Distribution and transferability of plasmids encoding trimethoprim resistance in urinary pathogens from Greece

A. TSAKRIS, A. P. JOHNSON*†, R. C. GEORGE†, S. MEHTAR and A. C. VATOPOULOS‡

Department of Microbiology, North Middlesex Hospital, London N18 1QX, †Division of Hospital Infection, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT and ‡Department of Microbiology, Hippocraton General Hospital, Athens 11527, Greece

Summary. Of 505 strains of Enterobacteriaceae responsible for significant bacteriuria and isolated from hospital patients in two Greek cities in 1989, 151 strains (30%) were resistant to trimethoprim (MIC $\geq$ 4 mg/L) and 220 (44%) were resistant to sulphamethoxazole (MIC $\geq$ 64 mg/L); 127 (84%) of the trimethoprim-resistant strains exhibited high-level resistance (MIC $>$ 1024 mg/L) and 121 (80%) were additionally resistant to four or more other antibiotics. Plasmids were detected in 141 (93%) of the trimethoprim-resistant strains. Trimethoprim resistance was encoded on self-transmissible plasmids in 79 (52%) of the resistant strains, and in a further seven strains (5%), plasmids coding for trimethoprim resistance could be mobilised by X⁺ factor. Co-transfer of various other antimicrobial resistances with trimethoprim resistance was observed, tetracycline resistance being the most common. The low degree of linkage observed between trimethoprim resistance and resistance to streptomycin and spectinomycin suggests that Tn7 is relatively uncommon in Greece. Classification of trimethoprim-resistance plasmids on the basis of their antimicrobial-resistance patterns and molecular mass revealed 39 different profiles. Overall, these findings differ from those from other European countries where the prevalence of transferable high-level trimethoprim resistance is low and where chromosomal Tn7-encoded trimethoprim resistance is common.

Introduction

Trimethoprim exerts its antibacterial activity by inhibiting the bacterial enzyme dihydrofolate reductase (DHFR) which is involved in the synthesis of tetrahydrofolate, an essential precursor of several amino acids and nucleotides.¹ Trimethoprim acts synergically with sulphonamides, which also inhibit tetrahydrofolate biosynthesis but at an earlier stage in the synthetic pathway.¹ However, resistance to trimethoprim has been documented in various clinically important bacteria. High-level trimethoprim resistance (MIC $>$ 1024 mg/L), which may be encoded on plasmids, transposons or chromosomal DNA, usually results from the production of a modified form of DHFR which is unaffected by trimethoprim.² Low-level resistance (MIC 4–1024 mg/L) also occurs and may similarly result from the production of modified DHFR or from other mechanisms such as reduced bacterial permeability to trimethoprim and other bacterial agents.^{2,3}

When trimethoprim was introduced into clinical use in 1968, it was available only in combination with sulphamethoxazole and this mixture was given the

generic name co-trimoxazole. In 1972, trimethoprim became available for use as a single agent in Finland and this occurred in other countries soon afterwards. In Greece, however, the combination of trimethoprim and sulphamethoxazole is still used in preference to trimethoprim alone, and, for this reason, antibiotic sensitivity testing in Greek hospitals is performed exclusively with disks containing a combination of these two antimicrobial agents.⁴ Therefore, it is impossible to assess from these data the incidence of resistance to trimethoprim alone. Because of this lack of information on the epidemiology of trimethoprim resistance in Greece, we have surveyed a collection of urinary tract pathogens isolated in two Greek cities during 1989 for resistance to this antibiotic. We report here the incidence of both high- and low-level resistance to trimethoprim, the frequency of plasmid-mediated resistance and the basic characteristics of the R-plasmids involved.

Materials and methods

Bacteria

During the first 6 months of 1989, 505 strains of Enterobacteriaceae belonging to six different genera

were obtained from separate patients with significant bacteriuria ($>10^5$ cfu/ml) in five Greek hospitals (three in Athens, two in Thessaloniki).

Escherichia coli strains 20R764 (*lac*⁺ *rif*^R) and 26R793 (*lac*⁻ *rif*^R) (kindly provided by Dr E. J. Threlfall, Division of Enteric Pathogens, Central Public Health Laboratory) were used as recipients in resistance-transfer experiments, and *E. coli* strain 18R951 containing the X⁺ fertility factor⁵ was used in experiments to mobilise non-self-transferable plasmids encoding trimethoprim resistance. For the estimation of the size of plasmids, *E. coli* strains 39R861 and V517 which harbour plasmids of known molecular mass, were used as standards.^{6,7}

Determination of MIC

MICs of trimethoprim and sulphamethoxazole were determined by an agar dilution method with Iso-Sensitest Agar (Oxoid) supplemented with lysed blood 5% v/v. Serial doubling concentrations of each antimicrobial agent were incorporated into the agar, and plates were inoculated with a multipoint inoculator (Denley Instruments) with an inoculum of 10^4 cfu/spot. High-level resistance to trimethoprim was defined as MIC >1024 mg/L and low-level resistance as MIC 4–1024 mg/L.⁸ Resistance to sulphamethoxazole was defined as MIC ≥ 64 mg/L. The MICs of other antibiotics (ampicillin, cephalothin, chloramphenicol, tetracycline, amikacin, gentamicin, netilmicin, streptomycin, spectinomycin and tobramycin) were determined (as described above) for trimethoprim-resistant strains only. The levels of resistance were defined according to the recommendations of a Working Party of the British Society for Antimicrobial Chemotherapy.⁹

Resistance-transfer experiments

Bacterial strains resistant to trimethoprim were tested for their ability to transfer resistance to recipient strains. Overnight broth cultures of donor and recipient cultures were mixed in a ratio of 1:10 and about 300 μ l of each mixture was spread over the surface of each of two nutrient-agar plates which were incubated

for 18 h at 37°C and 28°C respectively. The organisms were washed from the agar surface with phosphate-buffered saline, and transconjugants were selected by plating serial dilutions of the organism suspensions on nutrient-agar plates containing trimethoprim 2 mg/L and rifampicin 90 mg/L.

Mobilisation of plasmids

Strains which failed to transfer trimethoprim resistance were investigated further for the presence of non-self-transferable plasmids, by mobilisation with the transfer factor X⁺ employing the method described by Young *et al.*¹⁰

Isolation of plasmid DNA

Trimethoprim-resistant clinical isolates and transconjugants (one per donor strain) were analysed for plasmids by the method of Kado and Liu.¹¹ Plasmids were separated by electrophoresis in agarose 0.8% w/v gels, at 100V for 2.5 h, stained with ethidium bromide and visualised by UV transillumination.

Results

Incidence of trimethoprim and sulphamethoxazole resistance

Of the 505 clinical isolates, 57% were *E. coli*, 16% *Proteus* spp., 12% *Klebsiella* spp., 10% *Enterobacter* spp., 4% *Serratia* spp. and 0.6% *Citrobacter* spp.; 151 isolates (30%) were resistant to trimethoprim and 220 (44%) were resistant to sulphamethoxazole; 132 strains (26%) were resistant to both drugs (table I). Among the 151 trimethoprim-resistant strains, 127 (84%) exhibited high-level resistance. When strains from Athens and Thessaloniki were compared, there was no appreciable difference either in the overall incidence of trimethoprim resistance (29% in Athens versus 33% in Thessaloniki) or in the incidence of trimethoprim resistance in the various genera, with the exception of *Enterobacter* spp. (53% in Athens versus 88% in Thessaloniki).

Table I. Proportion of strains resistant to trimethoprim, sulphamethoxazole or both antibiotics

Species	Number of strains tested	Number (%) of strains resistant to			
		Tp (2 mg/L)	Tp (1024 mg/L)	Su (32 mg/L)	Tp (2 mg/L) + Su (32 mg/L)
<i>E. coli</i>	289	64(22)	57(20)	118(41)	56(19)
<i>Proteus</i> spp.	81	21(26)	15(19)	25(31)	16(20)
<i>Klebsiella</i> spp.	62	24(39)	21(34)	35(52)	22(35)
<i>Enterobacter</i> spp.	50	32(64)	27(54)	34(68)	29(58)
<i>Serratia</i> spp.	20	9(45)	6(30)	10(50)	8(40)
<i>Citrobacter</i> spp.	3	1(33)	1(33)	1(33)	1(33)
Total	505	151(30)	127(25)	220(44)	132(26)

Tp, trimethoprim; Su, sulphamethoxazole.

Resistance to other antibiotics

Of the trimethoprim-resistant strains (MIC ≥ 4 mg/L), 80% were also resistant to four or more other antimicrobial agents. Resistance to sulphamethoxazole was the most common other resistance character (87%) amongst the trimethoprim-resistant strains followed by ampicillin (79%), tetracycline (72%), cephalothin (66%), chloramphenicol (61%), streptomycin (51%) and spectinomycin (39%); 30% of the trimethoprim-resistant strains were resistant to both streptomycin and spectinomycin. More strains were resistant to tobramycin (43%) and netilmicin (40%) than to amikacin (30%) and gentamicin (24%).

Transfer of trimethoprim resistance

Transfer of trimethoprim resistance was observed in 86 (57%) of the 151 resistant strains (table II) and the incidences of transferable resistance in Athens and Thessaloniki were virtually identical. However, six of these 86 strains (five strains of *Enterobacter* spp.

and one *E. coli* strain) transferred resistance only at 28°C. Seven strains of *E. coli* contained non-self-transferable plasmids and required the X⁺ factor for their mobilisation. Transfer of trimethoprim resistance was higher among *E. coli* and *Enterobacter* spp. than in the other species examined (table II).

Of the 24 strains with low-level trimethoprim resistance, only two (both *Klebsiella* spp.) had transferable resistance (table II). The MIC of trimethoprim for both of these strains was 256 mg/L. Among the 127 strains with high-level resistance to trimethoprim, 84 (66%) exhibited transferable resistance (table II). Overall, the frequency of transfer of trimethoprim resistance varied from 1.6×10^{-2} to 2.9×10^{-8} transconjugants per donor, with the majority of strains exhibiting a transfer frequency of 10^{-3} – 10^{-4} .

Linkage of trimethoprim resistance with resistance to other antimicrobials

Resistance profiles were determined for 70 transconjugants obtained from different donors that carried single plasmids identical in size to plasmids present in their respective donors. Only 31 (44%) were resistant to sulphamethoxazole despite the fact that 90% of the donor strains exhibited resistance to this drug. Less than half of the transconjugants showed linkage of trimethoprim resistance with resistance to ampicillin (47%) or chloramphenicol (39%). Linkage was highest with tetracycline resistance (57%) (table III). Co-transfer of trimethoprim and streptomycin resistance determinants was demonstrated in 23% of the transconjugants and with spectinomycin resistance in 20%. However, co-transfer of trimethoprim resistance with resistance to both streptomycin and spectinomycin was found in only 11% of the transconjugants. Resistance to tobramycin was present in 46% of the transconjugants, 39% were resistant to netilmicin, 31% to amikacin, but only 20% exhibited resistance to gentamicin (table III).

Table II. Transferability of trimethoprim-resistance in trimethoprim-resistant (Tp^R) strains

Species (n)	Number of strains of donor resistance phenotype			
	High level Tp ^R		Low level Tp ^R	
	tra +	tra -	tra +	tra -
<i>E. coli</i> (64)	40*	17	0	7
<i>Proteus</i> spp. (21)	6	9	0	6
<i>Klebsiella</i> spp. (24)	12	9	2	1
<i>Enterobacter</i> spp. (32)	22	5	0	5
<i>Serratia</i> spp. (9)	4	2	0	3
<i>Citrobacter</i> spp. (1)	0	1	0	0
Total	84	43	2	22

tra +, Transferable; tra -, non-transferable.

* Seven *E. coli* strains required X⁺ factor for transfer.

Table III. Co-transfer of trimethoprim resistance with resistance to other antimicrobial agents in transconjugants that gained only a single plasmid*

Resistance character	Number of strains of each species from which indicated antimicrobial resistance was co-transferred					
	<i>E. coli</i> (n = 28)	<i>Proteus</i> spp. (n = 6)	<i>Klebsiella</i> spp. (n = 14)	<i>Enterobacter</i> spp. (n = 18)	<i>Serratia</i> spp. (n = 4)	Total (%) (n = 70)
Sulphamethoxazole	11	4	3	11	2	31(44)
Ampicillin	12	3	6	10	2	33(47)
Cephalothin	0	0	6	2	0	8(11)
Chloramphenicol	11	2	2	11	1	27(39)
Tetracycline	20	0	13	5	2	40(57)
Streptomycin	11	0	0	5	0	16(23)
Spectinomycin	3	1	0	10	0	14(20)
Gentamicin	2	0	2	9	1	14(20)
Tobramycin	2	0	12	17	1	32(46)
Netilmicin	2	0	12	12	1	27(39)
Amikacin	1	0	12	8	1	22(31)

* Transconjugants in which plasmid differed in size from plasmids present in donor strain were not included.

Characterisation of *R*-plasmids

Plasmids were detected in 141 (93%) trimethoprim-resistant strains. Although all strains with transferable resistance carried one or more plasmids, 85% of strains with non-transferable resistance also contained plasmids. At least one plasmid was visualised in each of 86 transconjugants tested; 75 of the transconjugants contained a single plasmid which, in all but three, were in the size range 60–130 Mda—the remaining three plasmids were *c.* 30 Mda in size. Seven transconjugants obtained by mobilisation of the donor plasmid with the X⁺ factor were classified as containing a single transferred plasmid, even though the X⁺ factor was also present. In transconjugants that exhibited resistance to six or fewer antimicrobials, the plasmids frequently were in the size range 60–100 Mda, whereas in those that exhibited resistance to more than six antimicrobials, the plasmid was ≥ 100 Mda in size. In five transconjugants, the plasmid differed in size from those identified in their donors, and in a further 11 transconjugants, two or more plasmids were co-transferred. These two groups of transconjugants were not included in subsequent analyses.

Analysis of the antimicrobial resistance patterns and molecular sizes of plasmids in trimethoprim-resistant transconjugants revealed 39 different profiles. The most commonly observed plasmid profiles are shown in table IV. Interestingly, plasmids with the same profile were sometimes seen in transconjugants derived from donor strains belonging to different genera. Furthermore, trimethoprim-resistance plasmids with the same profile occurred in donor strains obtained from both Athens and Thessaloniki (table IV).

Discussion

Trimethoprim, in combination with a sulphonamide, has been used extensively in Greece during the last 20 years, mainly for the treatment of urinary tract infections. The results of our study show that, currently, 30% of Enterobacteriaceae isolates from

urinary tract infections in Greece are resistant to trimethoprim, similar rates of resistance being seen in two cities, namely Athens and Thessaloniki. This high incidence of resistance is comparable to that seen in France¹² and Finland¹³ but appears lower than that seen in developing countries, such as India,¹⁰ Nigeria¹⁴ or South Africa,¹⁵ where more than half of the isolates are resistant. The association of resistance to trimethoprim and multi-resistance, which has been reported previously,¹⁶ was confirmed in the present study, in that 80% of the trimethoprim-resistant strains were also resistant to four or more other antibiotics.

A further point of interest is that, among trimethoprim-resistant strains from Greece, a high proportion (84%) exhibited high-level resistance. Similar proportions of strains with high-level resistance have been reported from India¹⁰ and Chile,¹⁷ although lower proportions have been observed in other surveys.^{15,18,19} Several European studies^{19–22} have shown that only a small proportion of strains with high-level trimethoprim resistance exhibit transferable resistance, possibly due to the location of the resistance gene on the chromosome.²³ In contrast, in the present survey, 66% of the strains with high-level trimethoprim resistance showed transferable resistance. The observation that two strains with low-level resistance (MIC 256 mg/L) to trimethoprim could also transfer their resistance, confirms that this form of resistance can be plasmid-mediated.^{24,25}

Analysis of transconjugants revealed that other antimicrobial resistances were co-transferred with resistance to trimethoprim. Tetracycline resistance showed the highest linkage, possibly reflecting the high consumption of tetracycline in Greece for a large number of common infections. In contrast, linkage of trimethoprim resistance and sulphamethoxazole resistance was surprisingly low, despite the fact that almost all the trimethoprim-resistant clinical isolates were resistant to the latter drug. This suggests that, despite the exclusive use of co-trimoxazole in Greece, resistance to trimethoprim and to sulphamethoxazole have evolved separately.

The low level of co-transfer of trimethoprim resistance with resistance to both streptomycin and

Table IV. Characterisation of plasmids* most commonly found in trimethoprim-resistant transconjugants

Number of transconjugants	Donor strains (source)	Resistance pattern	Approximate size (Mda)
6	<i>E. coli</i> (A), <i>Serratia</i> (T)	Tp	80
3	<i>E. coli</i> (A)	Tp Te	82
3	<i>E. coli</i> (A, T)	Tp Te	95
9	<i>Klebsiella</i> spp. (A), <i>Enterobacter</i> spp. (T)	Tp Te Tb Nt Ak	65
4	<i>Enterobacter</i> spp. (T)	Tp Su Ch Tb Nt Ak	70
6	<i>E. coli</i> (A, T)	Tp Su Ap Ch Te Sm	100
4	<i>E. coli</i> (A), <i>Klebsiella</i> spp. (A) <i>Serratia</i> spp. (T)	Tp Su Ap Ch Te Gm Tb Nt Ak	120
3	<i>Enterobacter</i> spp. (A, T)	Tp Su Ap Ch Sm Sp Tb Nt Ak	120

* Plasmids observed in transconjugants derived from three or more donor strains.

A, Athens; T, Thessaloniki. Ak, Amikacin; Ap, ampicillin; Ch, chloramphenicol; Gm, gentamicin; Nt, netilmicin; Sp, spectinomycin; Sm, streptomycin; Su, sulphamethoxazole; Te, tetracycline; Tb, tobramycin; Tp, trimethoprim.

spectinomycin suggests that the transposon Tn7 is not a major determinant of trimethoprim resistance in Greece. DNA hybridisation experiments, with a Tn7 probe, confirm this suggestion (unpublished observations). Resistance to tobramycin, netilmicin and amikacin was co-transferred with trimethoprim resistance more frequently than was gentamicin resistance. This may, at least in part, reflect the relatively low usage of gentamicin compared to other aminoglycosides in Greece. In addition, the most common aminoglycoside-modifying enzyme found in a recent survey of Greek isolates of *Enterobacter cloacae* was AAC (6')I (unpublished observations) which is active against tobramycin, netilmicin and amikacin.²⁶ Therefore, it is possible that the current findings reflect, in some instances, linkage of trimethoprim resistance with resistance to aminoglycosides mediated by production of AAC (6')I.

In Greece, plasmid-mediated resistance seems to be the prevalent cause of resistance to trimethoprim

among urinary tract pathogens. Analysis of trimethoprim-resistant transconjugants revealed a variety of plasmids, which differed on the basis of their molecular size and antimicrobial-resistance patterns. Plasmids with identical antimicrobial-resistance patterns and molecular sizes were observed both in transconjugants derived from donors belonging to different genera of bacteria, and in transconjugants derived from donors isolated from the two different cities included in the study, indicating that such plasmids are possibly widespread. However, it remains to be confirmed that plasmids with the same resistance profile and molecular size are, in fact, identical. Clearly, further molecular and genetic studies will be required to determine the relationship, if any, between these plasmids. It is hoped that such studies will result in an increased understanding of the epidemiology of resistance to trimethoprim.

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