REVIEW ARTICLE

Trimethoprim resistance; epidemiology and molecular aspects

—a review based upon a Symposium held on 19 April 1989 at the 4th European Congress of Clinical Microbiology, Nice, France

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Introduction. Trimethoprim resistance was first recognised in gram-negative bacteria 20 years ago. Workers in several research centres have studied the epidemiology and molecular aspects of trimethoprim resistance over the intervening years, initially in gram-negative bacteria and more recently in gram-positive bacteria. The introduction of this completely novel synthetic antimicrobial provided a unique opportunity to study the full evolution of bacterial resistance to an antimicrobial drug, without the pre-existing influence of resistance genes selected by other related compounds. Consequently, the model provided by trimethoprim resistance has considerable relevance to our understanding of the evolution of bacterial resistance to antimicrobial agents in general. Recent years have seen exciting and major advances in the use of modern DNA technology to study the epidemiology of trimethoprim resistance. Considerable progress has been made in evaluating the importance of the different resistance mechanisms, especially those carried by resistance plasmids. Nevertheless, in most countries resistance to trimethoprim has only recently reached clinically significant proportions and it remains a widely used and valuable component of the antimicrobial armamentarium. The papers presented in this review are based on a Symposium held at the 4th European Congress of Clinical Microbiology in April 1989. The Symposium provided a forum for the various aspects of trimethoprim resistance to be brought together and resulted in the collation of research material which forms this review.

Evolution and spread of trimethoprim resistance in gram-negative bacteria

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Trimethoprim, in association with sulphonamides, has been used for 20 years in human and veterinary medicine and is still one of the most widely used antibiotics in the world. The reasons for its success are: a broad spectrum of activity, a marked synergic effect on both sulphonamide-

susceptible and -resistant strains, excellent clinical results and, particularly important, a very low price. This success has nevertheless one drawback, namely the emergence of bacterial resistance.

Epidemiology of trimethoprim resistance

Comparisons of the incidence of bacterial resistance are difficult because many factors such as methodology, selection and choice of specimens and strains, type of patients and local epidemics greatly influence the percentage of resistant strains. For these reasons, assessment of changes in trimethoprim resistance are really only significant if they come from long-term follow-up studies within a single laboratory.

Resistance to trimethoprim has been carefully monitored for a long period in Finland, the United

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Kingdom and France. In Finland, from 1978 to 1984 resistance to trimethoprim in *Escherichia coli* isolates from outpatients increased slowly (e.g., 5.4% to 9.2% in Turku).¹ A sharper increase has been observed in the last 3 years and is best illustrated by data from the town of Rovaniemi, where resistance to trimethoprim was stable at about 5% between 1980 and 1986, but rose dramatically to 14.7% in 1987 and has remained at that level since (P. Huovinen, personal communication). In Paris, resistance to trimethoprim in *E. coli* increased slowly from 1972 to 1979 and then very sharply to 1982 (fig. 1). From 1983 to 1988, resistance to trimethoprim decreased in all groups of isolates. On the other hand, in Nottingham, in a study involving nearly 74 000 bacterial isolates from 1978 to 1988, trimethoprim resistance increased progressively throughout the study period² (K. J. Towner, personal communication). In *E. coli*, resistance rose from 2.0% to 15.5% and in *Proteus* spp. from 7.2% to 24.9%. A marked increase in resistance was found between 1979 and 1980 and between 1983 and 1984. Between 1983 and 1984 this sharp increase was found in both hospital strains (*E. coli* from 9.3% to 16.2%, *Proteus* spp. from 11.0% to 22.0%) and community isolates (*E. coli* from 8.5% to 13.7%, *Proteus* spp. from 7.3% to 17.5%).² More recently, between 1987 and 1988, another profound increase has been observed in

community isolates of *Klebsiella/Enterobacter* spp. (15.2% to 24%) and *Proteus* spp. (18.2% to 25.2%) (K. J. Towner, personal communication). In complete contrast, in the USA, the incidence of resistance to trimethoprim has remained very low both in the community as well as in nosocomial strains. The highest incidence amongst hospitalised patients has been 13%.³

In developing countries, the incidence of trimethoprim resistance is very disquieting. In Thailand, trimethoprim-resistant bacteria represented 40% of urinary isolates.⁴ Similarly, Young *et al.*⁵ found that 64% of urinary isolates in India in 1984 were resistant to trimethoprim. In Johannesburg Hospitals (South Africa), 50% of enterobacterial species isolated in 1986–1987 were resistant to trimethoprim.⁶ The same problems exist in South America where, in 1987, 25–41% of strains isolated from the community in Santiago de Chile were resistant to trimethoprim.⁷

The importance of antibacterial drug resistance in general, and trimethoprim resistance in particular, becomes acute when considering specific pathogens such as *Shigella* spp., which are responsible for a high rate of morbidity and mortality. Data from several countries indicating an increase in the percentage of trimethoprim-resistant strains among isolates of shigellae are summarised in table I. What are the reasons for this situation?

Fig. 1. Proportion of trimethoprim resistance in *E. coli* in the St Joseph Hospital in Paris and its relation to the usage of trimethoprim. ●—● Percentage of *E. coli* resistant to trimethoprim. ○—○ Consumption of trimethoprim.

Table I. Resistance to sulphamethoxazole-trimethoprim among *Shigella* spp.

Country	Year	Tp-R (%)	Total strains tested
Bangladesh	1980	0	1018
	1984	55	890
Mexico	1980	0	55
	1984	6	23
Thailand	1979-1984	35	1033
	1986-1988	61.1	211
Chile	1983	25	85
	1987	37	100
Brazil	1983	23	57

Resistance to trimethoprim, or to any other antibiotic, will spread if three favourable conditions are met: (1) the emergence of stable resistance genes; (2) the presence of resistance vectors such as epidemic strains, plasmids or transposons; (3) continuous or periodic selection pressure.

Emergence of stable trimethoprim resistance genes

Low level resistance to trimethoprim usually results from mutational events such as decreased permeability and quantitative or qualitative modifications of the bacterial target of trimethoprim, dihydrofolate reductase (DHFR).⁸ Plasmid-mediated low level resistance has been described, but only on a few occasions (see section by Young and Thomson in this review). Impermeability mutants are particularly interesting because of the cross-resistance to trimethoprim and other antibiotics, especially quinolones, which can act as selectors. This type of mutation has been seen in *Klebsiella*, *Enterobacter* and *Serratia* and is much more prevalent than it is often assumed to be.⁹

High level resistance to trimethoprim is usually specified by plasmids which encode an additional trimethoprim-resistant DHFR (reviewed by Amyes¹⁰). Trimethoprim resistance plasmids were first isolated in London in 1971¹¹ and have quickly spread to all enterobacterial species, *Vibrio cholerae* and even *Acinetobacter* and *Pseudomonas*, although these latter species are intrinsically resistant to trimethoprim. Trimethoprim resistance plasmids have been found in various enterobacteria. This is especially true of *Salmonella* spp., some of which have been implicated in human infections (for review, see Goldstein *et al.*¹²).

The main characteristics of a successful resistance plasmid are to be transferable from one bacterium to another and to encode resistance to

several unrelated antibiotics. Sequential studies from different centres have shown fluctuating levels of transferable resistance to trimethoprim among high level resistant strains. For example, in Edinburgh in 1982-83, a sharp decrease (50.7% to 37.2%) in the transferability of high-level trimethoprim resistance genes (normally associated with plasmid-mediated resistance) was observed, even though the incidence of high level resistance had remained constant.^{13,14} Similarly at the St Joseph Hospital in Paris, transferable trimethoprim resistance reached a peak during 1980-81 (62.6% of highly resistant strains) then decreased to 45-50% by 1988.

These fluctuations in the transferability of high-level resistance can be explained, at least in part, by the presence of transposable elements encoding resistance to trimethoprim which can jump from one plasmid to another plasmid or on to the bacterial chromosome.¹⁵ Transposon Tn7, which encodes resistance to streptomycin, spectinomycin and trimethoprim (DHFR Ia), was the first and the most common of the trimethoprim transposons described.¹⁶ Its presence has been more or less demonstrated or presumed in many bacterial species by hybridisation studies with a probe encoding the DHFR Ia gene.¹² Other transposons, very similar and probably identical to Tn7, have been isolated from enterobacteria and *Vibrio cholerae*.¹⁷ A second group of transposons, clearly different from Tn7, is represented by Tn4132; these encode resistance to trimethoprim alone.¹⁸ Finally, a third group of transposons, represented by Tn402, encode a type II DHFR.¹⁹

Extensive studies of the occurrence of Tn7 in enterobacteria have been performed in Finland in 1980-81 and in 1983.²⁰ A general increase in the percentage of enterobacteria harbouring Tn7 was observed between the two study periods (47.3% in 1980-81 and 56.1% in 1983); however, the percentage of *Klebsiella* strains harbouring Tn7 was significantly lower than in other enterobacteria during the study periods (12.9% in 1980-81 and 23.5% in 1983). All these fluctuations in the transferability rates can be explained by the movement of Tn7 into the bacterial chromosome. In a study from Glasgow the percentage of chromosomal Tn7 rose from 38% to 70% in hospital isolates during two study periods, 1979-80 and 1982.²¹ The percentage of chromosomal Tn7 was also very high in community isolates from Glasgow. Therefore, a general phenomenon can be proposed: after the initial "infection" of a bacterial cell by a plasmid carrying Tn7, the transposon will jump and remain on the chromosome even when the initial plasmid is cured spontaneously.

Several other trimethoprim-resistance transposons have been found on transferable plasmids; an epidemic with DHFR type II has been described in Boston.²² In Paris, we found that 36% of unrelated plasmids isolated during 1981–84 were able to hybridise with a DHFR II specific gene probe. However, there is no evidence yet to suggest that a transposon carrying the type II DHFR gene has migrated into the bacterial chromosome.

Selection pressures

The last factor responsible for the increase in trimethoprim resistance is selection pressure. As seen from fig. 1, the percentage of trimethoprim-resistant *E. coli* did not always follow the consumption of sulphonamide-trimethoprim. After 1982, with the increased use of new cephalosporins and fluoroquinolones, the consumption of sulphonamide-trimethoprim clearly decreased and so did the percentage of trimethoprim-resistant strains. A very interesting study concerning the effects of selection pressure has been done in Nottingham.² With the increased use of trimethoprim alone, the percentage of trimethoprim-resistant strains which are susceptible to sulphonamides increased dramatically, especially in *Proteus* spp., such that by 1988, 67% of the *Proteus* strains isolated in the community were susceptible to sulphonamides (K. J. Towner, personal communication).

In several studies, Amyes and other authors demonstrated that ampicillin, which is widely used in many clinical settings, may select bacteria harbouring plasmids encoding joint resistance to ampicillin and trimethoprim.^{23,24} Indeed, in our own hospital, between 53 and 87% of the plasmids encode joint resistance to both trimethoprim and ampicillin.

Conclusions

As inferred from epidemiological and genetic studies, resistance to trimethoprim is borne by many different replicons and can be mediated by epidemic strains, plasmids or transposons. These factors, combined with a widespread use in man and animals of trimethoprim, related substances and even unrelated antibiotics, may explain the emergence and spread of trimethoprim resistance in different species and provides a unique epidemiological model of the interaction between genetic elements, antibiotic policy and the resulting emergence, spread and stabilisation of resistance to an individual antibacterial drug.

Trimethoprim resistance in staphylococci

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Plasmid-mediated trimethoprim resistance in gram-negative bacteria emerged within 3 years of the clinical introduction of the drug.¹¹ The plasmid carriage of trimethoprim resistance undoubtedly contributed to the spread of resistance and the high proportion of resistant gram-negative isolates in certain areas of the world.⁵

Emergence of trimethoprim resistance in staphylococci

In gram-positive bacteria, the story is quite different. Amongst staphylococci, the incidence of trimethoprim resistance is very low, despite the continuous challenge with the drug for 20 years. Indeed, until the advent of methicillin-resistant *Staphylococcus aureus* (MRSA), trimethoprim resistance was virtually unheard of. In MRSA, the first trimethoprim-resistant strains were characterised by a low minimum inhibitory concentration (MIC) ranging from 10 to 250 mg/L.^{25–29} This resistance was presumed to be encoded by genes located on the bacterial chromosome as no transfer of the resistance determinants could be achieved. The mechanism of this moderate level of resistance is still unknown, although it has been suggested that it results from an over-production of the bacterial DHFR.³⁰ This type of chromosomal resistance has been found in both eastern and western Australia, the USA and Europe.^{25–29} It is likely that this chromosomal resistance represents an initial stage in the development of high-level resistance to trimethoprim in MRSA.

Plasmid-mediated trimethoprim resistance in staphylococci did not emerge until 1983, when it was found in Australian MRSA isolates.^{28,31} They were characterised by their high MIC of trimethoprim (> 512 mg/L). At that time, 10% of *S. aureus* strains in the USA were found to be trimethoprim resistant, but this resistance had not then been shown to be plasmid-mediated.²⁹ Archer *et al.*²⁹ demonstrated that strains from Virginia and from Pennsylvania were able to transfer their trimethoprim resistance genes and strains from both areas carried trimethoprim resistance plasmids (pG01 and pG05). Although the plasmids from the two

areas were different, they did show common restriction sites which indicated their relatedness to one another.²⁹

The trimethoprim resistance plasmids isolated from staphylococci from different parts of Australia were compared and were also shown to have similar restriction patterns to one another, suggesting that they too came from a common source. However, these restriction patterns were, by and large, different from those found in the plasmids obtained in the USA.³⁰ The one exception was the restriction pattern around the trimethoprim resistance gene, which was common to plasmids from both continents.³⁰ Thus, although the trimethoprim resistance gene appeared ubiquitous, the plasmids on which it was carried were quite different. In Australian MRSA, 50% of the high-level trimethoprim resistance was mediated by the 28.4-kb plasmid, pSK1. The trimethoprim resistance gene could be located to a 2.75-kb region of plasmid pSK1 by comparing it to spontaneous deletion mutants which had become trimethoprim-sensitive.³² A 5.1-kb *Eco*RI fragment of pSK1 was cloned into plasmid pACYC184, to form the hybrid plasmid pSK407. This hybrid plasmid was shown to express high-level trimethoprim resistance in *Escherichia coli*. Transposon mutagenesis, with Tn5, of the trimethoprim resistance gene showed that it comprised between 0.55 and 0.75 kb, suggesting it could code for a protein of up to 20 Kda.³³

The type SI DHFR

The pSK1-containing clinical strain encoded a high level of DHFR (Specific Activity = 129.6 nmol dihydrofolate reduced/min/mg of protein). However, staphylococci generally encode much higher levels of chromosomal DHFR than their gram-negative counterparts. The high background enzyme activity made the initial identification of a plasmid-encoded DHFR much more difficult. However, the 5.1-kb *Eco*RI fragment of pSK1, which encoded the trimethoprim resistance gene, had been cloned by Lyon *et al.*³³ into the multi-copy plasmid pACYC184 in *E. coli*. The specific activity of the chromosomal DHFR in this strain was much lower than in staphylococci. Indeed, the DHFR activity encoded by the recombinant plasmid in *E. coli* was more than 20 times higher than that of the same host containing the same plasmid lacking the insert. The plasmid DHFR, which had originally been encoded by pSK1, was partially purified by ammonium sulphate precipitation and gel filtration. The molecular size of the enzyme, when estimated

by Sephadex G-75 gel filtration, was 19.7 Kda,³⁴ which was very close to the 20 Kda predicted by Lyon *et al.*³³ from transposon mutagenesis of the gene. From these results, the enzyme appears to be monomeric.

The pSK1-encoded enzyme is far less susceptible to inhibition by trimethoprim, as 50 μ M of the drug is required to inhibit the enzyme by 50% compared with 40 nM for the *S. aureus* chromosomal enzyme. This 1000-fold difference in inhibitory capability is matched by the difference in the MIC of trimethoprim for *S. aureus* strains containing a trimethoprim resistance plasmid and a fully sensitive plasmid-free *S. aureus* strain. Surprisingly, the plasmid-encoded DHFR confers no resistance to the dihydrofolate analogue, methotrexate. All the gram-negative plasmid-encoded DHFRs which confer high-level trimethoprim resistance (MIC > 1000 mg/L) are also resistant to the inhibitory action of methotrexate. Considering the close structural similarity of methotrexate and dihydrofolate, the gram-negative enzymes are remarkable in their ability to distinguish these two compounds by resisting methotrexate, but retaining a high affinity for the substrate dihydrofolate. This makes it virtually impossible to devise a new drug that more closely mimics dihydrofolate than methotrexate but is also selective. However, as the gram-positive enzyme is methotrexate sensitive, the possibility exists to design a new DHFR inhibitor with the inhibitory properties of methotrexate, but the selectivity of trimethoprim, and still be able to overcome the plasmid-encoded enzyme.

The DHFR encoded by pSK1 retained a high affinity for the substrate dihydrofolate, showing a higher binding capability ($K_m = 10 \mu$ M) than the *S. aureus* chromosomal enzyme ($K_m = 80 \mu$ M). Furthermore, although the plasmid enzyme is competitively inhibited by trimethoprim, it has a much higher K_i (11.6 μ M) than that of the *S. aureus* chromosomal enzyme ($K_i = 6.5$ nM). All the properties show that the plasmid-encoded enzyme from Australia was different from the *S. aureus* chromosomal enzyme and from the 12 plasmid-encoded DHFRs found in gram-negative bacteria. We have thus designated this enzyme the type S1.³⁴

In the USA, plasmid pG01 was also subsequently shown to encode a DHFR which, by examination of protein production with [³⁵S] methionine in minicells, was estimated to be 18.5 Kda.³⁵ In this study, the enzyme was less susceptible to inhibition by trimethoprim than the chromosomal DHFR with an ID₅₀ of 7.3 μ M. This was around 2000-fold less susceptible than the staphylococcal chromosomal enzyme. These results strongly suggested a

link between the trimethoprim-resistant DHFR encoded by the Australian and the American plasmids. In our hands, the American DHFR had a Mr of 21 000 and very similar kinetic and inhibition properties to the type S1 from Australia.

The *dfrA* gene, encoding the type S1 DHFR, has also been found in coagulase-negative staphylococci isolated in Australia. In particular, when the 0.9-kb *EcoRI-EcoRV* restriction fragment of plasmid pSK407 (which consists almost entirely of the trimethoprim resistance gene) was used as a probe, the DNA of four multi-resistant coagulase-negative bacteria hybridised.³² In three strains (two *S. epidermidis* and one *S. hominis* I), the gene was located on a plasmid. In each case the plasmids were different from each other and from either pSK1 or pG01, the prototype trimethoprim resistance plasmids in *S. aureus*. In the final strain, *S. epidermidis* SK683, no plasmid location for the *dfrA* gene could be found and it was presumed to be located on the bacterial chromosome. The biochemical properties of the DHFRs were all very similar to the type S1 with the exception of the specific activity. In the case of the three plasmid-containing strains, the activity ranged between 27 and 101 nmol dihydrofolate reduced/min/mg of protein, which is high and consistent with the view that the genes are located on small, multi-copy plasmids. On the other hand, the specific activity in *S. epidermidis* SK683 was 7.7 nmol dihydrofolate reduced/min/mg of protein, which is compatible with the location of the gene on the limited copy-number chromosome.

The similarity of the biochemical properties suggest that all the DHFRs are closely-related. However, DHFRs with less than 70% DNA homology have been shown to display identical properties.¹⁰ Conventional DNA-DNA hybridisation studies, performed under stringent conditions, will reveal DNA homologies of greater than 75–85%. Without sequencing the gene, it is impossible to state that two genes or gene products are identical. However, isoelectric focusing is extremely sensitive in its ability to reveal differences of just one amino acid in proteins consisting of 200 residues or more. However, the magnitude of the differences in pI gives no indication of the actual difference between the proteins, merely a demonstration that they are not the same. Conversely, it is rare for two enzymes with different amino acid sequences to have exactly the same isoelectric focusing pattern. The DHFRs encoded by not only the Australian and American *S. aureus* plasmids, but also the Australian and American coagulase-negative staphylococcal plasmids, were examined

by isoelectric focusing, employing a modification of the method of Broad and Smith.³⁶ In every case, the plasmid-encoded DHFR band co-focused at pI 6.54, suggesting that the *dfrA* gene is ubiquitous in staphylococcal strains on both continents (S. Tait *et al.*, unpublished results).

Sequence of the dfrA gene and the type S1 DHFR

The *dfrA* gene in plasmid pSK1 has been ascribed to a 0.75-kb region of the plasmid.³² This region is flanked by three directly repeated copies of an insertion sequence, IS257.³⁷ Their presence suggests that the *dfrA* gene and the flanking insertion sequences form a composite transposon, Tn4003.³⁰ The ubiquitous nature of the type S1 DHFR and the location of the *dfrA* gene in many different replicons, strongly supports a transposon location of the gene. Furthermore, there is a close similarity of the restriction endonuclease map of Tn4003 from the Australian plasmid pSK1 with that of the trimethoprim resistance region on the American plasmid pG01, even though the rest of the plasmid is quite different from the American plasmids.³⁸ This suggests that the American plasmid also has the flanking IS257 insertion sequence as well as the trimethoprim resistance gene.

DNA sequence analysis has confirmed that the trimethoprim resistance gene is located on a unique composite class I transposon, which is flanked by three rather than the normal two insertion sequences, namely IS257.³⁹ The direct orientation of the three insertion sequences has been shown to confer instability to the carriage of the transposon, by promoting spontaneous deletions of Tn4003. Plasmids with these specific deletions have been found both spontaneously within the laboratory and within clinical isolates.

The open reading frame for the *dfrA* gene has been found, by sequence analysis, to be 486 bp, corresponding to a protein of 162 amino acids. The active site of the DHFRs is at the N-terminal, the first 50 amino acids of which are shown in fig. 2. There are some similarities between this sequence and the type Ia plasmid-encoded enzyme in gram-negative bacteria. A closer similarity was noted between the sequence of the gram-positive chromosomal DHFRs from *Lactobacillus casei*, *Streptococcus faecium* and *Bacillus subtilis*.^{39–42} However, a recent automated amino acid sequence of the N-terminal of the *S. aureus* trimethoprim-sensitive chromosomal DHFR suggests that the origin of the plasmid-encoded enzyme may lie in the same species in which it was first discovered.⁴³

Type Ia	Met	Lys	Leu	Ser	Leu	Met	Val	Ala	Ile	Ser	Lys	Asn	Gly	Val	Ile	Gly	Asn	Gly	Pro	Asp	Ile	Pro	Trp	Ser	Ala
Type S1	Met	Thr	Leu	Ser	Ile	Ile	Val	Ala	His	Asp	Lys	Gln	Arg	Val	Ile	Gly	Tyr	Gln	Asn	Gln	Leu	Pro	Trp	His	Leu
<i>S. aureus</i>		Thr	Leu	Ser	Ile	Leu	Val	Ala	His	Asp	Leu	Gln	Arg	Val	Ile	Gly	Phe	Glu	Asn	Gln	Leu	Pro	Trp	His	Leu
							5				10					15						20			25

Type Ia	Lys	Gly	Glu	Gln	Leu	Leu	Phe	Lys	Ala	Ile	Thr	Tyr	Asn	Gln	Trp	Leu	Leu	Val	Gly	Arg	Lys	Thr	Phe	Glu	Ser
Type S1	Pro	Asn	Asp	Leu	Lys	His	Ile	Lys	Gln	Leu	Thr	Thr	Gly	Asn	Thr	Leu	Val	Met	Ala	Arg	Lys	Thr	Phe	Asn	Ser
<i>S. aureus</i>	Pro	Asn	Asp	Leu	Lys	His	Val	Lys	Lys	Leu	Ser														
							30				35					40						45			50

Fig. 2. Comparison of the N-terminal amino acid sequence of the type S1 plasmid-encoded dihydrofolate reductase with the sequence of the *S. aureus* chromosomal enzyme.

Conclusions

Plasmid-encoded trimethoprim resistance in staphylococci appears to be mediated by one gene, *dfrA*, which encodes the type S1 DHFR. The location of this gene on a transposon (Tn4003) has ensured that it has spread widely, not only geographically but also within the species of the genus *Staphylococcus*. The spread of the gene encoding the type S1 DHFR by Tn4003 appears to be very reminiscent of the spread of the type Ia DHFR gene by transposon Tn7 amongst the plasmids and chromosome of gram-negative bacteria.

Plasmid-encoded trimethoprim-resistant dihydrofolate reductases in gram-negative bacteria

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Plasmid-encoded trimethoprim resistance in gram-negative bacteria was first reported in 1972¹¹ and was originally associated with extremely high minimum inhibitory concentrations (MICs) of trimethoprim (>1000 mg/L). The mechanism of resistance was later shown to result from the plasmid-mediated production of an additional, trimethoprim-resistant DHFR.^{44,45} So far a total of 12 different plasmid-encoded DHFRs belonging to seven major groups (types I–VII) have been identified in gram-negative bacteria (table II).

DHFR types I and II, encoded by plasmids R483 and R388 respectively, were the original plasmid DHFRs described and have now been reclassified

as DHFR types Ia and IIb.¹⁰ Both these enzymes are much larger than the chromosomal enzyme with a molecular size of 35 Kda, but differ from each other in their subunit composition—the type Ia enzyme being composed of two identical, inactive sub-units of 18 Kda, whereas the type IIb enzyme is made up of four identical, inactive sub-units of 8.5 Kda.⁴⁶ The type II enzymes are also much more heat stable and are, at least, one thousand times more resistant to the inhibitory action of trimethoprim than the type I enzymes.^{47,48}

In 1986, a second sub-class of type I DHFR was identified, the type Ib, coded by plasmid pUK163. This enzyme has very similar biochemical properties to the type Ia enzyme encoded by R483, differing only in its molecular size (24.5 Kda). The sub-unit composition of this enzyme has not yet been determined, but it has been postulated that the type Ib DHFR may comprise two partial type Ia sub-units.¹⁸ Both the type Ia and Ib DHFR genes are carried on resistance transposons, Tn7 and Tn4132 respectively.

The amino acid sequence of the type Ia DHFR has been determined and compared with the *E. coli* chromosomal DHFR sequence.⁴⁹ The two enzymes show 29% identity, increased to 44% when chemically similar residues are included, with the greatest regions of homology being in the amino terminal region which comprises most of the active site of the enzyme.

A number of sub-groups have also been distinguished among the type II DHFRs: the type IIa enzyme, encoded by plasmid R67,⁴⁸ the original R388 encoded type IIb enzyme,⁴⁶ and the type IIc enzyme coded by plasmid R751.⁵⁰ All three DHFR type II enzymes are very similar in their biochemical properties and share extensive (>78%) amino acid sequence homology.^{51–53} However, sequence comparisons with other DHFRs reveal them to be quite unlike other plasmid or bacterial DHFRs. Indeed it has been postulated that the type II DHFRs may

Table II. Properties of the plasmid-encoded dihydrofolate reductases

Plasmid type	DHFR sub-type	MIC for Tp (mg/L)	Ki for Tp (μ M)	Mr (Kda)	Countries where reported
I	a	> 1000	7.4	35.0	All continents
	b	> 1000	41	24.4	UK
II	a	> 1000	6100	35.0	Europe, America, Asia
	b	> 1000	150	35.0	Europe, America, Asia
	c	> 1000	400	34.0	UK
III	a	64	0.019	16.9	New Zealand, UK
	b	128	0.15	17.0	USA
	c	512	0.64	22.0	USA
IV		10	0.063	46.7	India
V		> 1000	3.2	36.0	Sri Lanka, UK
VI		> 1000	162	9.0	South Africa
VII		> 1000	7.0	11.5	UK
SI		> 1000	11.6	19.7	Australia, USA, UK

have been derived from an oxidoreductase which originally acted on some other substrate.^{46, 51}

The type III DHFR, which has now been reclassified as the type IIIa, was first described by Fling *et al.*⁵⁴ and is characterised by its low molecular size (16.9 Kda) and unusually low resistance to trimethoprim inhibition.⁵⁵ As a result, organisms producing this enzyme exhibit only a moderate level of resistance to trimethoprim (MIC 64 mg/L). Until recently, this enzyme had only been reported on one occasion in a clinical strain of *Salmonella typhimurium* isolated in New Zealand. However, in 1988, C. J. Thomson and colleagues (unpublished observation) identified the type IIIa enzyme in a clinical strain in Nottingham. Recently developed techniques for isoelectric point determination of DHFRs³⁶ have lead to the identification of two further sub-groups of type III enzymes, types IIIb and IIIc, encoded by plasmids from *Shigella* strains isolated in the USA (N. Barg *et al.*, unpublished observation). These enzymes also confer only a moderate level of trimethoprim resistance on their host, are very similar biochemically to the type IIIa enzyme, but differ markedly in their isoelectric points.

Sequence analysis of the type IIIa enzyme has shown it to share 51% homology with the *E. coli* chromosomal DHFR, suggesting that the type IIIa gene may have originated in either *E. coli* or in a closely related gram-negative organism.⁵⁶ Preliminary sequence data on the type IIIb DHFR, however, indicates it to be quite distinct from both the sensitive *E. coli* DHFR and the type IIIa plasmid enzyme (C. J. Thomson and S. G. B. Amyes, unpublished results). Thus, despite the similarities in biochemical properties between the two plasmid enzymes, their origin and evolution

appear to have occurred independently of each other.

The type IV plasmid DHFR, identified in a number of different clinical isolates in South India, is larger than other plasmid DHFRs (46.7 Kda), is only 10 times more resistant to the inhibitory action of trimethoprim than the *E. coli* chromosomal DHFR, but is unique in its ability to be induced in the presence of increasing concentrations of trimethoprim.⁵⁷ Bacteria producing the type IV DHFR appear almost sensitive to trimethoprim when tested on conventional sensitivity test agar, although in complex broth containing trimethoprim these organisms show a significant level of resistance to the drug, similar to that exhibited by strains producing type III DHFR.⁵⁸ The mechanism of resistance conferred by the type IV DHFR differs from other plasmid DHFRs and is believed to occur by a "swamping" mechanism which involves the removal of trimethoprim through binding of the drug to the large number of type IV enzyme molecules induced in its presence. Amino acid sequence data has been obtained for the first 50 residues of the amino terminal region of the type IV DHFR. Comparison with the amino terminal regions of other DHFRs shows that the type IV enzyme shares 40% and 34% homology with the *E. coli* chromosomal enzyme and the type Ia plasmid enzyme respectively (C. J. Thomson and S. G. B. Amyes, unpublished results). This may indicate a common ancestry for the genes encoding these enzymes.

The type V plasmid DHFR was originally identified in clinical isolates from Sri Lanka.⁵⁹ This enzyme shows many biochemical similarities to the type Ia plasmid DHFR, although it differs significantly in molecular size, being only 17.5 Kda—a

similar molecular size to the type Ia subunit. The type V DHFR has now been identified in clinical bacteria in Nottingham (K. J. Towner *et al.*, unpublished results). The complete amino acid sequences of the types Ia and V DHFRs have been compared.⁶⁰ The two enzymes share 75% homology over their entire amino acid sequence and differ in only six amino acids within the first 50 residues forming the active sites of the enzymes. It seems likely, therefore, that these two enzymes share a common ancestral gene which, in each case, has followed a slightly different evolutionary path.

Finally, two further plasmid-encoded enzymes have recently been identified. The type VI plasmid DHFR has been found in only one clinical strain of *Proteus mirabilis* isolated in South Africa. This enzyme has a low molecular size (10 Kda) and, like the type II enzymes, requires very high concentrations of trimethoprim to inhibit its activity by 50%. These characteristics might indicate a possible relationship with an active type II DHFR subunit. Hybridisation studies, however, appear to indicate that extensive sequence homology does not exist between these two genes.⁶ A type VII DHFR has recently been identified in an *E. coli* isolate of animal origin in Nottingham.⁶¹ This enzyme, like the type VI enzyme, is also small and heat labile. However, it has similar inhibitory profiles to the types Ia and V enzymes.

It is evident from the numerous recent reports of new plasmid-encoded trimethoprim-resistant DHFRs that these enzymes are continuing to emerge and evolve in a manner comparable to the β -lactamase enzymes. With advances in molecular and biochemical technologies it is to be expected that novel plasmid-encoded DHFRs will continue to be identified and, hopefully, relationships between, and the origins of, those already distinguished will become clearer.

DNA probes for trimethoprim-resistant dihydrofolate reductases

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As described earlier in this review, seven major types of trimethoprim-resistant DHFRs encoded by plasmids have now been characterised in gram-negative bacteria, with several of these major groups being further divided into related sub-

types.¹⁰ Trimethoprim resistance in gram-positive bacteria has been slower to evolve, but at least one distinct trimethoprim-resistant DHFR has been identified in multi-resistant *Staphylococcus aureus*.³⁴ These enzymes can be distinguished from each other, and from the host chromosomal DHFR, on the basis of several biochemical and biophysical criteria,^{18,36,48,55} but such identification is impracticable to perform for large scale epidemiological studies, primarily because of the time required to extract and purify each individual enzyme.

An alternative method of characterisation, which is simple to perform and can potentially be carried out on a large scale in routine microbiology laboratories, involves the use of non-radioactively-labelled DNA probes specific for particular DHFR genes. Carter *et al.*⁶² demonstrated that a biotin-labelled DNA probe could be used to rapidly screen a large number of trimethoprim R plasmids for the presence of the DHFR type I gene. This method can be adapted for use with any antibiotic resistance gene for which a suitable DNA probe is available and, consequently, efforts have been made to develop specific DNA probes for each of the known DHFR types. The DNA probes which are currently available for these genes are listed in table III. Information on their specificity is reviewed and updated in the following sections.

DHFR I

The type I DHFR probe consists of a 499-bp *HpaI* fragment of pFE872, derived originally from R483.⁴⁹ This DNA fragment is comprised almost

Table III. DNA probes for specific dihydrofolate reductase genes

DHFR type	Probe
I	499 bp <i>HpaI</i> fragment of pFE872 ⁴⁹
II	850 bp <i>EcoRI</i> fragment of pFE364 (or pFE700) ²² 280 bp <i>Sau3A/EcoRI</i> fragment of pWZ820 ⁶³
III	855 bp <i>EcoRI/HindIII</i> fragment of pFE1242 (M. E. Fling, personal communication) 700 bp <i>PstI/EcoRI</i> fragment of pUN972 (K. J. Towner, unpublished results)
IV	1700 bp <i>ClaI</i> fragment of pUK1148 ⁶⁴
V	500 bp <i>HincII</i> fragment of pLK09 ⁶⁴
VI	no probe currently available
VII	300 bp <i>EcoRV</i> fragment of pUN1042 (K. J. Towner and G. I. Carter, unpublished results)
SI	500 bp <i>EcoRI/HindIII</i> fragment of pG018 ³⁵

entirely of the structural gene and forms an extremely useful probe for screening purposes. When biotinylated and used in conjunction with the high stringency wash conditions described by Carter *et al.*,⁶² hybridisation is detected only with DHFR enzymes belonging to subtypes Ia and Ib. If low stringency wash conditions are used (two post-hybridisation washes only, each for 15 min in $2 \times \text{SSC}/0.1\%$ SDS), hybridisation can additionally be detected with the type V DHFR gene (K. J. Towner and G. I. Carter, unpublished results). This is not surprising since DNA sequence analysis has shown that there is 68% similarity at the nucleotide level between the type Ia and type V enzymes.⁶⁰

DHFR II

There are at least three subtypes within the DHFR II group, all of which seem to be closely related at the hybridisation level. Fling and colleagues produced a series of probes derived from R67 (subtype IIa), culminating in the use of a 850-bp *EcoRI* fragment from pFE364 or pFE700.²⁰ This probe (and its relatives) will readily eliminate any resistance genes which do not belong to any of the DHFR II subtypes (i.e., there are no "false-negatives"). Unfortunately, it also includes several hundred bases upstream of the type II structural gene, including a region of homology contained within the widely distributed transposon Tn21.^{60,65} This probe consequently generates fairly frequent "false-positives" when used to screen trimethoprim R plasmids for the presence of the type II gene. Putative positive hybridisation results should therefore be confirmed either biochemically or by use of alternative probes.

One possible alternative probe for the type II DHFR group is the 280-bp *Sau3A/EcoRI* fragment of pWZ820, derived originally from R388 (subtype IIb). Sequencing data shows that the DHFR IIb structural gene is only 234 bp long,⁶⁶ of which 153 bp of the 3'-terminal end are included in the probe. When biotinylated and used under high stringency conditions⁶² we have observed hybridisation of this probe only with plasmids which have also been subsequently shown by conventional biochemical criteria to belong to one of the DHFR group II subtypes.

DHFR III

The type III structural gene is contained within a 808-bp *TaqI* partial DNA fragment derived from the prototype III DHFR R plasmid pAZ1.⁵⁶ This fragment has been inserted into pUC9 to form the

recombinant plasmid pFE1242. When used in conjunction with high stringency wash conditions, the 855-bp *EcoRI/HindIII* fragment of pFE1242 forms a reliable probe for eliminating resistance genes which do not belong to the type III group (i.e., there are no "false-negatives"). The probe does, however, contain approximately 350 bp external to the structural gene and therefore generates occasional "false-positive" hybridisation results.

The recent recognition of the type III resistance gene in *E. coli* responsible for urinary infection in the UK has enabled an alternative probe to be identified following cloning of the resistance gene into the recombinant plasmid pUN972 (K. J. Towner, unpublished results). A 700-bp *PstI/EcoRI* fragment of pUN972 readily detects genes belonging to the type III group and contains less DNA extraneous to the structural gene than the probe derived from pFE1242. Further evaluation is in progress.

DHFR IV

The gene responsible for the type IV DHFR has been cloned into pBR322 to form the recombinant plasmid pUK1148.⁶⁴ The 1.7-kb *ClaI* fragment of pUK1148 forms a useful probe for the type IV gene. This probe also contains DNA sequences external to the type IV structural gene and, therefore, although it was very specific when tested against "control" DHFR plasmids, it can be anticipated that occasional "false-positives" may be generated when it is used to screen trimethoprim R plasmids on an epidemiological scale. To date, however, such "false-positives" have not been observed and the type IV gene has been detected only in bacterial isolates from Southern India.¹⁰

DHFR V

The type V DHFR gene was cloned into pBR322 by Sundström *et al.*⁵⁹ to form the recombinant plasmid pLK09. It was subsequently demonstrated by Towner *et al.*⁶⁴ that a 500-bp *HincII* fragment of pLK09 could be used successfully as a probe for genes belonging to the type V group. This probe consists almost entirely of the structural gene, but because of the 68% similarity at the nucleotide level between the type Ia and type V enzymes⁶⁰ it is important that it is used in conjunction with high stringency wash conditions. When biotinylated and used as a probe under the conditions described by Carter *et al.*⁶² we have detected no cross-hybridisation with any of the other recognised DHFR groups.

DHFR VI

A probe is not yet available for this DHFR gene, which has to date only been detected on R plasmids isolated in South Africa.¹⁰ However, no hybridisation has been observed between the plasmid carrying the prototype DHFR VI gene (pUK672) and the probes available for the other DHFR groups (K. J. Towner, unpublished results).

DHFR VII

This new enzyme group has recently been characterised in a trimethoprim-resistant strain of *E. coli* isolated in the UK. It was originally recognised on the basis of the failure of the trimethoprim R plasmid contained within the strain to hybridise with the probes for any of the other known DHFR types.⁶¹ The type VII gene has now been cloned into pUC18, forming the recombinant plasmid pUN1042, and a 300-bp *EcoRV* fragment used as a preliminary probe (K. J. Towner and G. I. Carter, unpublished results). Unfortunately this probe contains only part of the structural gene. The type VII enzyme has very similar biochemical properties to the type I and type V groups,⁶¹ and some cross-hybridisation was indeed detected between the type VII probe and the type V gene (but none of the other groups). Enzymes belonging to the type VII group can, however, be clearly distinguished as neither the type I nor the type V probes show any hybridisation with the type VII gene. Development of a more specific type VII probe is currently in progress.

DHFR S1

The type S1 DHFR is the only trimethoprim-resistant DHFR reported to date from gram-positive bacteria.¹⁰ The gene has been cloned into pBR322 by Coughter *et al.*,³⁵ who also reported the use of a 500-bp *EcoRI/HindIII* fragment of the recombinant plasmid pG018 as a probe. This probe should be an intragenic fragment of the structural gene and was shown to fail to hybridise, even under low stringency conditions, with the type I, II or III genes.³⁵ It has now also been shown that there is a lack of hybridisation between the type S1 gene and the probes capable of recognising the other known DHFR types (K. J. Towner and G. I. Carter, unpublished results).

Conclusions

New groups of trimethoprim-resistant DHFRs are now being reported on a regular basis and it

seems that DNA hybridisation procedures offer the best approach for monitoring the evolution and distribution of these important resistance genes on an epidemiological scale. The use of cloning procedures enables DNA probes to be constructed for a particular need. Although requiring a certain amount of specialist expertise, these procedures are relatively inexpensive to carry out. When combined with the development of non-radioactive detection systems for use in hybridisation experiments, it is now certainly feasible for studies on the epidemiology of trimethoprim-resistant DHFR genes to be carried out in most routine microbiology laboratories.

Clinical importance of trimethoprim resistance in staphylococci isolated in Europe

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Among the gram-positive bacteria, staphylococci are the most important pathogens in both community- and hospital-acquired infections.^{67,68} The two most important species are *Staphylococcus aureus* and *S. epidermidis* and this communication focuses on these two species only. *S. aureus* is more virulent than *S. epidermidis* and may cause a number of serious infections including wound infections, abscesses, urinary tract infections, endocarditis, pneumonias and bacteraemias with a high mortality.^{69,70}

S. epidermidis was, in the past, often regarded as a culture contaminant, but it is now well established that it is a pathogen. Most *S. epidermidis* infections are hospital-acquired and related to the ability of this organism to colonise skin and indwelling foreign devices.⁷¹ The most serious consequences are bacteraemias and, in the USA, coagulase-negative staphylococci are currently the leading cause of nosocomial bacteraemias, with *S. epidermidis* accounting for 60–80% of cases.⁷²

Antibiotic therapy

The frequency and severity of staphylococcal infections demands suitable antibiotic therapy. For non- β -lactamase producing staphylococci, penicil-

lin G or V are the drugs of choice. Since nearly all staphylococci nowadays produce β -lactamase, a penicillinase-resistant penicillin such as methicillin or cloxacillin is required, or alternatively, a cephalosporin, imipenem or clavulanate potentiated amoxycillin can be used. These agents, however, are rendered useless once methicillin-resistant staphylococci (MRS) prevail. Since MRS are almost uniquely multi-resistant, i.e., also resistant to gentamicin, erythromycin, tetracycline, and many other antibiotics, few alternatives are left.^{68,72,73} Vancomycin is the drug of choice in these instances. Co-trimoxazole and, recently, the fluoroquinolones have proved to be valuable drugs against MRS.^{74,75} Resistance to the fluoroquinolones, however, is being acquired at a surprisingly fast rate, especially by MRS.

Resistance to trimethoprim

Trimethoprim resistance in staphylococci has been reported since the early 1980s.^{26,32} Trimethoprim resistance genes reside on large plasmids or in the chromosome and code for a novel, trimethoprim-insensitive DHFR.^{32,34} In view of the importance of trimethoprim-sulphonamide combinations such as co-trimoxazole in the treatment of infections by MRS, we undertook a study to evaluate the relationship between resistance to trimethoprim, methicillin and sulphonamide, the level of trimethoprim resistance, its mobility and its mechanism.

In this study a total of 269 strains was investigated—196 strains of *S. aureus* and 73 strains of *S. epidermidis*. Only strains exhibiting resistance to one or more of the following antimicrobials were included: trimethoprim (Tp), methicillin (Mt), co-trimoxazole (TpSu) or gentamicin (Gm). All strains came from University Hospitals in Hungary (54 *S. aureus*, 21 *S. epidermidis*), Germany (30 *S. aureus*), Switzerland (43 *S. aureus*, 19 *S. epidermidis*), the UK (30 *S. aureus*) and Spain (39 *S. aureus*, 33 *S. epidermidis*). All strains were identified by growth on mannitol-salt agar, their DNAase and coagulase reaction, the API-Staph system (API SYSTEM S.A., Montalieu Vercieu, France) and the Staph-rapid test (Roche). The susceptibility to eight antimicrobials—Tp, sulphamethoxazole (Su), Mt, penicillin (Pc), chloramphenicol (Cm), Gm, erythromycin (Em) and tetracycline (Tc)—was determined on Mueller Hinton Agar (Difco) at 30°C. The breakpoints for resistance were chosen according to the NCCLS recommendations⁷⁶ and were ≥ 16 mg/L for Tp, Mt and Gm, and ≥ 512 mg/L for Su.

Among the *S. aureus* strains, 117 (60%) were

resistant to Mt, 51 (26%) to Tp, 68 (35%) to Su, 85 (43%) to Gm, 186 (95%) to Pc, 54 (28%) to Cm, 118 (60%) to Em and 134 (68%) to Tc; 36 strains (18%) were resistant to MtTp, 48 (24%) to TpSu and 35 (18%) to MtTpSu. Coupled resistance to Mt plus Gm was more frequent; it was present in 66 strains (34%).

Large differences were observed if the results with strains from different sources were analysed separately. Of the 54 *S. aureus* strains from Hungary, 49 were resistant to Mt, 5 to Tp, 15 to Su, 16 to Gm, 4 to MtTp, 5 to TpSu and 4 to MtTpSu. Of the 39 isolates from Spain, 14 were resistant to Mt, 3 to Tp and only 1 to Su. None of these exhibited simultaneous resistance to MtTp or triple resistance to MtTpSu, whereas 14 were resistant to Mt plus Gm. Coupled resistance to MtTp was also rare in the isolates from Switzerland (1 strain), and none was resistant to MtTpSu. The isolates from Germany were generally the most resistant. Of 30 *S. aureus* strains studied, 22 were resistant to Mt, 25 to Tp, 28 to Su, 24 to Gm, 21 to MtTp, 25 to TpSu and 21 to MtTpSu. A rather high proportion of strains resistant to MtTp, TpSu and MtTpSu was also present in the *S. aureus* isolates from the UK (10, 11 and 10 strains, respectively, out of 30).

In contrast to *S. aureus*, the majority of *S. epidermidis* strains were resistant to Tp (56 out of 73, 77%); 27 (37%) were resistant to Mt, 45 (62%) to Su, 48 (66%) to Gm, 73 (100%) to Pc, 25 (34%) to Cm, 44 (60%) to Em and 55 (75%) to Tc. As in *S. aureus*, double resistance to MtTp occurred in 18 strains (25%), to TpSu in 43 (59%) and triple resistance to MtTpSu in 13 (18%). Analysis of the strains of different origin again showed large variation. Of the 21 *S. epidermidis* from Hungary, 6 were resistant to Mt, 17 to Tp, 10 to Su, 4 to MtTp, 10 to TpSu and 4 to MtTpSu, 6 to MtGm. Fifteen of the 19 Swiss isolates were resistant to Tp, 2 to Mt, 11 to Su, 5 to Gm, 2 to MtTp, 11 to TpSu, 1 to MtTpSu and none to MtGm. Twelve of the 33 isolates from Spain were resistant to MtTp, 22 to TpSu and 8 to MtTpSu.

From these data it seems that resistance to trimethoprim occurs more frequently in *S. epidermidis* than in *S. aureus*, and is also more frequently connected to methicillin resistance in *S. epidermidis*. The occurrence of strains resistant to MtTp or MtTpSu varies widely from country to country but is generally low. This underscores the role of co-trimoxazole as a valuable alternative in the treatment of multi-resistant staphylococcal infections.⁷⁴

As regards the mechanism of trimethoprim resistance, the level of resistance was of interest. As shown in fig. 3, the majority of *S. aureus* strains

Fig. 3. Distribution of MICs of trimethoprim in *S. aureus* (196 strains; ■) and *S. epidermidis* (73 strains; ▨).

resistant to trimethoprim have MIC values of 64 and 128 mg/L. Only 7 of 196 strains tested exhibited high level resistance with MICs > 512 mg/L. In contrast, there is a clear tendency in *S. epidermidis* towards high level trimethoprim resistance, with 30 strains exhibiting MICs of > 512 mg/L. These differences in the level of trimethoprim resistance may reflect differences in the mechanism of resistance and in the genetic localisation of the responsible trimethoprim resistance gene.³⁸

Transfer of *Tp*-resistance

Plasmid DNA was isolated from 46 trimethoprim-resistant strains as described by Bennett⁷⁷ with slight modifications. Agarose gel electrophoresis showed plasmids in all strains and generally two or more plasmids were present. Protoplast transformation was then carried out with *S. aureus* 113 (NCTC 8325 r⁻) following the procedure from Goetz and coworkers⁷⁸ with some modifications. No transformants could be obtained after selection on trimethoprim 8 mg/L from the 21 *S. aureus* strains exhibiting MICs for trimethoprim between 32 and 256 mg/L. In the four strains with MICs of 512–1024 mg/L, trimethoprim resistance was transferable. With *S. epidermidis* only two strains with MICs for trimethoprim of 32 and 256 mg/L were tested and trimethoprim resistance was not trans-

ferable. In contrast, in 8 of the 19 strains with MICs of 512–1024 mg/L, trimethoprim resistance could be transferred. Details of those strains which showed transferable trimethoprim resistance are shown in table IV. Generally, only one plasmid was transferred to the host, ranging in size from 8.5 to 45 kb. In addition to trimethoprim, resistance to penicillin, cadmium nitrate (Cd), HgCl₂ (Hg), sulphonamides or ethidium bromide (EtBr) was often cotransferred. In no case, however, was resistance to gentamicin transferred, in contrast to findings with Australian isolates.⁷⁹ Occasionally a small plasmid carrying chloramphenicol (Cm) resistance was simultaneously co-transferred. Overnight growth at 44° in Mueller Hinton broth cured the majority of strains with transferable trimethoprim resistance and loss of trimethoprim resistance always coincided with loss of the large plasmid, whereas the small plasmids (< 5 kb) conferring chloramphenicol resistance could not be cured.

Trimethoprim-resistant DHFR

The trimethoprim-resistant clinical isolates exhibited DHFR activities in the crude extract which ranged from 38 to 226 mU/mg of protein, in most cases above 100 mU/mg of protein. This is higher than the basal chromosomal enzyme level, i.e., in *S. aureus* ATCC 25923 34 mU/mg of protein or *S.*

Table IV. Resistance profiles in staphylococci with transferable Tp resistance

Strain	Origin	MIC for Tp ($\mu\text{g/L}$)	Resistance patterns	
			original isolate	transformant
<i>S. aureus</i> 157/4696	CH	> 1024	Tp Su Pc Cm Tc Cd	Tp Pc Cd
<i>S. aureus</i> 853	D	512	Tp Su Mt Pc Em Tc Cd Hg	Tp Pc Cd Hg
<i>S. aureus</i> V21571	D	1024	Tp Su Mt Gm Pc Cm Em Tc Cd	Tp Pc Cd
<i>S. aureus</i> 1075	CH	> 1024	Tp Su Pc Cm Tc Cd	Tp Pc Cd
<i>S. epidermidis</i> 955	CH	> 1024	Tp Su Gm Pc Em Tc EtBr	Tp EtBr
<i>S. epidermidis</i> 961	CH	> 1024	Tp Su Gm Pc Em EtBr	Tp EtBr
<i>S. epidermidis</i> Hub	CH	> 1024	Tp Su Pc Cm	Tp Pc Cm
<i>S. epidermidis</i> H8915	H	> 1024	Tp Gm Pc Cm Em Tc EtBr	Tp EtBr
<i>S. epidermidis</i> H15043	H	> 1024	Tp Su Gm Pc Em EtBr	Tp Su EtBr
<i>S. epidermidis</i> SP26	SP	1024	Tp Su Pc	Tp
<i>S. epidermidis</i> SP33	SP	1024	Tp Su Mt Gm Pc Em Tc	Tp Pc
<i>S. epidermidis</i> Mur78	SP	> 1024	Tp Su Gm Pc Em	Tp Su

CH = Switzerland; D = Germany; H = Hungary; SP = Spain.

aureus 113 (NCTC 8325r⁻) 50 mU/mg of protein. As far as investigated, two enzymes with DHFR activity were found in these strains.^{34,43} DHFR activity in the transformants was 1.5–2-fold higher than in the original isolate (with only two exceptions), probably due to the presence of only one plasmid resulting in a higher copy number. The trimethoprim resistance gene from *S. aureus* strain 157/4696 has, meanwhile, been cloned into the pUC18 vector and its nucleotide sequence is presently being determined.

Conclusions

A considerable number of MRS were found also to be resistant to trimethoprim. *S. epidermidis* strains were more generally resistant than *S. aureus*, as observed elsewhere.^{38,72} However, the frequency of strains with resistance to both methicillin and trimethoprim varies widely among strains of different origin, but is generally low. Thus, trimethoprim-sulphonamide combinations may often constitute a valuable alternative treatment of infections due to MRS. As observed by others,³² trimethoprim resistance could be transferred only from strains with a high MIC for trimethoprim. The clear tendency towards high level trimethoprim resistance in *S. epidermidis*, in contrast to *S. aureus*, is in line with previous observations and supports the idea that *S. epidermidis* constitutes a reservoir of resistance genes and that the trimethoprim resistance gene may have evolved in *S. epidermidis*.^{38,72}

The dissemination of trimethoprim resistance plasmids in a large isolated community

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Perth, the capital of Western Australia, is a large modern city with a population in excess of one million people. It is, however, nearly 3000 km from the nearest city of similar size and thus is ideal for a study of the appearance and spread of R plasmids in a large isolated community. Trimethoprim, in the form of co-trimoxazole was first introduced into Australia for general clinical use in February 1973. Six years later, records at our hospital showed that there was a low incidence of trimethoprim-resistant enterobacteria. However, 1 year later, in 1980, a marked increase in the number of such isolates was noted.

This paper describes the results of surveys for trimethoprim resistance in human clinical isolates of enterobacteria carried out in 1980–81 and in 1985. In both surveys, isolates were examined for resistance pattern and transferability, together with the size and incompatibility group of any R plasmid conferring trimethoprim resistance. In the first survey, trimethoprim-resistant isolates from pigs were also included although trimethoprim had

Table V. Clinical isolates with trimethoprim resistance

Year	Source	Number of isolates						Total
		<i>E. coli</i>	<i>K. pn.</i>	<i>K. ox.</i>	<i>Ent.</i>	<i>Citro.</i>	<i>Proteus</i>	
1981	man	33	11	5	3	1	0	53
	pigs	59						59
1984	man	19	24	2	9	1	4	59

K. pn. = *Klebsiella pneumoniae*; *K. ox.* = *Klebsiella oxytoca*; *Ent.* = *Enterobacter* spp.; *Citro.* = *Citrobacter* sp.

never been used for growth promotion or therapeutic purposes in the pig farm. These isolates were part of the normal faecal flora of healthy weaned piglets and were obtained at very low frequencies. Trimethoprim-resistant human clinical and porcine isolates are listed in table V. More than 75% of the human clinical isolates came from urine specimens and *Escherichia coli* and *Klebsiella* spp. accounted for the majority.

A high proportion (86%) of the porcine isolates were capable of transferring trimethoprim resistance, whereas in the human clinical isolates, a lower proportion of transferable resistance was detected (table VI). The incompatibility groups for the transferable trimethoprim plasmids indicate that *IncFIV* and *IncN* R plasmids were initially important in the appearance of trimethoprim resistance in both the human and porcine isolates. Four years later, however, *IncC* R plasmids had virtually replaced *IncFIV* plasmids in the human isolates.

IncN trimethoprim R plasmids

In the first survey of trimethoprim resistance, the *IncN* R plasmids ranged in size from 51 to 57 kb. They comprised two categories (SuTpHg resistance and SmSpSuTp resistance) and were transferred

from various genera among the human clinical isolates. Representatives of each category had characteristic restriction endonuclease digest patterns and plasmids conferring SmSpSuTp resistance hybridised to the type I DHFR probe used (pFE506) whereas plasmids conferring SuTpHg resistance hybridised to the type II DHFR (pFE420). Both types of *IncN* plasmids were also detected in isolates from the pigs.

By 1985 the *IncN* plasmids had changed. They were larger in size (65–77 kb) and hybridised to the type I DHFR probe. Two thirds conferred resistance to ApTcSmSuTp and were detected in a *K. pneumoniae* strain causing nosocomial infections in the hospital. The remaining six plasmids in this survey came from a variety of hosts including *E. coli*, *Enterobacter* spp. and a *Citrobacter* sp. They were also larger and had some additional resistance determinants. The *IncN* R plasmids had successfully maintained themselves in our community over that period.

IncFIV trimethoprim R plasmids

The *IncFIV* R plasmids are of great interest. They had rarely been reported in other parts of the world prior to their isolation by Mee and Nikolett.⁸⁰ They ranged in size from 125 kb to 175 kb (table VII). The plasmids from human isolates exhibited a variety of resistance patterns, only some of which included resistance to Hg, whereas all the plasmids from porcine isolates conferred resistance to Hg. The plasmid collection was divided into six sets, labelled A–F, based mainly on their patterns of resistance.⁸¹ Digestion of the porcine and human *IncFIV* plasmids, using the restriction enzyme *EcoRI*, showed that plasmids in sets A, B and C shared 13 restriction fragments >1 kb in size amongst all representatives, while three other restriction fragments were common to all but one of the plasmid representatives.

The Cm plasmids from man and pigs (Sets D +

Table VI. Transferable trimethoprim resistance

Year	Number tested	Number of isolates in Inc group				
		Tra ⁺ (%)	FIV	N	C	Uc*
1981	59 pig	51 (86)	30	3	†	1
	53 man	19 (36)	11	8	0	0
1985	59 man	35 (59)	1	21	10	3

*Uc = unclassified, i.e., compatible with all plasmids tested.

† = undetected among the number studied.

Tra⁺ = transfer positive.

Table VII. Characteristics of the *IncFIV* R plasmids

Origin	Resistance pattern	Size (kb)	Set
Man	SuTp	155	A
Man	TcSuTp	164	B
Man	TcSmSuTp	170-175	C
Man	TcCmSuTpHg	141-158	D
Pigs	TcCmSuTpHg	147-162	D'
Pigs	SuTpHg	125	E
Pigs	TcSuTpHg	131	F

Su = sulphonamide; Tp = trimethoprim; Tc = tetracycline; Sm = streptomycin; Cm = chloramphenicol; Hg = mercury.

D') shared 14 fragments in common and another four restriction fragments were present in all but one of the representatives. Between these two groups, however, only three restriction fragments were common—14.6, 8.36 and 4.44 kb. Another four fragments were shared by some members of both groups. The R plasmids from pigs without Cm^r (sets E and F) were almost identical in their restriction fragment patterns. The Tc^r plasmids of set E had one extra restriction fragment of 5.55 kb. These plasmids shared only two of the three common restriction fragments (14.6 and 4.44 kb).

Thus the *IncFIV* R plasmids with trimethoprim resistance comprised three major groups. They had various biotypes of *E. coli* as the original host, but differed in size, patterns of resistance and restriction endonuclease digest patterns. All, however, hybridised with the type II DHFR probe used and showed no reaction to the type I DHFR probe.⁸¹

The Tp^r genes from the epidemiologically related *IncFIV* plasmids were cloned into plasmid pACYC184. The Tp^r gene was present on an *EcoRI* fragment > 20 kb for plasmids of sets A, B and C, a 5.28-kb fragment for sets D and D', and an 8.81-kb fragment for sets E and F.⁸² Despite the size variation, the DHFR gene itself was highly conserved on all plasmids and map differences in the flanking regions provided evidence that the most recent exchange of trimethoprim R plasmids between pigs and man had occurred relatively recently and had originated in the animals. These D and D' R plasmids had identical *E. coli* hosts, based on biotyping, indicating that the organism, together with its R plasmid, had spread from pigs to man.

Conclusions

The *IncFIV* trimethoprim R plasmids arose in Perth and exhibited considerable variation in their

struggle to establish themselves. Other work has shown that they contain several replication regions and they rearranged their composition, losing and acquiring resistance determinants, during a relatively short time span. The extent of variation within the *IncFIV* R plasmids is unusual. Most studies of R plasmids conferring resistance to a particular antibiotic and belonging to a particular incompatibility group have shown a considerable degree of structural stability of the plasmid itself for long periods sometimes exceeding a decade. The variation seen in the case of the *IncFIV* trimethoprim R plasmids may reflect the difficulties of establishing in the local community. Within a few years they had been overtaken by "fitter" *IncN* and *IncC* trimethoprim R plasmids. During their stay they produced plasmids of sets A, B and C in man, sets E and F in pigs, and sets D and D' in both man and pigs. All three groups represent unique lines of evolutionary development and probably arose from a prototype *IncFIV* plasmid. It is not possible to ascertain whether this original *IncFIV* plasmid arose in pigs or man, but the data indicate that plasmid exchange between man and animals is occurring relatively frequently.

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Editors' conclusions

The study of trimethoprim resistance has been greatly facilitated by the advent of improved technology. The use of specific DNA probes for the known plasmid-encoded trimethoprim resistance genes has allowed a much more rapid evaluation of the relative importance and dissemination of these different genes. Furthermore, DNA-DNA hybridisation studies have revealed the emergence of a plethora of new plasmid-encoded resistance genes with quite distinct biochemical properties. The advent of rapid automated nucleotide and amino acid sequencing procedures may soon provide us with even more efficient methods of studying possible inter-relationships between these different genes and understanding the evolution of trimethoprim resistance on a wider basis. Further collaboration between molecular biologists and protein biochemists will undoubtedly continue to provide exciting and illuminating advances in the study of trimethoprim resistance in the future.

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