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Restriction endonuclease characterization of resistant plasmids in Enterobacteriaceae isolated from children in the Sudan

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SUMMARY

The investigation of plasmid similarity is an important component in the surveillance of antimicrobial resistance and in the detection of epidemic plasmids. The use of restriction endonucleases in the classification of transferable, multiply-resistant plasmids from faecal Enterobacteriaceae isolated at the Children's Emergency Hospital, Khartoum was investigated. Twenty-four transconjugant plasmids, coding for 11 different resistance patterns, each of molecular weight 62 MDa. were studied using four restriction enzymes; *Pst* I, *Eco*R I, *Hind* III and *Ava* II. Fifteen different digest profiles were obtained. Restriction profiles discriminated between plasmids with differing resistance patterns and demonstrated homology of plasmids with common resistance patterns. Restriction endonuclease digest patterns provide a potentially rapid and reproducible method of plasmid classification, that could contribute towards surveillance systems in tropical countries with a high prevalence of antimicrobial resistance.

INTRODUCTION

Several studies have demonstrated a high prevalence of bacterial resistance to commonly used antimicrobial agents in tropical countries (1–5). Genetic investigations during outbreaks of bacterial enteric diseases have demonstrated transferable antimicrobial resistance, and the spread of potentially epidemic plasmids both within and between bacterial species (6–8). Measures to reduce the spread of resistance, and to predict likely resistance problems in future outbreaks, require effective methods for the surveillance and the classification of resistance plasmids (9). While incompatibility grouping has been the basis of plasmid classification (10), and has been shown to be of value in comparing plasmids from outbreaks in different geographical locations (11), recent studies have shown the value of restriction endonuclease characterization in the study of transferable-resistance plasmids (12, 13). As part of a study of antibiotic resistance in enteric flora isolated from children in Khartoum, Sudan, we have investigated the use of restriction endonuclease digestion to characterize resistant transconjugant plasmids.

Table 1. *Plasmid numbers and resistance patterns*

Plasmid no(s).	Resistance pattern
pKc 1–8	Ap Su Tm Sm
pKc 9, 10	Tc C
pKc 11	Ap Tm
pKc 12–15	Tm
pKc 16	Ap Tc
PKc 17	Ap Su Tm Sm C
pKc 18, 19	Ap Tc Su Tm Sm
pKc 20	Ap Su Tm
pKc 21	Ap Tc Su Tm Sm C
pKc 22	Ap Tc C
pKc 23, 24	Ap

PATIENTS AND METHODS

Transferable resistance

Resistance to commonly used antimicrobial agents (ampicillin, tetracycline, sulphonamide, trimethoprim, streptomycin and chloramphenicol) was studied in Enterobacteriaceae isolated from stool specimens from 87 patients attending the Children's Emergency Hospital, Khartoum. Transferable resistance was demonstrated by broth and surface conjunction methods, and plasmid profiles determined using the method of Kado & Liu (14). Details of the methods used in this part of the study have been published previously (15).

Restriction endonuclease characterization of transconjugant plasmids

The conjugation experiments demonstrated 11 different resistance patterns transferred by single plasmids, in all cases of molecular weight 62 MDa, and in each case originating from *Escherichia coli* isolated from faecal specimens. Twenty-four of these plasmids, including each of the 11 resistance patterns, were used in the current study. The plasmid numbers and their resistance patterns are shown in Table 1. For the digests, purified plasmid DNA was prepared using the method of Birnboim & Doly (16), including an additional washing step with acetate-MOPS (0·1 M sodium acetate, 0·05 morpholinopropane sulphonic acid) before final precipitation with ethanol. The common molecular weights of the transconjugant plasmids were confirmed by running the undigested DNA on adjacent gel tracks; these gels were calibrated by using control plasmids of the following molecular weights: 62 Md, 38 Md, 25 Md, 3·0 Md. Four restriction enzymes [each used separately] were used: *Pst* I, *Eco*R I, *Hin*d III and *Ava* II (Northumbria Biologicals, UK). Digests were performed using 15 μl of purified plasmid DNA and 10 units of enzyme in a total volume of 40 μl with the appropriate buffer. Digests were incubated at 37 °C for 3 h. Digested plasmid DNA was run on horizontal. 0·7 % agarose gels at 120 V for 2·5 h, and visualized by staining with ethidium bromide for 20 min. A *Hin*d III digest of phage lambda was used for gel calibration. Gels were photographed using Polaroid 665 film.

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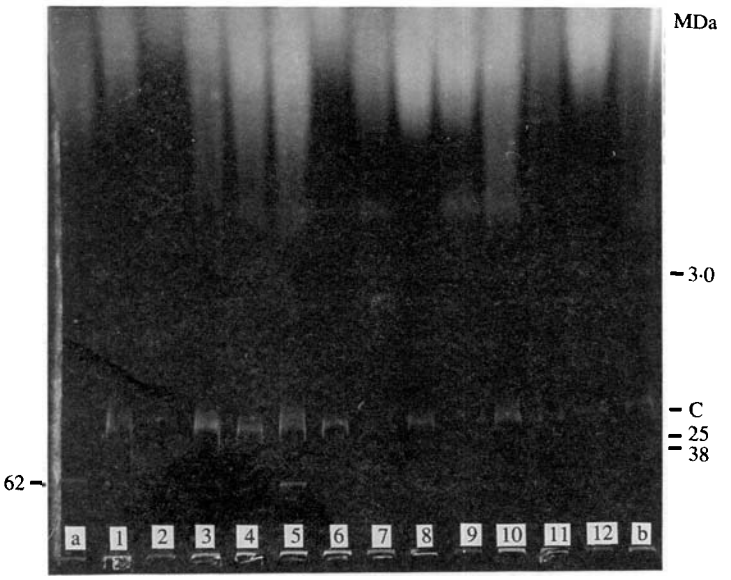

Fig. 1. Gel photo of undigested plasmid DNA for plasmids 1–12. Lanes 1–12, plasmids 1–12. Lanes a and b, standard plasmids. C, chromosomal DNA.

RESULTS

Transconjugant plasmids

The undigested plasmid DNA is shown in Figs. 1 and 2. In Fig. 1 there appears to be some variation in the distance travelled by different plasmids, but if compared to the position of the corresponding chromosomal DNA this difference is seen to be due to gel variability rather than a true difference in plasmid mobility. The Figures confirm that each of the 24 transconjugant plasmids are of a similar molecular weight, in the range 60–65 Md, and estimated to be 62 Md from a logarithmic plot of the standards.

Digest patterns

Figs. 3–6 show the digest patterns for the four enzymes based on the gel photographs. Figs. 7 and 8 are representative gel photographs showing *Hin*d III and *Ava* II digests of plasmids pKC-1 to pKC-12. No plasmids with different resistance patterns have common digest patterns. Plasmids pKC-11,16,17,20,21 and 22, each being the only plasmids in their resistance group, have unique digest patterns. Considerable similarity of digest patterns exist among plasmids with common resistance patterns. Among the eight plasmids with the resistance pattern Ap Su Tm Sm, there appears to be a high degree of DNA homology. For *Pst* I and *Ava* II the eight plasmids have almost identical digest patterns. For *Eco*R I and *Hin*d III there are some variations, but each has common bands. No plasmids of other resistance groups have patterns similar to those of plasmids 1–8. The two plasmids with the resistance pattern Tc C (plasmids 9 and 10) have identical digest patterns for each of the enzymes, indicating a very high degree of DNA homology. The four plasmids with the resistance pattern Tm (plasmids 12–15) do not show a high degree of DNA homology. Plasmids 13 and 14 had

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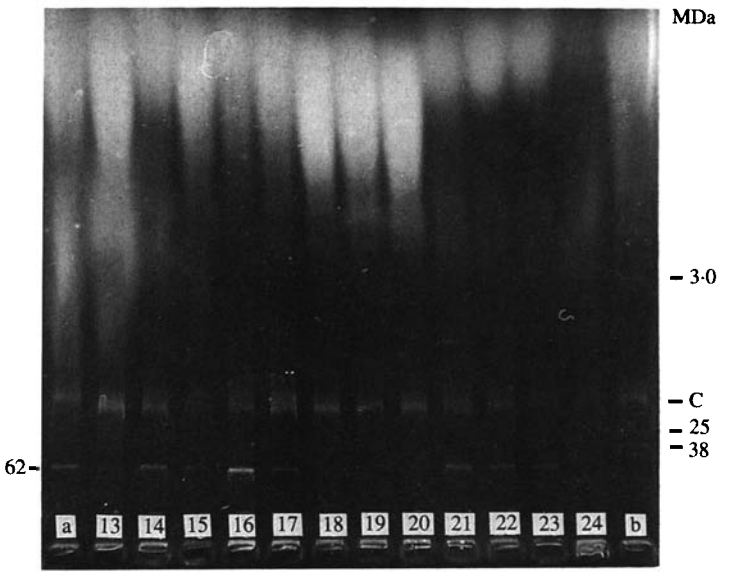
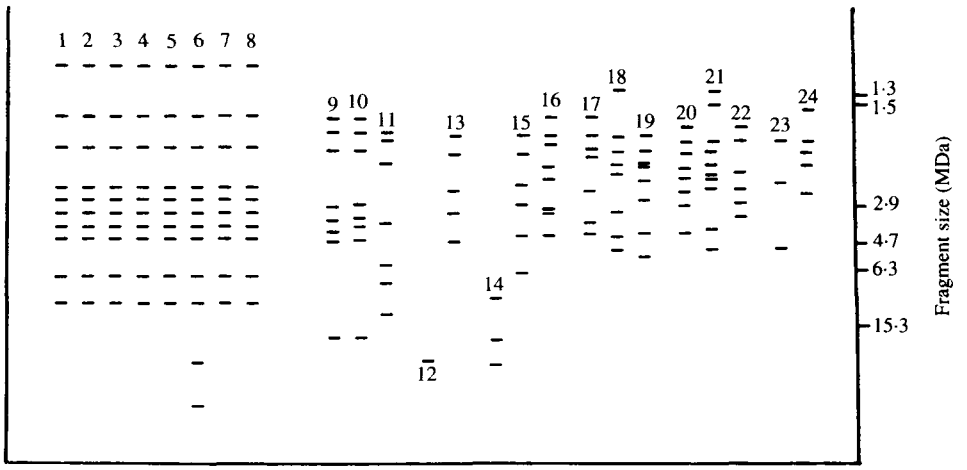


Fig. 2. Gel photo of undigested plasmid DNA for plasmids 13–24. Lanes 13–24, plasmids 13–24. Lanes a and b standard plasmids. C, chromosomal DNA.



Plasmids 1–24

Fig. 3. *Pst* I digests of plasmids 1–24.

similar digest patterns with *Pst* I, and some similarity with plasmid 15 with *Eco*R I, but were different with the other two enzymes. The other Tm resistant plasmid, plasmid 12, appeared to be distinct in each digest. In the two remaining resistance groups, Ap Tc Su Tm Sm (plasmids 18 and 19) and Ap (23 and 24), each plasmid pair had some bands in common, but for none of the enzymes were the patterns within each group identical. Thus, based on the digest patterns, 15 different plasmid types are apparent.

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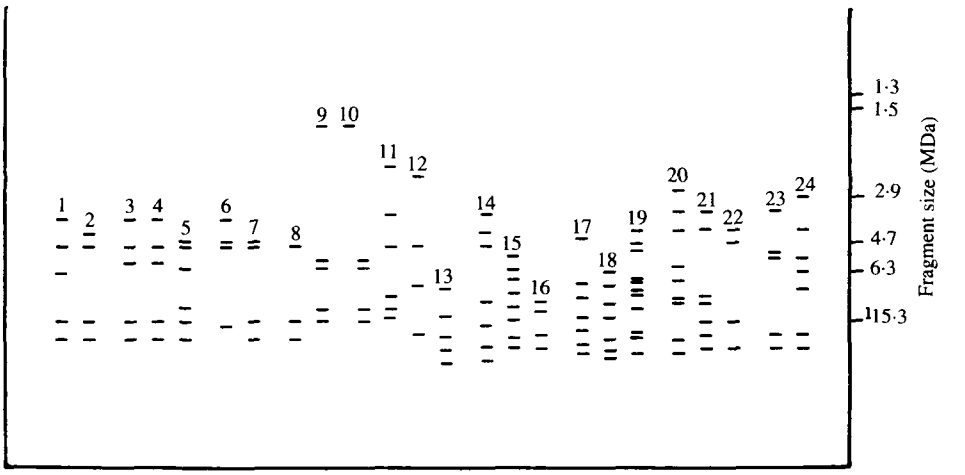

Fig. 4. *Eco*R I digests of plasmids 1–24.

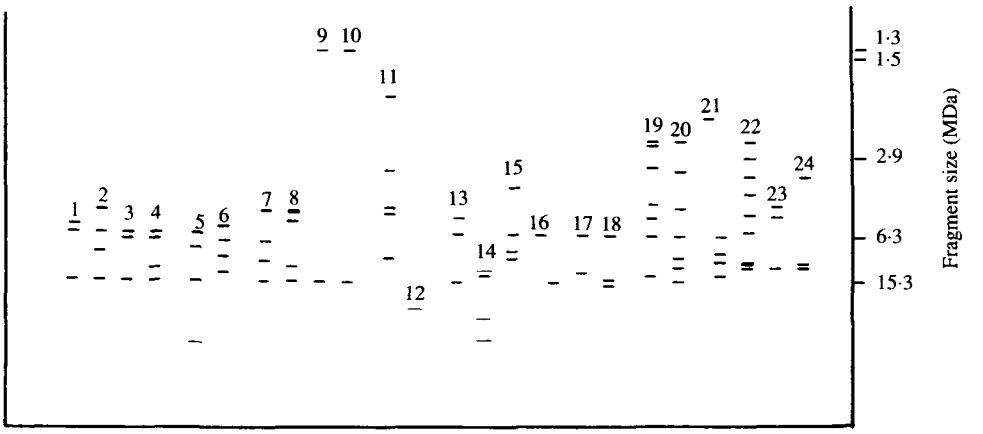

Fig. 5. *Hin*d III digests of plasmids 1–24.

Fragment number and size distribution

Table 2 shows the number of fragments and the plasmid molecular weight estimated from each digest. *Pst* I and *Ava* II produced larger numbers of fragments (mean values 13 and 14 respectively) than did *Eco*R I and *Hin*d III (means values 7 and 5 respectively). There is considerable overlap of fragment number for a given enzyme between plasmids of differing resistance patterns, suggesting that fragment number alone would not be of value in classifying plasmids.

For most plasmids, the sum of fragment sizes do not equal the total plasmid molecular weight as shown in Figs. 1 and 2. Such anomalous findings may result

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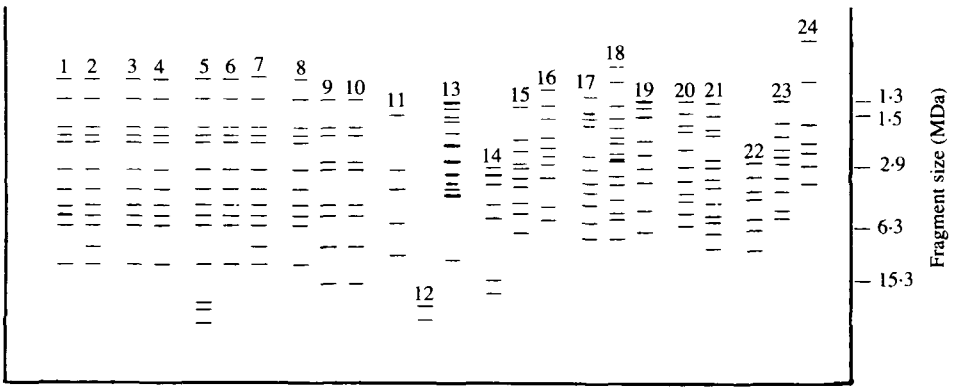

Fig. 6. *Ava* II digests of plasmids 1–24.

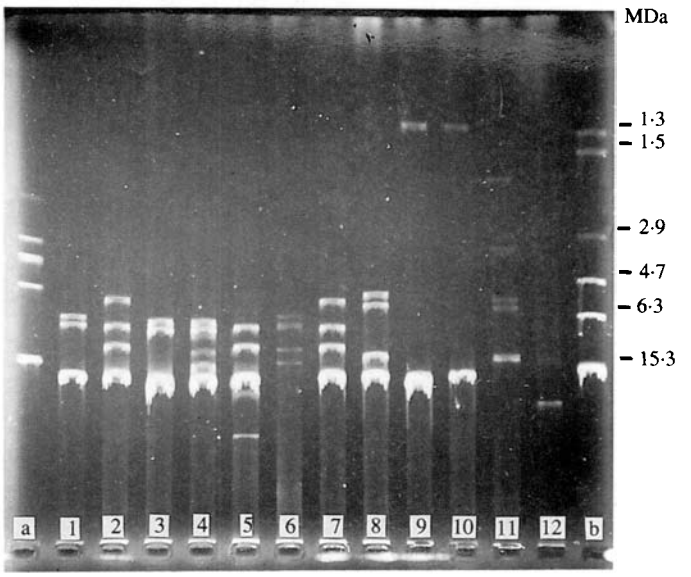

Fig. 7. Gel photo of *Hind* III digests of plasmids 1–12. Lanes 1–12, plasmids 1–12. Lane a, phage lambda *Eco*R I digest; lane b, phage lambda *Hind* III digest.

from multiple fragments of a common size existing as single fragment bands, a lack of differentiation of fragments of similar sizes and a lack of visualization of all DNA fragments. Where the calculated molecular weight exceeds 62 MDa, more than one plasmid may have been present, although only a single plasmid was apparent in each original DNA extraction. In addition to being used to compare plasmid profiles, the determination of fragment sizes may illustrate the presence of transposons with known internal digest patterns. *Pst* I digestion of the ampicillin resistance transposon Tn3 yields two internal fragments of molecular weights 0·4 and 1·9 Ma (17). Because fragment sizes less than 1·0 MDa were poorly visualized in the gels, it was not possible to establish the presence of this fragment

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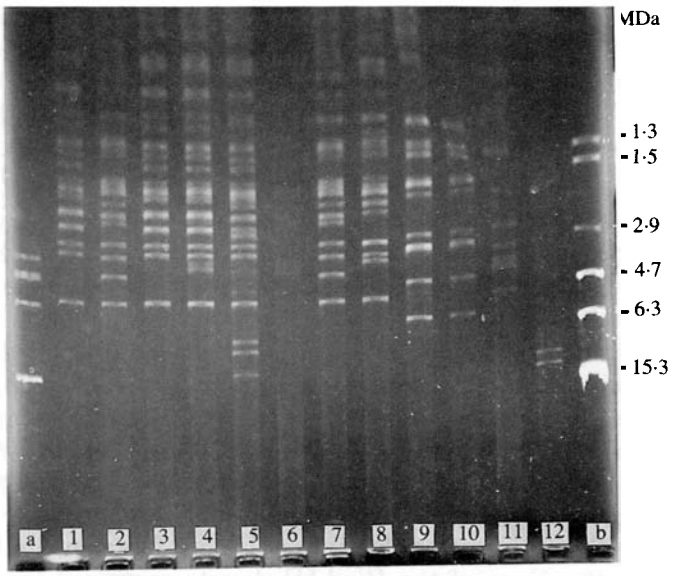

Fig. 8. Gel photo of *Ava* II digests of plasmids 1–12. Lanes 1–12, plasmids 1–12. Lane a phage lambda *Eco*R I digest. Lane b phage lambda *Hin*d III digest.

Table 2. *Fragment numbers and estimated molecular weights from digest patterns*

	Fragment no.				Estimated mol. wt (MDa)			
Plasmid	*Pst* I	*Eco*R I	*Hind* III	*Ava* II	*Pst* I	*Eco*R I	*Hind* III	*Ava* II
1	16	5	3	14	44·3	44·8	31·8	27·5
2	16	4	4	15	44·0	39·3	41·6	32·0
3	16	5	3	14	44·0	44·8	33·2	27·5
4	16	5	5	16	44·0	44·8	44·2	35·2
5	20	6	4	16	68·0	46·5	36·0	70·4
6	16	6	4	14	57·0	39·3	36·8	27·5
7	16	4	4	15	44·0	39·3	41·6	32·0
8	15	3	4	14	37·0	35·5	40·2	24·3
9	10	5	2	11	33·0	38·9	19·4	25·8
10	10	5	2	11	33·0	38·9	19·4	25·8
11	10	7	5	9	27·0	42·3	21·8	23·5
12	1	5	1	2	48·0	28·5	30·0	27·7
13	10	5	3	14	25·3	45·1	39·3	36·1
14	4	8	4	12	77·0 (+62)	45·2	77·0 (+62)	60·4
15	11	10	4	13	39·5	61·7	49·7	36·7
16	15	5	2	14	36·8	43·1	33·0	30·2
17	14	7	2	16	33·2	48·7	31·0	36·8
18	15	7	6	20	43·4	55·0	58·0	51·9
19	15	13	10	18	41·1	66·8	58·2	32·7
20	14	13	9	22	41·3	54·9	94·4	30·6
21	15	10	5	19	43·1	50·2	80·0	37·9
22	10	11	16	18	51·8	47·2	71·5	30·2
23	9	7	4	12	21·1	29·3	49·8	28·5
24	10	10	6	11	17·7	45·4	58·9	18·4

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Table 3. *Plasmid classification using* Pst *I*/Eco*R I*

Major group	*Pst* I fragments	*Eco*R I fragments	Subgroup	Plasmid in sub-groups
A	> 11	1–6	A_1	1–8, 16
		7–10	A_2	15, 17, 18, 21
		> 10	A_3	19, 20
B	5–10	1–6	B_1	9, 10, 13
		7–10	B_2	11, 23, 24
		> 10	B_3	22
C	1–5	1–6	C_1	12
		7–10	C_2	14
		> 10	C_3	

pair. However, three plasmids (11, 21 and 24) each had well-defined fragments of 1·9 MDa from *Pst* I digests, and were ampicillin-resistant, and so may contain the Tn3 transposon. Tn7, a transposon coding for resistance to trimethoprim, streptomycin and spectinomycin, has internal digest fragments of 1·7 and 1·4 MDa with *Hind* III (18). None of the *Hind* III digests contained fragments of these sizes.

Combined enzyme patterns

The combination of digest results from more than one enzyme has been shown to be a possible basis for plasmid grouping (12). Table 3 shows the grouping of the plasmids based on a combination of the fragment numbers using *Pst* I and *Eco*R I. The plasmids are divided into three major groups, A, B and C, based on the number of fragments generated with *Pst* I. Each major group is divided into three minor groups, A1-3, B1-3 and C1-3, based on the number of fragments generated by *Eco*R I. The system shows a potentially useful classification of plasmids. All eight plasmids coding for resistance group 1 (Ap Su Tm Sm) come within minor group A_1. All plasmids that code for resistance to four or more antimicrobials are in group A. The two plasmids in group C both code from trimethoprim alone.

DISCUSSION

The findings from our study confirm those of others (12, 13, 19) that restriction endonuclease digest patterns have a potential role in resistance plasmid classification. The 24 plasmids investigated are divisible into 15 types on the basis of digest pattern, with adequate discrimination between plasmids coding for different resistance patterns. Among plasmids of common antimicrobial patterns, some show a high degree of DNA homology as indicated by similar digest patterns (Ap Su Tm Sm plasmids pKC1–8, TcC plasmids pKC9–10). These plasmids occurred in trans-conjugants derived from isolates from different non-hospitalized children, indicating a spread of such plasmids within the community. In other cases, plasmids with the same resistance pattern have very dissimilar restriction digest patterns and therefore little DNA homology (Tm plasmids pKC12–15, Ap plasmids pKC23–24). These findings suggest the distribution of particular resistance genes in dissimilar plasmids, possibly as transposons. In our data, Tn3 may be present in a small number of isolates, and Tn7 appears not to be present.

However, other studies have shown that both of these transposons may occur in isolates from the tropics and may contribute to the widespread dissemination of resistant genes between species (8, 20). The number of fragments generated by single enzymes is not sufficiently discriminatory to be useful in plasmid classification, but combining the results from two different digests does provide a basis for classification.

Classification by restriction enzyme patterns may be complementary to incompatibility grouping. Incompatibility group is determined by only a part of the total DNA of a plasmid, and plasmids of the same incompatibility group may differ in both molecular weight and antibiotic resistance determinants (10). As the DNA responsible for incompatibility grouping is related to the plasmid replication mechanism, and is likely to be highly conserved (22), it may be regarded as a marker of common evolutionary origin (23). However, the exchange of DNA within plasmids, both by acquisition and loss of transposons, and by gene mutation and deletions (24) results in plasmids of the same incompatibility group having diverse total DNA. Various studies have demonstrated DNA variability within incompatibility groups by restriction endonuclease digests and DNA hybridization (25–28). While plasmid incompatibility provides evidence for a common origin, a high degree of DNA homology demonstrated by identical or similar digest patterns is a useful indicator of current identity.

Our earlier findings of a high prevalence of antimicrobial resistance in the Khartoum study group (5), and the genetic diversity of transconjugant plasmids demonstrated in this paper, suggest that there is a heterogeneous and mobile genetic pool in the faecal flora of this population. Data on resistance plasmids is an essential component in combating the spread of antimicrobial resistance in such communities. The data have shown that restriction endonuclease digests can help to identify common and potentially epidemic plasmids, and can provide a classification scheme to assist in plasmid surveillance.

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