DISTRIBUTION AND TRANSFERABILITY OF PLASMIDS IN TRIMETHOPRIM-RESISTANT URINARY STRAINS OF *ESCHERICHIA COLI*: A COMPARATIVE STUDY OF HOSPITAL ISOLATES

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SUMMARY. Trimethoprim-resistant urinary isolates of *Escherichia coli*, collected in 1982, were studied and the results compared with those obtained for isolates collected during the period 1979–1980. Ninety-eight (81%; a 10% increase) were resistant to trimethoprim 1024 µg/ml and 93 (95%) were also resistant to sulphamethoxazole 1024 µg/ml. The frequency distributions of plasmids were similar in both collections although there was a significant increase in the number of small plasmids (mol. wt $\leq 20 \times 10^6$) in the 1982 collection. Transfer of resistance was associated with isolates that carried larger numbers of plasmids.

A significantly smaller proportion of isolates in this, than in the earlier series, transferred trimethoprim resistance to *E. coli* K12 suggesting continued transposition of trimethoprim resistance on to the bacterial chromosome. Fifteen different trimethoprim resistance plasmids were identified, of which five, including that found most frequently, were common to both collections. Plasmids which transferred trimethoprim resistance without sulphonamide resistance were more common in the 1982 series. Plasmids which transferred linked trimethoprim and streptomycin resistances, in particular those not carrying other resistance markers, were less common in the 1982 series of isolates. Although the majority of *E. coli* isolates highly resistant to trimethoprim remained resistant to sulphonamide, our results suggested changes in the genetic location and linkage of the resistance markers.

INTRODUCTION

Since trimethoprim became available for prescription as a single agent late in 1979, after 10 years of use in combination with sulphamethoxazole, the incidence of trimethoprim-resistant bacteria has risen very little (Lacey, 1982; Brumfitt, Hamilton-Miller and Wood, 1983). This is similar to the experience in Finland where, though trimethoprim has been in use as a single agent since 1973, the incidence of trimethoprim-resistant organisms has been reported to be no higher than that in countries using only trimethoprim-sulphonamide combinations (Huovinen, Manty-

jarvi and Toivanen, 1982; Kasanen and Sundquist, 1982). However, an increasing proportion of trimethoprim-resistant bacteria possesses high-level resistance to the drug (MIC > 1000 µg/ml); this suggests the continuing spread of plasmid- or transposon-mediated resistance (Towner, 1982; Brumfitt *et al.*, 1983).

Following our report on isolates collected during 1979–1980 (Kraft, Platt and Timbury, 1983), trimethoprim-resistant urinary coliforms isolated during 1982 have been investigated by similar methods. In this paper, we present the characteristics of this second collection of isolates together with a detailed comparison of the results of the two studies with respect to plasmid distribution, transferable resistance and the genetic linkage of trimethoprim resistance to other resistance determinants.

MATERIALS AND METHODS

Bacteria. The strains of enterobacteria studied were isolated in significant numbers ($\geq 10^5$ organisms/ml) from specimens of urine from hospital patients and showed no inhibition zone around a disk containing trimethoprim 1.25 µg. They were collected from the routine diagnostic laboratory, Glasgow Royal Infirmary between January and July 1982. The organisms were identified in tests with the API 20E system (API Laboratory Products Ltd, Farnborough, Hants GU14 7QH). After initial screening, repeat isolates from the same patient were excluded unless they were distinguishable from previous isolates.

Media, antimicrobial sensitivity tests, resistance transfer procedure and detection of plasmid DNA were as described previously (Kraft *et al.*, 1983).

Retransfer of resistance. When an *E. coli* K12 transconjugant was isolated which had acquired a single resistance marker, retransfer of that marker and the relevant plasmid to another appropriate strain of *E. coli* K12 was used to distinguish between plasmid-mediated resistance and the possible selection of a mutant. Retransfer was also used to separate plasmids from crosses that generated only multiplasmid transconjugants.

Statistical analysis. The plasmid-frequency distributions in the two collections of organisms were compared in a χ^2 test. Other data from the two collections were compared in a χ^2 (2×2) contingency test incorporating Yates's correction for continuity (Siegel, 1956).

RESULTS

Characterisation of the clinical isolates

One hundred and forty-seven isolates of *E. coli* were collected that were resistant to a 1.25-µg trimethoprim disk; 81% of them were highly resistant to trimethoprim (MIC > 1024 µg/ml), an increase of 10% compared with the first collection (Kraft *et al.*, 1983). That difference, however, was not statistically significant ($p > 0.05$). We obtained from six different patients nine isolates which were moderately resistant to trimethoprim (MIC > 64 but < 1024 µg/ml). None transferred trimethoprim resistance although all but one carried plasmids.

Our detailed characterisation was restricted to 98 highly resistant *E. coli* isolates obtained from 91 patients. A large proportion of these isolates was multi-resistant, although resistance was not detected to drugs other than those listed in our previous report, namely, trimethoprim (Tp), sulphamethoxazole (Su), streptomycin (Sm), tetracycline (Tc), ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), and nalidixic acid (NaI). Resistance to three or more drugs was found in 96% of the isolates and 90% were resistant to four or more. However, the proportions of isolates resistant to five or more drugs were smaller than in the first collection (table I). There

TABLE I

Antimicrobial resistance of clinical isolates of E. coli resistant to trimethoprim 1024 µg/ml

Number of antibiotics* to which resistance was detected	Percentage† of isolates resistant from collection of	
	1979-1980	1982
1	0	0
2	2	4
3	11	5
4	14	31
5	28	36
6	22	17
7	18	6
8	5	1

*Antibiotics, and their concentrations per disk: trimethoprim 1.25 µg; sulphamethoxazole 25 µg; streptomycin 10 µg; tetracycline 10 µg; ampicillin 10 µg; chloramphenicol 10 µg; kanamycin 30 µg; and nalidixic acid 30 µg.

†The numbers of isolates in the 1979-1980 and 1982 collections were, respectively, 88 and 98.

was no decrease in the proportion of isolates resistant to sulphamethoxazole. All except four isolates (i.e., 94 strains) showed no zone around a 25-µg sulphamethoxazole disk and the MIC of this drug for 93 isolates (95%) was > 1024 µg/ml.

Transfer of resistance

Significantly fewer (34) isolates from this collection transferred trimethoprim resistance to *E. coli* K12 than the 44 from the 1979-1980 collection, a decrease of 15% ($p < 0.05$). Altogether, 52% of the isolates from the 1982 series transferred antimicrobial resistance to *E. coli* K12, 9% fewer than in the earlier collection, a difference that was not statistically significant ($p > 0.2$).

The proportion of isolates that transferred resistance to both trimethoprim and sulphamethoxazole had decreased from 35% to 22% whereas the proportion that transferred resistance to one of the drugs in the absence of resistance to the other remained similar (table II). With the exception of one isolate that transferred trimethoprim resistance only, transfer of resistance to trimethoprim, or sulphonamide,

TABLE II

Clinical isolates of E. coli that transferred resistance to trimethoprim and sulphamethoxazole to strains of E. coli K12

Drug resistance transferred	Percentage of isolates* from collection of	
	1979-1980	1982
Trimethoprim and sulphamethoxazole	35	22
Trimethoprim but not sulphamethoxazole	15	13
Sulphamethoxazole but not trimethoprim	7	12
Other antimicrobials excluding trimethoprim and sulphamethoxazole	4	5
None	39	48

* See footnote, table I.

was generally accompanied by transfer of resistance to at least one other antimicrobial agent.

Seventeen different patterns of resistance cotransferred with trimethoprim resistance. Isolates in the earlier (1979–1980) collection cotransferred 16 different patterns, and 10 patterns were common to both collections.

The commonest patterns transferred by isolates in the 1982 collection were Tp Su Sm Ap (six isolates) and Tp Su Sm Tc and Tp Su Sm Tc Ap (four isolates each), patterns which had also been observed with the 1979–1980 collection; conversely the three commonest patterns in the 1979–1980 collection were also present in the 1982 collection. The twelve strains that transferred resistance to sulphonamide but not to trimethoprim transferred five different patterns of resistance, two of which were common to both collections, including the most frequent pattern Su Sm Ap (seven isolates from the 1982 collection and one from the 1979–1980 collection).

Plasmid distributions in the clinical isolates

Plasmids were detected by gel electrophoresis in 97 (99%) of the isolates, a frequency similar to that found in the previous collection. Seventy-seven per cent of the isolates in the present series compared with 60% in the previous one carried two or more plasmids. The distribution of plasmid numbers in the two collections is shown (fig. 1a). There was an increase in the number of isolates that harboured three or more plasmids although the difference between the two distributions was not statistically significant ($p > 0.2$). However, this increase appeared to be due to an increase in the number of isolates that harboured multiple small plasmids of mol.wt $\leq 20 \times 10^6$ and this difference was statistically significant ($p < 0.001$) (fig. 1b). Further comparison of the plasmid distributions in the two collections was, therefore, restricted to plasmids of mol. wt $> 20 \times 10^6$ (fig. 2). The frequency distribution of plasmids of mol. wt $> 20 \times 10^6$ was similar in the two collections (fig. 2a). However, the small numbers of isolates in more than 20% of the categories precluded statistical comparison in a χ^2 test.

There was the same tendency in both collections for resistance transfer to be associated with the presence of more than one plasmid in the isolate concerned. Among isolates that did not transfer resistance, the modal plasmid number was one plasmid per isolate (fig. 2b); among isolates that transferred resistance, the modal plasmid numbers were two to three per isolate from the 1979–1980 collection and two per isolate from the 1982 collection (fig. 2c).

Characterisation of plasmids

Twenty-four isolates generated a total of 25 trimethoprim-resistant transconjugants with a single plasmid which corresponded in mol.wt to one in the donor. In addition, trimethoprim-resistant transconjugants obtained from four clinical isolates acquired a plasmid which did not correspond to any detected in the donor. In the case of these four isolates, the transconjugant plasmid was of mol.wt $(8-10) \times 10^6$ larger than a donor plasmid and also specified low-level streptomycin resistance. One isolate (strain GRI15279) from the 1979–1980 collection behaved similarly (table III). Sixteen isolates transferred single plasmids that coded for resistance to sulphonamide but not to trimethoprim.

FIG. 1.—Frequency distribution of plasmids in clinical isolates of *E. coli* resistant to trimethoprim 1024 $\mu\text{g/ml}$: (a) all plasmids; (b) plasmids of mol. wt $\leq 20 \times 10^6$. $\square$ = 88 isolates from 1979–1980, $\blacksquare$ = 98 isolates from 1982.

FIG. 2.—Frequency distribution of plasmids of mol. wt $> 20 \times 10^6$ in clinical isolates of *E. coli* resistant to trimethoprim 1024 $\mu\text{g/ml}$: (a) all isolates; (b) isolates that did not transfer antimicrobial resistance; (c) isolates that transferred antimicrobial resistance to *E. coli* K12. □ = 88 isolates from 1979–1980, ▨ = 98 isolates from 1982.

TABLE III

Plasmids in clinical isolates of E. coli that appeared to acquire trimethoprim and streptomycin resistance on transfer to E. coli K12

Isolate, GRI no.	Donor plasmids			Transconjugant plasmid	
	Mol. wt ($\times 10^6$)	Transfer	Resistance*	Mol. wt ($\times 10^6$)	Resistance*
15279	< 10	—	ND		
	40	—	none	47	Tp Sm
1482	30	—	ND		
	40	+	Ap	53	Tp Sm Ap
14682	5	—	ND		
	40	—	none	48	Tp Sm
	85	—	ND		
26582	< 15	—	ND		
	< 20	+	not known†		
	20	—	none	30	Tp Sm
	42	—	ND		
	65	+	Su Sm Ap		
	70	—	ND		
27582	< 15‡	+	Su Sm†		
	25	—	none	36	Tp Sm
	55	+	not known†		
	70	+	Tc		

* See text for key to abbreviations. In the case of non-transferring plasmids, the absence of resistance markers was deduced from the resistance carried by the larger transconjugant plasmid.

† Plasmids cotransfer only.

‡ Four different plasmids.

ND=not determined.

Genetic linkage of transferable trimethoprim and sulphonamide resistance was similar to that in the 1979–1980 collection. Eight isolates transferred resistance to both trimethoprim and sulphonamide on the same plasmid, 6% fewer than previously, whereas 17 isolates transferred trimethoprim resistance on a plasmid without sulphonamide resistance, a corresponding increase of 6%. Of these 17 isolates, eight also transferred sulphonamide resistance but on a separate plasmid, a proportion similar to that in the first collection.

Fifteen different trimethoprim-resistance plasmids were identified on the basis of mol. wt and resistance pattern, three more than in the 1979–1980 collection. However, only five of these plasmids were common to both collections (table IV). The plasmid found most frequently in both collections transferred the resistance pattern Tp Su Sm Ap and had a mol. wt of $c. 50 \times 10^6$. Altogether, trimethoprim- and sulphonamide-resistance determinants were linked on four different plasmids, the same number as with the 1979–1980 isolates, but only one was common to both collections. Eleven different plasmids specified trimethoprim resistance without sulphonamide resistance, three more than in the 1979–1980 isolates, and four of these were common to both collections.

Significantly fewer plasmids carried linked trimethoprim and streptomycin resistance. Among the 1982 isolates, 53% of the plasmids analysed carried both these markers compared with 86% in strains from 1979–1980 ($p < 0.01$). In particular there were fewer plasmids with the resistance pattern Tp Sm in the 1982 collection and one Tp Sm plasmid of mol. wt $c. 49 \times 10^6$ that was transferred by eight isolates in the

TABLE IV

Characteristics of plasmids that transferred resistance to trimethoprim, or to sulphamethoxazole without trimethoprim

Antimicrobial resistance* pattern transferred	Mean plasmid mol. wt ($\times 10^6$)	Number of donor isolates that transferred plasmid from collection of	
		1979-1980	1982
Tp	46	0	1
Tp Sm†	35	3	2
Tp Sm	49	8	0
Tp Sm†	60	2	1
Tp Sm	73	1	0
Tp Tc†	32	2	3
Tp Tc	86	0	1
Tp Km	42	0	1
Tp Km†	64	3	2
Tp Sm Tc	48	3	0
Tp Sm Ap	49	0	1
Tp Sm Km	74	1	0
Tp Su Sm Tc	31	1	0
Tp Su Sm Ap†	50	9	5
Tp Sm Tc Ap	62	0	1
Tp Tc Ap Km	66	0	1
Tp Su Sm Tc Ap	46	3	0
Tp Su Sm Tc Ap	67	1	0
Tp Tc Ap Cm Km	65	0	3
Tp Su Sm Tc Ap Cm	62	0	1
Tp Su Sm Tc Ap Cm	92	0	1
Tp Su Sm Tc Ap Cm Km	56	0	1
Su Sm	5	0	3
Su Sm	33	1	0
Su Sm Tc	78	1	0
Su Sm Ap	34	3	0
Su Sm Ap†	64	3	7
Su Tc Ap	65	0	1
Su Sm Tc Ap	62	1	0
Su Sm Tc Ap Km	91	0	3
Su Sm Ap Cm Km	56	0	1
Su Sm Tc Ap Cm	90	0	1
Su Sm Tc Ap Cm Km	66	5	0

* See text for key to abbreviations.

† Plasmids common to isolates from both collections.

1979-1980 collection, did not appear at all. More plasmids were found in the 1982 series with the resistance patterns Tp Tc and Tp Km.

Six different plasmids transferred sulphonamide resistance without trimethoprim resistance and one of these, resistance pattern Su Sm Ap and mol. wt $c. 64 \times 10^6$, was common to both collections.

DISCUSSION

Our results showed that the proportion of trimethoprim-resistant *E. coli* with high-level resistance had increased in 1982 relative to 1979-1980. A similar increase has been reported by other workers (Brumfitt, Hamilton-Miller and Gooding, 1980; Towner, 1981) and suggests a continuing trend towards plasmid- or transposon-mediated resistance. Only high-level trimethoprim resistance was transferable and

plasmids specifying intermediate levels of trimethoprim resistance (Towner and Wise, 1983) have not appeared among our isolates.

In spite of the change in prescribing policy, 95% of the highly trimethoprim-resistant isolates were also highly resistant to sulphamethoxazole. Isolates highly resistant to trimethoprim but sensitive to sulphonamide still appear to be rare. This finding, which is consistent with the findings of other workers (Huovinen and Toivanen, 1981; Towner, 1982; Brumfitt *et al.*, 1983) may reflect the fact that trimethoprim as a single agent is not yet widely used. Over 84% of the trimethoprim prescribed in Scotland in 1982 was in the form of co-trimoxazole (Drug Information Centre, Ninewells Hospital, Dundee; unpublished data).

The reason for the decrease in multiple antibiotic resistance among our trimethoprim-resistant isolates is not immediately apparent, unless it reflects the further spread of a transposable element specifying trimethoprim resistance through a population of plasmids carrying few other resistance markers.

The decrease in the proportion of isolates that transferred trimethoprim resistance suggests a continuation of the process whereby resistance is transposed from a plasmid on to the bacterial chromosome and the plasmid subsequently lost. Alternatively, resistance may be carried on non-transferable (*tra*⁻) plasmids. Towner (1981) and Towner, Venning and Pinn (1982) mobilised trimethoprim resistance from, respectively, one and 18 strains of *E. coli* by means of plasmid RP4 and their results suggest that both mechanisms may be involved.

We suggested previously (Kraft *et al.*, 1983) that, because many isolates carried trimethoprim and sulphamethoxazole resistance on separate plasmids, there was potential for these markers to segregate after the removal of sulphonamide selection. Thus, trimethoprim-resistant, sulphonamide-sensitive strains might be expected to emerge having lost the sulphonamide plasmid but not the plasmid specifying trimethoprim resistance. As the majority of our isolates remained resistant to both drugs, this process does not thus far appear to be widespread. This may be due to the minimal change in prescribing. However, the frequent genetic linkage of sulphonamide and ampicillin resistance may also be contributory (see table IV) because the widespread use of the latter drug may co-select for the maintenance of the sulphonamide-resistance determinant in the bacterial population. There is circumstantial evidence that hospital patients previously treated with ampicillin may have an increased incidence of rectal carriage of trimethoprim-resistant coliforms (Grüneberg and Bendall, 1979) and of urinary-tract infection with such organisms (Amyes, 1983).

Our results, however, suggest that there have been changes in the genetic location of the resistance determinants. There were fewer isolates in 1982 that transferred plasmids carrying both trimethoprim and sulphonamide resistance, whereas a larger proportion transferred plasmids that carried one of these markers in the absence of the other. This suggests that not only has trimethoprim resistance, possibly in the form of the transposon Tn7, been transposed to a chromosomal location but also that sulphonamide resistance may behave similarly.

With the exception of the predominant plasmid type, the general lack of similarity between the plasmids from the two collections might be attributed to a change in the plasmid population. However, it seems more likely that these results simply represent two relatively small samples from a very large pool of different plasmids. Therefore, detailed comparisons should be made with caution. Nevertheless, they provide

evidence for the dissemination of trimethoprim resistance through a large variety of different plasmids.

The reduction in the proportion of plasmids carrying linked trimethoprim-streptomycin resistance (presumptive evidence of Tn7), and the appearance of plasmids carrying Tp, Tp Tc and Tp Km, suggests the possible spread of other trimethoprim transposons in the plasmid population. Towner and Wise (1983) have described plasmids that specified Tp and Tp Km resistance but they did not find one specifying Tp Tc. Transposon Tn 402, described by Shapiro and Sporn (1977), determines trimethoprim resistance without streptomycin resistance; so too does transposon Tn 78, considered to be a derivative of Tn7 (Datta *et al.*, 1979).

The five isolates described in detail in table III probably contained transposon Tn7 either on the chromosome or on a *tra*⁻ plasmid. In-vitro selection for trimethoprim resistance, imposed after broth mating, allowed the isolation of the transconjugants described, which appeared to contain a transferable (*tra*⁺) plasmid that had acquired transposon Tn7. This further emphasises the need to compare the mol. wt of donor and transconjugant plasmids. The low frequency of transfer obtained reflects, no doubt, the low level of transposition events in natural populations. Although these results may be analogous to the clinical situation in a patient receiving trimethoprim therapy, it is arguable whether the original clinical isolate contained a plasmid coding for transferable trimethoprim resistance.

The significant decrease in the prevalence of plasmids coding for linked resistance to trimethoprim and streptomycin between the two collections may be a result of the transposition of Tn7 on to the chromosome followed by loss of the plasmid. The much lower incidence of plasmids which transferred the resistance pattern Tp Sm only, supports this hypothesis. The absence of other resistance markers makes this group of plasmids the most likely to be lost after such a transposition event because antimicrobial selective pressure will no longer maintain them against the biosynthetic burden they impose on the host bacterium (Sherratt, 1982).

The findings, that the plasmid distributions in the two collections were similar and that transfer of resistance again tended to be associated with isolates which carried larger numbers of plasmids, suggest that this is a consistent property of this group of organisms. The relation also applies to plasmids of mol. wt $> 20 \times 10^6$. The increased incidence of small plasmids of mol. wt $\leq 20 \times 10^6$ in the second collection remains unexplained.

In conclusion, despite the introduction of trimethoprim as a single agent into clinical prescribing, there has been little reduction in the use of co-trimoxazole. Our finding that high-level trimethoprim resistance remains associated with high-level sulphonamide resistance in the majority of isolates supports the view that selective pressure from these drugs has changed only minimally. However, our results also suggest continuing change in the genetic location of the resistance determinants. Trimethoprim resistance may be transposed on to the bacterial chromosome; sulphonamide resistance may be showing a similar trend. This, together with the genetic linkage of trimethoprim or sulphonamide resistance with determinants of resistance to other drugs (e.g. ampicillin) which remain in common use, creates a complex dynamic situation subject to the effect of many selective pressures from within and external to the cell. Thus, alteration of a single factor, such as reduction of sulphonamide usage, may not necessarily produce rapid changes in bacterial resistance.

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