ANTIMICROBIAL RESISTANCE

Trimethoprim resistance in urinary pathogens in northern Scotland: epidemic spread of a resistance plasmid encoding the type Ib trimethoprim-resistant dihydrofolate reductase

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Summary. The prevalence of trimethoprim resistance in enterobacterial urinary pathogens from hospitalised patients in the Angus district of northern Scotland (22·8 %) was twice that found in similar isolates from patients attending general practitioners (11·2 %). Thirty-three of the 143 trimethoprim-resistant strains were shown to harbour transferable plasmids conferring high-level trimethoprim resistance. In total, 17 different plasmid types were distinguished. Two plasmids, pUK1184 and pUK1185, accounted for 36 % of the trimethoprim resistance plasmids and were shown by restriction endonuclease digestion fingerprints to be closely related to plasmid pUK28, previously demonstrated to be endemic in urinary pathogens in the Edinburgh area. Only 21 % of the plasmids were shown to encode the type Ia trimethoprim-resistant dihydrofolate reductase, whereas 70 % of the trimethoprim resistance plasmids were found to encode the type Ib dihydrofolate reductase. Hybridisation of the trimethoprim resistance plasmids identified in this study with gene probes specific for the integrase genes of transposons Tn7 and Tn21 indicates that the *dhfrIa* is rarely present within Tn7 or related transposons in these plasmids and may be more prevalent within Tn21-like transposons. In contrast, with the exception of the two endemic plasmids that harboured the *dhfrIb* gene within a Tn7-like transposon, the majority of *dhfrIb* genes were not found to be associated with either Tn7- or Tn21-like structures.

Introduction

Trimethoprim, both alone and in combination with sulphamethoxazole, continues to be used widely for the treatment of urinary tract infections. Resistance to trimethoprim is most commonly mediated by plasmid- or transposon-encoded dihydrofolate reductase (DHFR) enzymes which are insusceptible to the inhibitory action of trimethoprim.¹ To date, a total of 16 different gram-negative bacterial plasmid-encoded DHFRs have been described.^{2–5} The type Ia DHFR, commonly carried within the highly successful transposon Tn7,⁶ is generally reported to account for the majority of high-level trimethoprim resistance.⁷ Four other trimethoprim-resistant DHFRs, types Ib, V, VI and VII, share > 60 % DNA sequence homology with each other and the type Ia DHFR.^{8,9} The epidemiology of each of these genes is less well defined. The *dhfrV* and *VII* genes have been reported to occur in bacteria isolated in Europe but at a relatively low frequency.^{10–12} The *dhfrV* gene was also identified as the major mediator of plasmid-encoded trimethoprim resistance in enterobacterial isolates from Sri Lanka.¹³

The *dhfrIa*, *V* and *VII* genes have all been identified within integron structures commonly associated with Tn21-like transposons.^{8,14,15} The *dhfrVI* gene has so far been identified exclusively within pathogens isolated in South Africa.¹⁶ The *dhfrIb* gene, which has been shown to reside within a Tn7-like transposon, *Tn4132*,⁹ was originally identified within plasmids extracted from urinary pathogens isolated in Edinburgh, Scotland.¹⁷ Recently, this gene has been shown to be present in gram-negative commensal faecal flora obtained from healthy individuals in Southern India.¹⁸

In Scotland, several surveys have been conducted on both hospital and general practice isolates. These studies have been restricted to two major cities in the South of Scotland, Edinburgh and Glasgow.^{19–22} As in other parts of Europe, the incidence of plasmid-mediated resistance has decreased in both these areas during the 1980s.^{19–21} However, amongst the plasmid-mediated trimethoprim resistance, one particular resistance plasmid (R-plasmid), pUK28, was found to dominate and has persisted within hospital isolates, especially in the Edinburgh area.^{19,20} This plasmid belongs to an unusual incompatibility group, *IncA*, harbours a Tn7-like transposon carrying trimethoprim and streptomycin/spectinomycin resistance determin-

Table 1. Oligonucleotides employed as probes in hybridisations

Gene	Oligonucleotide sequence	Bases nos. in gene	Reference
<i>dhfrIa</i>	5'-CAAGTTTACATCTGACAATGAGAACGTAT	429-458	28
<i>dhfrIb</i>	5'-GTTGGACATCAAATGATGACAATGTAGTTG	197-226	9
<i>dhfrV</i>	5'-CCTGGACGGCCGATAATGACAACGTAATAG	1502-1531	14
<i>dhfrVII</i>	5'-GAATTTC AAGCTCAAATGAAAATGTATTAG	790-819	8

ants and a Tn3-like transposon carrying ampicillin resistance determinants. Resistance to sulphamethoxazole is also mediated by pUK28.²⁰

The purpose of the present study was to investigate the prevalence and nature of resistance to trimethoprim in urinary pathogens isolated in the Angus district of northern Scotland and to determine whether plasmid pUK28 was also responsible for plasmid-mediated trimethoprim resistance in this area.

Materials and methods

Isolation of clinical strains

Enterobacterial isolates were cultured from mid-stream urine specimens containing 10⁸ organisms/L as well as pus cells. They were collected from the diagnostic laboratory, Stracathro Hospital, Angus, between November 1987 and January 1988. All repeat specimens were excluded from the study.

Characterisation and sensitivity testing

Each isolate was identified to genus level by the MAST ID 15 system (Mast Laboratories Ltd, Merseyside). The minimum inhibitory concentration (MIC) of trimethoprim for each organism was determined by diluting a 6-h broth culture in saline 0.9% and plating c. 40-100 cfu on to the surface of IsoSensitest Agar plates (Oxoid) containing increasing concentrations of trimethoprim. The lowest concentration showing no visible growth after incubation for 18 h at 37°C was taken as the MIC. The sensitivity of each strain was also tested against a selection of antibacterial drugs by plating a similar dilution on to IsoSensitest agar plates containing 10 mg/L of drug, except in the cases of sulphamethoxazole and spectinomycin where 100 mg/L was used. The concentrations were chosen so that a direct comparison could be made with our earlier studies in Edinburgh, Glasgow and elsewhere.

Transfer of resistance plasmids

Bacterial strains resistant to trimethoprim 10 mg/L were tested for their ability to transfer trimethoprim resistance by the method described previously.¹⁹ Conjugation studies were performed at both 37°C and 30°C to allow detection of temperature-sensitive transferable plasmids. *Escherichia coli* K₁₂ strains J53-2 (F⁻ *pro met* rifampicin-resistant) and J62-1 (F⁻ *pro his trp* nalidixic acid-resistant)²³ were employed as recipients. The X⁺ transfer factor²⁴ was employed for mobil-

isation of non-self-transferable plasmids by a triple mating procedure described previously.¹⁹

Preparation of plasmid DNA and restriction enzyme analysis

Plasmid DNA was extracted from overnight nutrient broth cultures by the method of Birnboim and Doly²⁵ modified as described by Maniatis *et al.*²⁶ Plasmid DNA was digested with the restriction endonuclease *Hind*III (Life Technologies Ltd, Paisley) according to the manufacturer's instructions. Routinely, reaction mixtures contained 10 µl of plasmid DNA and 5-10 units of enzyme in a total volume of 20 µl. Incubation was for 1-2 h at 37°C. DNA fragments were analysed and their sizes were estimated by horizontal agarose gel electrophoresis. Bacteriophage λ DNA, digested with an appropriate restriction endonuclease, was included on each gel to provide mol. wt standards.

Preparation of Southern blots and DNA hybridisation procedures

Plasmid DNA isolated on agarose gels was depurinated, denatured, neutralised and transferred to Hybond N+ filters (Amersham International) by the method of Southern.²⁷ The filters were baked at 80°C for 2 h to immobilise the plasmid DNA before hybridisation. Oligonucleotide probes specific for the *dhfrIa*, *Ib*, *V* and *VII* genes (table 1) were 3'-end labelled with fluorescein-dUTP by the ECL oligo-labelling system (Amersham) in conditions recommended by the manufacturer. Pre-hybridisation and hybridisation were performed at 42°C with hybridisation buffers recommended by Amersham. After hybridisation, the filters were washed in 2 × SSC with SDS 0.1% twice for 15 min at room temperature and in 0.5 × SSC with SDS 0.1% twice for 15 min at 53°C. Detection of a positive hybridisation result was by means of an ECL detection kit (Amersham) with the conditions and protocols recommended by the manufacturer. Plasmids pFE872 (*dhfrIa*),²⁸ pUK1121 (*dhfrIb*),²⁹ pLK09 (*dhfrV*),¹³ pUK672 (*dhfrVI*)³⁰ and pUN1034 (*dhfrVII*)³¹ were used as controls. DNA gene probes specific for the integrase genes of Tn7 (*int₇*) and Tn21 (*int₂₁*) comprised a 1.7-kb *Ava*I/*Hpa*I fragment of plasmid pRSS021³² and a 1.3-kb *Kpn*I/*Bam*HI fragment of plasmid pLK026,¹⁵ respectively. The probes were labelled with fluorescein-11-dUTP with an ECL random prime labelling kit (Amersham) and

the conditions recommended by the manufacturer. Pre-hybridisation and hybridisation were performed at 60°C with hybridisation buffers recommended by Amersham. After hybridisation, filters were washed according to the manufacturer's recommendations and detection of a positive hybridisation result was by means of an ECL detection kit (Amersham) with the conditions and protocols recommended by the manufacturer.

Results

Characterisation of clinical strains

Eight hundred and forty-five strains of Enterobacteriaceae were isolated from specimens with significant bacteriuria during the period of study; of these, 417 strains were obtained from hospitalised patients and 428 from patients in the community. *Escherichia coli* comprised 74.7% of the total population studied; no significant difference was observed between the two populations. *Proteus* spp. were more prevalent amongst hospital isolates (15.9%) than amongst community isolates (9.1%). *Klebsiella* and *Enterobacter* spp. comprised c. 4% and 3% of the population, respectively, in both hospital and community isolates; 6% of the isolates did not belong to any of these genera.

Each isolate was tested for resistance to a range of antimicrobial agents and the results are summarised in the figure. As expected, the prevalence of resistance to all antimicrobial agents was lower in isolates obtained from general practice than in those obtained from hospitalised patients. The prevalence of trimethoprim

resistance (MIC > 10 mg/L) in hospital isolates (22.8%) was double that found in community isolates (11.2%). Similarly the prevalence of spectinomycin resistance within hospital isolates (14.3%) was significantly higher than that found in community isolates (4.9%) and may indicate an increased prevalence of Tn7-like transposons, which encode resistance to streptomycin/spectinomycin in addition to trimethoprim resistance.

Characterisation of trimethoprim-resistant isolates

The prevalence and nature of trimethoprim resistance within the two populations is summarised in table II. In both populations, *E. coli* accounted for > 70% of the strains studied. *Proteus* spp. accounted for 16% of the hospital isolates. In contrast, only 9.1% of general practice isolates belonged to this genus. However, the increased incidence of *Proteus* spp. among hospital specimens did not contribute to the different overall level of trimethoprim resistance observed in the two populations. The majority of trimethoprim-resistant bacteria were found to be resistant to 1000 mg/L (77.9% of trimethoprim-resistant hospital isolates and 81.2% of general practice isolates).

Each isolate resistant to 10 mg/L of trimethoprim was tested for the presence of transferable R-plasmids. The results (table II) show that only 23% of the resistant strains in each population transferred trimethoprim resistance. Three of the plasmids identified in hospital isolates, and one plasmid from a general practice isolate, required to be mobilised by the X⁺ transfer factor. In addition, one plasmid, from a general practice specimen, transferred only at the lower temperature of 30°C. All the trimethoprim R-plasmids mediated resistance to high levels of the agent (MIC > 1000 mg/L).

Characterisation of R-plasmids

The 33 trimethoprim R-plasmids (22 from hospital isolates and 11 from general practice isolates) were characterised by determination of their resistance profile, molecular size and *Hind*III restriction endonuclease fingerprint. Seventeen different plasmid types were distinguished; eight were found exclusively within the hospital strains (table III) and five exclusively within the community isolates (table IV).

Figure. Percentage of enterobacterial pathogens resistant to antimicrobial agents. □, Community isolates; ■, hospital isolates; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Na, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Sx, sulphamethoxazole; Tc, Tetracycline; Tp, trimethoprim.

Table II. Percentage of trimethoprim resistance amongst urinary bacteria in Angus district (Nov. 1987–Jan. 1988)

Source of isolates	Number of strains	Strains (%) with MIC of trimethoprim (mg/L)		Strains with trimethoprim R-plasmids (%)
		> 10	> 1000	
Hospitals	417	22.8	17.7	5.3
General practice	428	11.2	9.1	2.6
Total	845	16.9	13.4	3.9

Table III. Characteristics of trimethoprim R-plasmids from hospital isolates

Number	Resistance pattern	Size (kb)	Plasmid designation	Hybridisation with specific probes			
				<i>dhfrIa</i>	<i>dhfrIb</i>	<i>int₇</i>	<i>int₂₁</i>
6	ApSmSpSxTp	66	pUK1184	—	+	+	—
5	ApSmSpSxTp	67	pUK1185	—	+	+	—
2	ApSxTp	73	pUK1188	—	+	—	—
1	ApSxTp	122	pUK1187	—	+	+	—
1	SxTp	68	pUK1189	+	—	—	+
1	ApCmKmSpSxTcTp	63	pUK1192	—	—	—	+
1	ApCmSmSpSxTcTp	57	pUK1191	—	+	—	+
1	ApSmSxTp	69	pUK1194	—	—	—	—
1	SxTp	15	pUK1190	—	+	—	—
1	ApSxTp	71	pUK1195	—	+	—	—
1	SpTp	67	pUK1193	+	—	—	+
1	Tp	86	pUK1186	—	—	—	—

Abbreviations of resistance determinants: Ap—ampicillin; Cm—chloramphenicol; Km—kanamycin; Sm—streptomycin; Sp—spectinomycin; Sx—sulphamethoxazole; Tc—tetracycline; Tp—trimethoprim.

Table IV. Characteristics of trimethoprim R-plasmids from community isolates

Number	Resistance pattern	Size (kb)	Plasmid designation	Hybridisation with specific probes			
				<i>dhfrIa</i>	<i>dhfrIb</i>	<i>int₇</i>	<i>int₂₁</i>
1	ApSxTp	112	pUK1187	—	+	+	—
2	ApSxTp	12	pUK1276	—	+	—	—
2	SxTp	68	pUK1189	+	—	—	+
1	ApSmSpSxTp	67	pUK1185	—	+	+	—
1	ApSmSxTp	123	pUK1274	+	—	—	+
1	ApSxTp	71	pUK1279	—	+	—	—
1	KmTp	67	pUK1278	+	—	+	—
1	SxTp	15	pUK1190	—	+	—	—
1	Tp	79	pUK1280	+	—	—	+

Abbreviations as for table III.

Four plasmids, pUK1185, pUK1187, pUK1189 and pUK1190, were found in both hospital and community isolates.

In the hospital isolates, two plasmids, pUK1184 (identified on six occasions) and pUK1185 (identified on five occasions), were found to predominate. Both plasmids harbour resistance genes for ampicillin, sulphamethoxazole and streptomycin/spectinomycin in addition to trimethoprim. Digestion of plasmid pUK1184 with the restriction endonuclease *Hind*III resulted in a fingerprint identical to that obtained after *Hind*III digestion of plasmid pUK28, previously shown to predominate in urinary pathogens isolated in Edinburgh hospitals during 1981–1986.¹⁹ Plasmid pUK1185 showed a very similar restriction pattern, differing only in a single fragment. Plasmid pUK1185 was also found on one occasion in a general practice isolate. In all instances where plasmid pUK1185 was isolated, the host organism was identified as *E. coli*. Four of the five *E. coli* strains harbouring pUK1185 were isolated from different patients resident in the same ward of a long-stay hospital, suggesting that resistance in this ward was being spread by cross-contamination with a plasmid-bearing *E. coli*. In contrast, plasmid pUK1184 was identified in clinical

strains of *E. coli*, *Citrobacter* and *Proteus* spp. Furthermore, the isolates were obtained from patients in four different hospitals, ruling out the possibility of cross-contamination with a resistant pathogen or R-plasmid.

Characterisation of the trimethoprim resistance genes

Southern blots of the trimethoprim R-plasmids were hybridised with oligonucleotide probes specific for the type Ia, Ib, V and VII DHFR genes. The results are summarised in tables III and IV. None of the plasmids hybridised with probes specific for the *dhfrV* and *VII* genes. A total of seven plasmids hybridised with the *dhfrIa* oligonucleotide probe representing six different plasmid types. Twenty-three plasmids, representing nine different plasmid types and including the two endemic plasmids, pUK1184 and pUK1185, hybridised with the *dhfrIb* oligonucleotide probe. Three plasmids did not hybridise with any of the DHFR specific probes tested.

Prevalence of *Tn7* and *Tn21* integrase genes in trimethoprim R-plasmids

Each plasmid was tested for the presence of the *int₇*

and *int*₂₁ integrase genes. The majority of different plasmids carrying the *dhfrIa* gene showed positive hybridisation with the *int*₂₁ gene probe; only one plasmid, pUK1278, hybridised with the *int*₇ gene probe. This indicates that, amongst this population of trimethoprim R-plasmids, the *dhfrIa* gene is rarely found within a Tn7-like location and may be more associated with Tn21-like structures. In contrast, three different plasmids carrying the *dhfrIb* gene showed positive hybridisation with the *int*₇ gene probe, including the two endemic plasmids pUK1184 and pUK1185. Only one plasmid, pUK1191, showed positive hybridisation with the *int*₂₁ gene probe and the remaining five plasmid types harbouring the *dhfrIb* gene showed no hybridisation with either integrase gene probe.

Discussion

The prevalence of trimethoprim resistance amongst urinary pathogens appears to vary according to country. Most recent data indicate that trimethoprim resistance in enterobacterial urinary pathogens in northern Europe has levelled to around 12% in general practice isolates and 15–20% in hospital isolates.⁷ In contrast, surveys in tropical and developing countries have revealed that the prevalence of resistance to trimethoprim is very much higher than in industrialised countries, with 30–64% of urinary pathogens showing resistance to trimethoprim 10 mg/L.⁷ In most areas of the world, the proportion of high-level, non-transferable resistance increased dramatically in the 1980s and this has been shown to result from the migration of trimethoprim resistance genes, carried on resistance transposons, into the bacterial chromosome.^{19–21}

The results of this study show that resistance to trimethoprim continues to present a significant dilemma in the choice of treatment for urinary tract infections in Scotland. As might be expected, a notable difference was observed between the prevalence of

resistance amongst hospital isolates than amongst those from general practice. In Scotland, studies conducted in Edinburgh during the 1980s showed that following a peak incidence of 18.3% trimethoprim resistance (MIC > 10 mg/L) amongst hospital isolates of urinary pathogens, the incidence of resistance to trimethoprim gradually decreased to around 12% by 1984 and has remained at that level since.³³ Resistance levels in the Angus district of northern Scotland are significantly higher than in Edinburgh, although as has previously been reported in clinical strains in Edinburgh and Glasgow, the majority of the highly resistant isolates were unable to transfer their resistance,^{19–21} suggesting that a similar migration of trimethoprim resistance transposons into the bacterial chromosome has occurred in northern Scotland.

An examination of the trimethoprim R-plasmids identified in this survey has revealed that two very closely related R-plasmids, pUK1184 and pUK1185, were responsible for the majority of transferable trimethoprim resistance in this population. Both plasmids are clearly related to plasmid pUK28, the trimethoprim R-plasmid previously shown to be endemic in Edinburgh hospital and community isolates,^{19,20} and illustrate the epidemic spread and persistent maintenance of a single plasmid within bacterial populations separated by relatively large geographic distances. Plasmid pUK28 was also confirmed in this study to harbour a *dhfrIb* gene. Hence it would appear that this gene, and not the *dhfrIa* gene, is the dominant trimethoprim resistance gene amongst trimethoprim R-plasmids in Scotland. Although the *dhfrIb* gene was found to be present within Tn7-like structures on three occasions, the majority of *dhfrIb* genes do not appear to be associated with integron structures. Thus, other mechanisms of spread of this gene, which have contributed to its widespread occurrence in trimethoprim R-plasmids in Scotland, must exist and remain to be determined.

We thank Tayside Health Board for grant no. R267.

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