predominance of a given strain for a long time gives rise to the question as to whether any particular plasmid-encoded biological properties were responsible. To our knowledge, ours is the first report of isolates of Shiga's bacillus maintaining a uniform plasmid

profile for about a decade. However, among the genus *Salmonella*, *S. wien* was reported to have maintained a uniform plasmid profile for this length of time.^{24,25} This *Salmonella* species harboured an Inc FI_{me} plasmid, as did the Shiga's bacillus endemic in Ethiopia. However, the Inc FI_{me} plasmid in our isolates was atypical; it had a mol. wt of only 72 Mda and was incompatible with Inc FI_{me} and MP10, but was compatible with FI. Casalino *et al.*²⁵ reported the existence of natural derivatives of inc FI_{me} plasmids of 70–80 Mda. The authors classified these atypical strains as Inc FI_{me} despite their compatibility with MP10.

A common pattern of cryptic plasmids in Shiga's bacillus has been demonstrated in this study and in a previous report.²⁰ The fact that African strains of Shiga's bacillus contained significantly more plasmids than strains from Central America and Mexico²⁰ is interesting and deserves further study. Recent reports from Asia indicate that small plasmids are ubiquitous in *Shigella* isolates.^{22,26}

Isolates of Shiga's bacillus of PP-4 and R-type ACT overlapped with the appearance of the "Zairian strain" in Ethiopia. The loss of resistance of streptomycin and sulphadiazine in these strains corresponded with the loss of the 5.1- and 4.2-Mda plasmids as detected by agarose gel electrophoresis.

It is now well established that the 4.2-Mda plasmid coded for SSu resistance in isolates of Shiga's bacillus from Africa^{19,20} and so the 5.1-Mda plasmid may not be associated with SSu resistance.

Isolates of Shiga's bacillus of PP-3 and R-type CSSuT were temporally clustered in 1978-1980, and possessed plasmid pYH12 (58 Mda, Inc B). These isolates were of same R-type as those from Central America.¹ However, the plasmid size in Ethiopian strains is considerably smaller than the 80-Mda Inc B plasmids from Central America,²⁷ but is identical with that of Inc B plasmids from Sri-Lanka.²⁰

Despite the wide use of trimethoprim for the treatment of enteric and other infections, Shiga's bacillus has remained almost uniformly sensitive to this drug in Ethiopia.^{4,6} The only strain resistant to trimethoprim in this study was the "Gimira strain" with PP-9. This epidemic strain showed the basic plasmid profile of the "Zairian strain" and was isolated when the latter had established itself in Ethiopia. It is most likely that the "Gimira strain" is a subclone of the "Zairian strain".

The single isolate of Shiga's bacillus with trans-

ferable Su resistance carried a 70-Mda Inc B plasmid (PP-7). Davey and Pittard²⁸ analysed five *Shigella* strains harbouring transferable plasmids of 42-59 Mda coding for sulphonamide resistance. By contrast, the non-conjugative sulphonamide-resistance plasmids have been reported to be of 3.5-3.9 Mda.²⁹

The above discussion clearly illustrates the clonal nature of isolates of Shiga's bacillus from Ethiopia. The fact that the plasmid profile in Shiga's bacillus has remained constant over many years limits the usefulness of plasmid profiles for epidemiological investigation. However, when the usual profile is known for a given area, identification of a different pattern, as has been demonstrated in this study, becomes easy and epidemiologically useful.

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