R-factor mediated trimethoprim resistance: result of two three-month clinical surveys

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SUMMARY   All urinary tract isolates were monitored in the Whittington Hospital, London for trimethoprim resistance over a three-month period in 1975; this survey was repeated 18 months later in 1977. In the later survey the incidence of trimethoprim resistance had increased significantly, and the proportion of strains carrying R-factors conferring trimethoprim resistance had nearly doubled. The pattern of resistances associated with R-factor trimethoprim resistance also changed between these two surveys.

The combination of trimethoprim and sulphamethoxazole in the chemotherapeutic preparation co-trimoxazole (Septrin, Bactrim) was first used clinically in 1969. At that time virtually all coliforms, but not pseudomonas, were sensitive to the combination. In those days 23-30% of organisms were already resistant to sulphonamides, but in most cases this did not appear to diminish the successful use of co-trimoxazole. In 1972, organisms were isolated that showed very high resistance to trimethoprim, and the gene which determined this resistance resided on an R-factor (Fleming *et al.*, 1972; Datta and Hedges, 1972). This was disturbing, for the clinical efficiency of co-trimoxazole often seems to depend on the bacteria being sensitive to the trimethoprim moiety. Nevertheless, between 1972 and 1974, Grüneberg (1976) reported that, in domiciliary patients, the incidence of trimethoprim resistance remained constant among urinary pathogens. However, among hospital isolates he found that the incidence of trimethoprim resistance had increased from 3% to 8% during this period. No mention was made of the contribution of R-factor-determined resistance to this overall figure.

We performed two clinical surveys to investigate trimethoprim resistance. Both were conducted over three-month periods on urinary tract isolates. The first survey was in 1975 and the second in 1977. In addition to testing for trimethoprim resistance, we also tested each trimethoprim-resistant isolate for the

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presence of R-factors conferring trimethoprim resistance.

Material and methods

INITIAL ISOLATION

All urines were screened for significant bacteriuria using the filter paper strip method of Leigh and Williams (1964). Urines giving less than 20 colonies per inoculum were regarded as giving no significant growth; those yielding more were further investigated and a disc sensitivity test was performed. The bacteria isolated were identified according to the taxonomic criteria of Cowan (1974).

DISC SENSITIVITY TEST

All disc sensitivity tests were performed on Oxoid Diagnostic Sensitivity Test Agar containing 7% lysed horse blood, using a modification of the Stokes (1968) method, as described by Pearson and Whitehead (1974). The test culture was spread over the inner half of an agar plate using a swab while the plate itself was rotating on a turntable. A swab of the control culture, *Escherichia coli* NCTC 10418, was spread on the outer half. This method of plating left a narrow circular band between these two cultures, and on to this were placed discs containing the antibiotics normally used in the treatment of urinary pathogens. A resistant strain was defined as one showing a reduced zone of inhibition, the radius of which was at least 3 mm smaller than the radius of inhibition exhibited by *E. coli* NCTC 10418 on the

other side of that disc, as described by Stokes and Waterworth (1972).

MINIMUM INHIBITORY CONCENTRATION AND TESTS FOR ANTIBIOTIC RESISTANCE TRANSFER
The minimum inhibitory concentration experiments were performed on Wellcotest Sensitivity Test Agar, as previously described (Amyes and Smith, 1974). To test for transferable resistance, each strain was cultured overnight in Oxoid no. 2 nutrient broth; 0·1 ml was mixed with 1 ml of either *E. coli* strain J6-2-1 (nalidixic acid resistant) or *E. coli* J6-2-2 (rifampicin resistant) (Clowes and Rowley, 1954) and maintained at 37°C for five hours. The cultures were vortexed and centrifuged, and the pellets were resuspended in Davis-Mingioli basal medium (Davis and Mingioli, 1950). The suspensions were then plated, in various dilutions, on Davis-Mingioli plates containing the supplements for *E. coli* J6-2 with either nalidixic acid or rifampicin (20 μg/ml) and trimethoprim (10 μg/ml). Recipients exhibiting trimethoprim resistance were purified and their resistance markers were checked.

Results

From July to September 1975, all trimethoprim-resistant isolates from urinary tract infections examined at the Whittington Hospital were collected and tested for the presence of transferable trimethoprim resistance. From January to March 1977, a second survey of trimethoprim-resistant strains was made, again using urinary isolates collected at the Whittington Hospital. These were tested for transmissible trimethoprim resistance as before. In both surveys the isolates were divided into those obtained from inpatient wards of the hospital and those from the outpatient community in the Archway area of London. In the 1975 survey the total number of strains isolated was 788, of which 604 were from inpatients and 184 from outpatients. In 1977 the total number of strains examined was 863, 622 being from inpatients and 201 from outpatients.

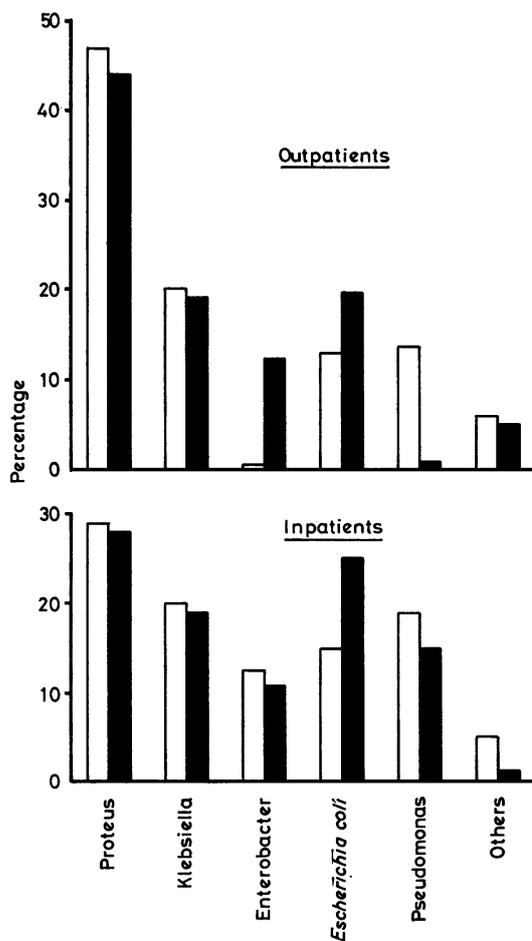The results in Table 1 show the percentage of

Figure *Proportion of each species as a percentage of the trimethoprim-resistant isolates.*
☐ *1975 survey—No. of outpatients 14*
No. of inpatients 49
■ *1977 survey—No. of outpatients 16*
No. of inpatients 77

trimethoprim-resistant isolates in each group using the standard disc sensitivity test technique. In 1975

Table 1 *Trimethoprim resistance among urinary isolates*

| | Fraction and percentage resistant | | | | | |
	Inpatients		Outpatients		Total	
July-Sept 1975	$\frac{49}{604}$	8·1	$\frac{14}{184}$	7·6	$\frac{63}{788}$	8·0
Jan-March 1977	$\frac{77}{662}$	11·6	$\frac{16}{201}$	8·0	$\frac{93}{863}$	10·8
x^2 (1975 compared with 1977)		4·28		0·02		3·73
Probability		< 0·05		0·80-0·90		0·05-0·10

there was no significant difference between in- and outpatients regarding the frequency of trimethoprim resistance ($\chi^2 = 0.05$, P $= 0.80$-0.90). In 1977 the same was true when in- and outpatients were compared ($\chi^2 = 2.17$, P $= 0.10$-0.20). Table 1 shows that when the frequency of trimethoprim resistance was compared between years a significant increase had occurred in the percentage of resistant isolates from inpatients within the 18-month period, the rise being from 8·1 % to 11·6 %. The frequency of trimethoprim resistance among outpatients had not increased significantly during the same period (Table 1).

In order to determine if there was any trend in this rise in resistance, we looked at the individual species in each sample. Some species, notably *Proteus* and *Pseudomonas*, show a higher percentage of resistance than other Gram-negative bacilli, and it was our purpose to determine whether any other group was becoming predominant. In the Figure it can be seen that in 1975 *Proteus* species comprised the major group of trimethoprim-resistant organisms, especially among outpatients. *Klebsiella* and *Pseudomonas* species also made up a sizable proportion. *Escherichia coli*, although making up the major proportion of urinary isolates, showed a relatively low incidence of resistance.

In 1977, however, almost every species showed a small reduction in their percentage of trimethoprim-resistant strains among the isolates from both in- and outpatients. The notable exception was *E. coli*, a species normally highly sensitive to trimethoprim, which showed a substantial increase in the frequency of trimethoprim resistance from both in- and out-patients. The only other species to show increased

trimethoprim resistance was *Enterobacter* isolated from outpatients.

The level of resistance to trimethoprim for each isolate was also measured by estimating the minimum inhibitory concentration of trimethoprim. Levels of trimethoprim resistance in excess of 500 μg/ml have been associated with either R-factor conferred resistance or a thymineless (thy⁻) character (Amyes and Smith, 1975). None of these strains was found to require thymine so they were all examined for the presence of R-factors.

By using the standard mating technique described, of the isolates obtained in 1975, six exhibited transfer of trimethoprim resistance, and all these were the strains which exhibited trimethoprim resistance levels exceeding 500 μg/ml. This has been expressed as a percentage in Table 2. The percentage of transferable trimethoprim resistance in the general practice population was greater than among inpatients. When the isolates of 1977 were examined, there was an increase in the percentage of transferable trimetho-prim resistance from both in- and outpatient isolates. The incidence of transferable trimethoprim resistance from outpatient isolates did not increase as much as that from inpatient isolates, where the incidence doubled. Table 2 shows that none of these increases is significant; however, this is not surprising in view of the relative rarity of transferable trimethoprim resistance among these strains. One worrying aspect is that the rate of increase in the frequency of transferable trimethoprim resistance exceeds the rate of increase of all types of trimethoprim resistance over the 18-month period for either in- or outpatients (compare Table 2 with Table 1).

Table 2 *Transferable trimethoprim resistance*

	Fraction and percentage transferring					
	Inpatients		*Outpatients*		*Total*	
Among trimethoprim resistant strains						
July-Sept 1975	$\frac{4}{49}$	8·2	$\frac{2}{14}$	14·3	$\frac{6}{63}$	9·5
Jan-March 1977	$\frac{9}{77}$	11·7	$\frac{3}{16}$	18·7	$\frac{12}{93}$	12·9
χ^2 (1975 compared with 1977)		0·41		0·11		0·42
Probability		0·50-0·70		0·70-0·80		0·50-0·70
Among all isolates						
July-Sept 1975	$\frac{4}{604}$	0·7	$\frac{2}{184}$	1·1	$\frac{6}{788}$	0·8
Jan-March 1977	$\frac{9}{662}$	1·4	$\frac{3}{201}$	1·5	$\frac{12}{863}$	1·4
χ^2 (1975 compared with 1977)		1·51		0·12		1·51
Probability		0·20-0·30		0·70-0·80		0·20-0·30

Recipient bacteria, which had received trimethoprim resistance, were examined for the presence of resistance to other antibiotics, including those not used at the Whittington Hospital for the treatment of urinary tract infections. In addition, tests were made to see whether resistance transfer could occur in the absence of cell-to-cell contact. In all cases the mechanism of transfer required cellular contact, and hence the mechanism of transfer seems to depend on conjugation, that is, the transfer was due to the presence of R-factors in all cases. As regards the presence of resistance to antibiotics other than that to trimethoprim in 1975, sulphonamide resistance was carried on almost all the R-factors (Table 3), and the only other resistance gene carried by these R-factors was that conferring streptomycin/spectinomycin resistance. In the later 1977 survey, kanamycin, ampicillin, and chloramphenicol resistance were found in association with trimethoprim resistance.

Table 3 *Resistance pattern of trimethoprim resistant R-factors*

	1975		1977	
	Inpatient	*Outpatient*	*Inpatient*	*Outpatient*
Sulphonamide	3/4	2/2	7/9	1/3
Streptomycin/ spectinomycin	1/4	1/2	1/9	0/3
Kanamycin	0/4	0/2	2/9	1/3
Ampicillin	0/4	0/2	1/9	2/3
Chloramphenicol	0/4	0/2	1/9	0/3

Discussion

Two clinical surveys were performed to test for changes in trimethoprim resistance. In the four years prior to 1975, little change in the level of trimethoprim resistance among urinary tract isolates was reported (Grüneberg, 1976). In our surveys (1975 and 1977) there was a statistically significant increase in the percentage of trimethoprim-resistant organisms in the isolates from inpatients, whereas there was no significant change among outpatient isolates. This is perhaps to be expected because the selection pressure to produce trimethoprim resistance is probably more acute in the hospital environment. In addition, there was quite a sharp increase in the incidence of R-factor conferred trimethoprim-resistance, the increase being more marked with inpatients, doubling in just 18 months. It is in this light that the future of the efficacy of co-trimoxazole should be considered. When co-trimoxazole is administered, high concentrations of trimethoprim are excreted in the urinary tract. This could encourage the selection of the type of high-level trimethoprim resistance that is conferred by plasmids. In the earlier survey, R-factor

trimethoprim resistance was associated mainly with sulphonamide resistance while in the latter survey it was quite often without sulphonamide resistance. The reason for this is obscure. It could be thought that as the level of active sulphamethoxazole in the urine is low there may be no strong selective pressure for the presence of sulphamethoxazole resistance. However, as all the clinical strains that did not transfer sulphonamide resistance with trimethoprim resistance were nevertheless resistant to sulphonamides this view seems to be incorrect. Thus resistance to both drugs seems to be essential for bacterial survival in the face of co-trimoxazole therapy. It seems that the nature of the genes for sulphonamide resistance has changed within the 18 months of these two surveys. The reason for the change may be because sulphonamide resistance has become so widespread among urinary isolates in recent years. Consequently, the selective pressure for R-factors to confer resistance to this drug may have diminished to the extent that plasmids are no longer required to carry resistance to it as well as to trimethoprim in order to enable bacteria to withstand co-trimoxazole therapy.

Our results seem to contradict a recent report (Brumfitt *et al.*, 1977) on the emergence of trimethoprim resistance. However, our work reports the results of sequential surveys in one hospital, and it seems that single surveys cannot monitor accurately the emergence of resistance. In support of this view is the fact that other sequential surveys (Grüneberg, 1976; Marks *et al.*, 1977) reach conclusions similar to ours as regards the increase in the proportion of trimethoprim-resistance among bacteria. In addition, we show that R-factor mediated trimethoprim resistance is also increasing. It is interesting that the bacterial population resistant to trimethoprim is changing. Overall, between 1975 and 1977, the greatest increase in the frequency of trimethoprim-resistance occurred in *E. coli*, and there must be some considerable selective pressure to produce trimethoprim resistance in this normally sensitive species. Surprisingly, there was not a great increase in the number of R-factors coming from this species. In 1975, 50% of the R-factors were isolates from the *Klebsiella-Enterobacter* spp and by 1977 this figure had risen to 66·7%, while the percentage coming from *E. coli* remained the same.

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