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Trimethoprim resistant dihydrofolate reductases in normal faecal flora isolated in India

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SUMMARY

A high incidence of resistance to trimethoprim has been shown in the normal faecal flora in a population in south India. The dihydrofolate reductase (*dhfr*) genes mediating transferable resistance to trimethoprim have been identified. Unusually, in this study, the *dhfr*V was shown to be the predominant resistance gene (*dhfr*V 50% of transconjugants, *dhfr*Ia 30%), the *dhfr*Ib was also detected being distinguished from the *dhfr*V by an oligo-probe. However, when non-transferable resistance was considered, the *dhfr*Ia was the most prevalent of the *dhfr*s identified. All those plasmids harbouring the *dhfr*Ia were shown to possess Tn*7*. All the plasmids that probed positive for the *dhfr*V and the *dhfr*Ib were shown to be associated with the integrase of the Tn*21*-like transposons, but 8 of the *dhfr*V genes were not associated with the Tn*21* resolvase. The *dhfr*IV was shown to be present in all seven plasmids that produced low level trimethoprim-resistance. The *dhfr*V, first characterized in Sri Lanka, would seem to have a local distribution in this region of Asia but is distinguishable from the *dhfr*Ib only by the use of an oligo-probe.

INTRODUCTION

Trimethoprim, a selective inhibitor of bacterial dihydrofolate reductase (DHFR) [1], has been widely used throughout the world in the treatment of a number of infections, for over 20 years, either alone or in combination with a sulphonamide. Despite the emergence of resistance mediated by plasmid-encoded DHFRs insensitive to the action of trimethoprim [2], resistance has remained at relatively low levels in industrialized countries [3, 4]. In developing countries, however, resistance is a growing problem, with high levels being reported in Africa, South America and Asia amongst enterobacteria [5–7], including *Salmonella* spp. [8, 9] and *Shigella* spp. [10–12]. High levels of resistance have also been reported in the normal faecal flora from populations in Africa and Asia [13–16] and in the Netherlands [17, 18].

Plasmid-encoded resistance to trimethoprim is mediated by DHFR enzymes that are insensitive to inhibition by the drug. Sixteen such enzymes have been identified in the Enterobacteriaceae [19, 20]. The nucleotide sequences of many of

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these have been determined and the types Ia [21], Ib [22], V [23], VI [24] and VII [25] have been shown to be closely related. The DHFR type Ia is found associated with the transposon Tn*7*, which integrates at a high frequency into the *Escherichia coli* chromosome, at a specific site attTn*7* [26]. As a result, the type Ia is the most widespread and frequently isolated of the trimethoprim resistant DHFRs [4, 27] and has been increasingly identified in the chromosomes of trimethoprim resistant *E. coli* [28]. The type Ia has also been found, infrequently, in association with Tn*21*-like transposons [28, 29]. In this location it has become integrated at the recombination site contained within the integron region of the Tn*21* group of transposons [29]. The type Ia is bounded by regions that are similar to integron associated sequences; however, a functional integron is not present. Other resistance genes are found at this site, perhaps indicating past integron activity [30, 31].

The DHFR types Ia, IIc, V, VII and X have been found as gene-cassettes located at the integron-associated active recombination locus of Tn*21*-like transposons [25, 32]. The DHFR Ib has only been characterized as part of a Tn*7*-like transposon [33]. Despite the location as part of such a promiscuous group of transposons, the DHFR types V, VI, VII and X are far less frequently identified in trimethoprim resistant strains than the DHFR type Ia [3], and little is known of the epidemiology of the *dhfr* Ib [33]. The DHFR type IV, on the other hand, has only ever been identified in plasmids isolated from urinary pathogens in this area of India [34]. This DHFR confers only low level resistance, conferring an MIC of 4 mg/l as determined on IST agar [35]. It has, however, been shown to be inducible, under certain circumstances, that results in a much higher MIC [36, 37].

In 1990, almost universal carriage of enterobacteria resistant to ampicillin, chloramphenicol and trimethoprim was reported in south India [13] in the town of Vellore and 3 rural villages near to this town. In this present study, the nature of the resistance to trimethoprim in these strains is investigated and the genes mediating this resistance are identified.

MATERIALS AND METHODS

'Breakpoint' sensitivity tests were performed at 10 mg/l of antibiotic [38], and 50 mg/l of $HgCl_2$ [29], unless otherwise indicated. Minimum inhibitory concentrations were determined by doubling dilution in Isosensitest agar (Oxoid, Basingstoke, UK). Transfer of plasmids to the standard rifampicin resistant *E. coli* J62-2 was by the method of Amyes and Gould [38]. Plasmid DNA from the resulting transconjugants was prepared by the method of Takahashi and Nagano [39]. Subsequent digestion with restriction endonucleases was carried out under the conditions recommended by the manufacturers (Gibco BRL, Life Sciences, Paisley, Scotland). Plasmid DNA was separated in a 0·6% agarose gel while digests of plasmid DNA were separated in 0·8% agarose.

Plasmid DNA was transferred to Hybond C-Extra (Amersham, UK), by a modified method of Southern [40], as recommended by the manufacturer. Colony blotting was performed by the method described by Sundström and colleagues [41]. The probes for the various *dhfr* genes, used to identify the trimethoprim resistance mechanisms, and the transposon and integron open reading frames

Table 1. *Probes used in hybridization studies, and their origin*

Probe for	Derivation	Probe fragment	Reference
dhfr Ia	pFE872	*Hpa* I 499 bp	21
dhfr IIa	pFE700	*Eco* RI 800 bp	42
dhfr III	pUN972	*Eco* RI, *Pst* I 700 bp	(Gift of K. J. Towner)
dhfr IV	pUK1148	*Cla* I 1700 bp	43
dhfr V	pLK09	*Hinc* II 500 bp	43
dhfr VII	pUN1056	*Eco* RV 300 bp	44
**tnp*A of Tn*21*	pGS150	*Eco* RI 1·25 Kb	29
†In Tn*21*	pRSS011	*Ava* I, *Hpa* I 1·7 Kb	29
‡In Tn*7*	pLK026	*Bam* HI, *Kpn* I 1·29 Kb	29

* Resolvase gene of Tn*21*. †Integrase-like orf of Tn*21*. ‡Integrase-like orf of Tn*7*.

(ORFs), are as outlined in Table 1. The vectors carrying these various probes were prepared by the polyethylene glycol method described by Sambrook, Fritsch and Maniatis [45]. The probe fragments were isolated with the Geneclean (Bio101, La Jolla, CA) DNA preparation kit. The probes were labelled with biotin by nick translation (Gibco BRL, Life Sciences, Paisley, Scotland) for the preparation of probes for hybridization with plasmid blots or with dCTP[^{32}P] (Amersham, UK) by the random prime method (Gibco BRL, Life Sciences, Paisley, Scotland) colony hybridization.

An oligo-nucleotide probe, as characterized by Young and colleagues was used to distinguish the *dhfr* Ib in those plasmids that hybridized with the *dhfr* V gene probe. This has shown to be necessary to differentiate between these two *dhfr* genes because of sequence homology [33]. The probe was labelled and detected by the ECL kit (Amersham, UK) following the manufacturer's instructions.

Hybridization wash and detection conditions for biotinylated probes, for high stringency, were as described by the manufacturer (Gibco BRL, Life Sciences, Paisley, Scotland). Colony blots were hybridized and washed under conditions of high stringency as described by Sundström and colleagues [41].

RESULTS

Resistance in wild-type strains

One hundred and four trimethoprim resistant strains were tested for resistance to other antimicrobial agents and the MIC of trimethoprim was determined in both wild-type and transconjugant strains. Rates of resistance to those antimicrobials tested are outlined in Figure 1. In total, 62 isolates were resistant to 6 or more of the agents tested. No resistance was encountered to cefotaxime (1 mg/l), ceftazidime (2 mg/l), cefuroxime (4 mg/l), gentamicin (1 mg/l), or ciprofloxacin (0·1 mg/l) in any of the organisms tested. All but 8 of the wild-type strains had MICs of > 1024 mg/l for trimethoprim.

Plasmid-mediated resistance

Thirty-six of the 104 trimethoprim resistant strains were capable of transferring this resistance to *E. coli* K12 J62-2. All of these donors were identified as *E. coli* by the API 20E system. The antibiotic resistance profiles of the transconjugants

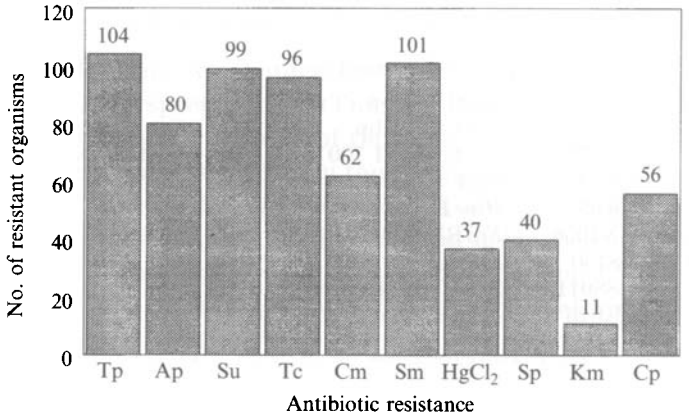

Fig. 1. Number of isolates resistant to other antimicrobial agents in the trimethoprim resistant strains. Tp, trimethoprim; Tc, tetracycline; Ap, ampicillin; Su, sulphamethoxazole; Sm, streptomycin; Sp, spectinomycin; Cm, chloramphenicol; $HgCl_2$, mercuric chloride; Km, kanamycin; Cp, cephaloridine.

Table 2. *Resistance profiles of the trimethoprim resistant transconjugants*

Resistance profile*	Transconjugant strain number
Tp	13, 91, 147, 185
Tp Tc	188
Tp Ap	166
Tp Su Sm	7, 32
Tp(L) Su Sm	179
Tp Tc Cm Hg	64
Tp Tc Cm Hg Ap	160
Tp Tc Cm Ap	111
Tp Su Tc Sm	190
Tp Su Tc Sm Ap	199
Tp(L) Su Tc Sm Ap	10, 16, 34, 35, 36
Tp(L) Su Sm Ap Km	118
Tp Tc Ap Sp Sm Km	163
Tp Su Tc Sm Ap	20, 120, 198
Tp Su Ap Sm Sp	217
Tp Su Tc Ap Sm Sp	6, 26, 29, 167, 208, 219
Tp Su Tc Cm Sm Ap Km Cp Hg Ag	186, 2, 9, 60

* L indicates low level resistance to trimethoprim; Ag, amoxicillin/clavulanic acid.

are shown in Table 2. Twenty-nine of these 36 transconjugants demonstrated high level resistance to trimethoprim with MICs at or above 512 mg/l. Seven of the transconjugants demonstrated low level resistance to trimethoprim, the donor strains of all 7 also had low MICs of trimethoprim, all with MICs between 4 and 64 mg/l.

Seventeen different antibiotic resistance profiles in the transconjugants were found (Table 2). However, on restriction endonuclease digest analysis of the plasmids prepared from the transconjugants, 27 quite distinct patterns could be identified. Thus it appears that 27 different plasmids are responsible for trimethoprim resistance, and a number of other resistance mechanisms, in this population of Gram-negative rods from normal faecal samples.

Table 3. *Characteristics of plasmids positive for the* dhfr *V probe*

Donor	Resistance profile	Plasmid sizes (Kb)	In*1**	*tnp*A*
13	Tp	69	+	−
91	Tp	46, 2	+	−
147	Tp	53, 13, 4	+	−
192	Tp	54, 8, 3	+	−
166	Tp, Ap	64, 13, 6	+	−
7	Tp, Su, Sm	82	+	−
32	Tp, Su, Sm	74, 17, 4, 2	+	−
64	Tp, Tc, Cm, Hg	80, 55	+	+
190	Tp, Su, Tc, Sm	100, 8, 3	+	−
160	Tp, Tc, Cm, Ap, Hg	80, 46	+	+
199	Tp, Su, Tc, Sm, Ap	82	+	+
20	Tp, Su, Tc, Sm, Ap	120	+	+
198	Tp, Su, Tc, Sm, Ap	52	+	+

* In*1* is the probe for ORF of the putative integrase, *tnp*A is the probe for the resolvase of Tn*21*. The largest plasmid carried the *dhfr* in all cases.

Table 4. *Characteristics of plasmids positive for the* dhfr*Ib oligo-probe*

Donor	Resistance profile	Plasmid sizes (Kb)	In*1**	*tnp*A*
120	Tp, Su, Tc, Sm, Ap	30	+	+
186	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	70, 63	+	+
2	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	89	+	+
9	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	100	+	+
60	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	108	+	+

* In*1* is the probe for ORF of the putative integrase, *tnp*A is the probe for the resolvase of Tn*21*. The largest plasmid carried the *dhfr* in all cases.

Hybridization with dhfr *probes*

When the transconjugants were hybridized with the various probes for the trimethoprim resistant DHFRs, positive results were obtained with 3 of the probes. None of the plasmids was negative for any of the probes tested, and none hybridized with more than one gene probe.

Eighteen of the plasmids hybridized with the probe for the type V DHFR (Table 3). However, 5 of these were subsequently shown to be *dhfr*Ib genes when the oligo-probe was used to distinguish between these 2 *dhfr* genes that showed close sequence homology (Table 4). These plasmids were then tested for positive hybridization with probes for the *tnp*A gene of Tn*21* and the gene coding for the integrase associated with the integron. All those plasmids harbouring the *dhfr*V and *dhfr*Ib gene also possessed the open reading frame (ORF) of the integrase. However, 8 plasmids encoding the *dhfr*V did not possess the *tnp*A for the Tn*21* transposase. This suggests the absence of the Tn*21* transposon in these plasmids (Table 3).

Some of the plasmids, encoding the *dhfr*V gene, were shown to be very similar when their restriction endonuclease digest patterns were compared. The plasmids from strains 13, 91, 147 and 192 were shown to be very similar, although not

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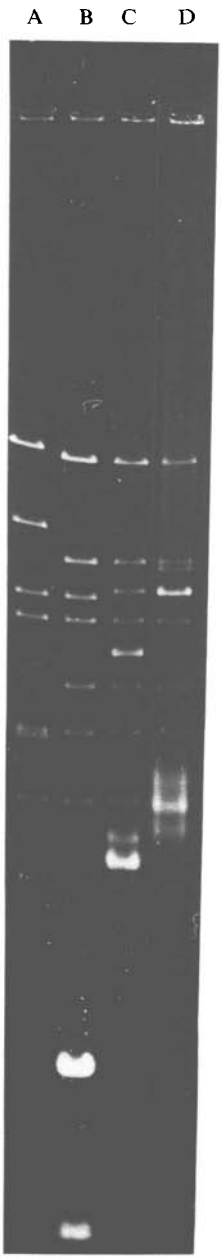

Fig. 2. A 0·8% agarose gel of a *Bam*H1 restriction endonuclease digest of plasmids isolated from the transconjugants 13, 91, 147 and 192, harbouring the *dhfr*V gene: A, 13; B, 91; C, 147; D, 192.

identical (Fig. 2), and were counted as one group. They were, however, isolated from different sites, 1 each from 2 of the villages (13, 19) and 2 from the town of Vellore itself (147, 192). These plasmids also encoded only trimethoprim resistance out of all the antimicrobials tested. The plasmids from strains 7 and 32 also had very similar restriction endonuclease digest patterns to each other and had the same antimicrobial resistance profile (Table 2), as did the plasmids from strains 9

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Table 5. *Characteristics of plasmids positive for the* dhfr*I probe*

Donor	Resistance profile	Plasmid size (Kb)
185	Tp	40
188	Tp. Tc	60. 85
111	Tp. Tc. Cm. Ap	77. 2
6	Tp. Su. Tc. Ap. Sm. Sp	48. 4
26	Tp. Su. Tc. Ap. Sm. Sp	48. 4
29	Tp. Su. Tc. Ap. Sm. Sp	94
208	Tp. Su. Tc. Ap. Sm. Sp	57. 7. 4
217	Tp. Su. Ap. Sm. Sp	35. 7. 4
219	Tp. Su. Tc. Ap. Sm. Sp	57. 7. 4
167	Tp. Su. Tc. Ap. Sm. Sp	77. 30. 2
163	Tp. Km. Tc. Ap. Sm. Sp	84

The largest plasmid carried the *dhfr* in all cases.

Table 6. *Plasmids positive for the* dhfr*IV probe in this study*

Plasmids	Resistance profile	Plasmid size (Kb)
pUK2005 (10)	Tp. Su. Tc. Sm. Ap	65
pUK2007 (16)	Tp. Su. Tc. Sm. Ap	65
pUK2012 (34)	Tp. Su. Tc. Sm. Ap	65
pUK2013 (35)	Tp. Su. Tc. Sm. Ap	65
pUK2014 (36)	Tp. Su. Tc. Sm. Ap	65
pUK2019 (118)	Tp. Su. Sm. Ap. Km	52
pUK2026 (179)	Tp. Su. Sm	77

Numbers in parentheses are the original donor strains.

and 60. harbouring the *dhfr*Ib. Both of these plasmids were isolated from the same village.

Many of the transconjugants harbouring the *dhfr*V were sensitive to sulphamethoxazole (Table 3) which is usually associated with Tn*21* and the integrase system of this transposon [46]. Only 3 of those plasmids that did not show hybridization with the *tnp*A probe were sulphamethoxazole resistant. Only 2 of the plasmids which gave positive hybridization with the *tnp*A gene did not carry resistance to sulphamethoxazole (Table 3).

The remaining 11 of the transconjugants, that demonstrated high level resistance to trimethoprim, all harboured the *dhfr*Ia gene (Table 5). All of these plasmids showed positive hybridization with the probe for the Tn*7* integrase ORF (Table 5).

All the plasmids that were responsible for mediating low level resistance to trimethoprim demonstrated positive hybridization with the probe for the *dhfr*IV gene (Table 6). Five of these plasmids were identical in both their antibiotic resistance and restriction endonuclease digest pattern although they were again isolated from different areas.

Of those strains that did not transfer trimethoprim resistance, 27 were shown to carry the *dhfr*Ia gene by colony blot and hybridization (Table 7). Only four strains that did not transfer trimethoprim resistance hybridized with the probe for the *dhfr*V (Table 8).

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Table 7. *Strains that demonstrated positive hybridization with the* dhfr*Ia gene probe but were not capable of transferring trimethoprim resistance*

Resistance profile	Strain number
Tp Su Ap Tc Cm Sm/Sp Hg Km	174
Tp Su Ap Tc Sm/Sp Hg Km	203
Tp Su Ap Tc Cm Sm/Sp	47, 54, 110, 112, 117, 141, 148, 152, 153, 161, 170, 193
Tp Su Ap Tc Cm Km	149
Tp Su Ap Cm Sm/Sp	195
Tp Su Ap Tc Cm	173
Tp Ap Tc Sm/Sp	45
Tp Su Tc Sm/Sp	61, 139, 155, 172, 194, 201
Tp Su Sm/Sp	162, 171
Tp	136

Table 8. *Strains that demonstrated positive hybridization with the* dhfr*V gene probe but did not transfer trimethoprim resistance*

Resistance profile	Strain number
Tp Su Ap Tc Sm	23
Tp Su Ap Tc Cm Sm Hg	37, 56, 191

DISCUSSION

Trimethoprim resistant Gram-negative rods were encountered at very high rates in the normal faecal flora in urban and rural populations in Tamil Nadu, south India [13]. Thirty-six percent of this resistance was due to transferable plasmid-mediated resistance to the drug. Along with resistance to trimethoprim, a number of other resistance determinants were co-transferred. All the transferable resistance determinants were shown to result from the presence of previously described trimethoprim resistant DHFRs. In this survey, however, the type V DHFR was marginally the most frequently isolated of the plasmid-mediated DHFRs, accounting for a third of the transferable resistance. The type Ia DHFR is usually found to be the most commonly isolated DHFR [27, 4]. In this case, only 11 of the transconjugants were shown to harbour the *dhfr*Ia gene.

The epidemiology of the *dhfr*Ib gene has not been well studied and its prevalence is not known. The probe for the *dhfr*V gene hybridized, under conditions of high stringency, with 5 plasmids (Table 4) that were later shown to hybridize with the oligo-probe for the *dhfr*Ib. This probe cannot therefore differentiate between the *dhfr*V and *dhfr*Ib and the use of this probe may mask the true extent of the *dhfr*Ib. The *dhfr*Ib has been characterized in a transposon Tn*4132* that is similar to Tn*7* [33]. In this study, however, the plasmids did not hybridize with the Tn*7* integrase-like ORF but did hybridize with the integrase probe for Tn*21* and the transposase of TN21. This may indicate the presence of the *dhfr*Ib on a Tn*21*-like transposon having been integrated to this location as part of a gene cassette, further work is underway in the characterization of these elements.

The *dhfr*V gene was first characterized in strains from Sri Lanka [41] and the region where these strains were collected is close to Sri Lanka. Other studies in which the *dhfr*V has been frequently found have also shown some association with

this part of Asia. In a study conducted in Sicily the type V DHFR was shown to be the most frequently isolated of the DHFRs in an outbreak of trimethoprim resistant *Shigella* sp. It was, however, thought that the strain had been introduced in immigrants from Sri Lanka [47]. In a study conducted in Finland, 4 or 5 *Shigella* carrying the *dhfr* V gene probably originated in Sri Lanka [48]. The DHFR type V may have originated in this area or this region may represent a focus for this resistance gene.

The *dhfr* Ia gene was the most common trimethoprim resistance gene in those strains that were not capable of transferring resistance. The movement of the *dhfr* Ia gene onto the chromosome via the action of Tn*7* has been noted on previous occasions. This reflects the preferred insertion of Tn*7* into the *E. coli* chromosome at the specific insertion site attTn*7* [26]. Here, as in other surveys, this has resulted in an increasing identification of the *dhfr* Ia gene as part of the *E. coli* chromosome [28, 50]. The *dhfr* Ia gene accounted for 53% of the identified trimethoprim resistance mechanisms, and 36% of all trimethoprim resistant isolates. The stability conferred on the gene by insertion into the chromosomes has probably resulted in the success of the *dhfr* Ia gene mediated by Tn*7*.

The *dhfr* V, encoded by Tn*21* has not met with the same success, although the Tn*21*-like group of transposons is responsible for a wide range of different resistance mechanisms [46]. The integron system associated with the Tn*21*-group of transposons has been responsible for the accumulation of resistance mechanisms to sulphonamides [45], β-lactams [50], aminoglycosides [51], heavy metals [52] and disinfectants [25] as well as the insertion of the *dhfr* genes encoding for the DHFR types IIc, V [23], VII [25], X [32]. Recently the *dhfr* Ia has been found in Tn*21* inserted as a gene-cassette that has been shown to be associated with Tn*7*; however, the integrase of Tn*7* seems to have undergone a deletion that has rendered it inoperative [29, 32].

All of the *dhfr* V genes identified in this study are associated with the integrase ORF of Tn*21*; however, 8 showed no positive hybridization with the probe for the transposase of Tn*21*. This indicates the insertion of the integron into the plasmid without the presence of an intervening transposon, has had been demonstrated in the case of the *dhfr* II of plasmid R388 [23]. Several of these plasmids also lack resistance to the sulphonamides, that is usually found associated with the *dhfr* V gene [29] and integron structures in general [46]. This indicates that sulphamethoxazole resistance has been deleted or is not being expressed. Further investigation of the structures involved in the integration of the *dhfr* V gene will need to be performed to confirm the genetic background of trimethoprim resistance in these plasmids.

The type IV DHFR was identified in all plasmids which conferred low level resistance to trimethoprim. Five of the transconjugants showed the presence of the same 65 Kb plasmid mediating resistance to trimethoprim, sulphamethoxazole, tetracycline, ampicillin and streptomycin. The other two plasmids were distinct from each other and this group. These plasmids all showed some similarities in resistance profile when compared with those plasmids previously characterized harbouring the *dhfr* IV gene (Table 6). The *dhfr* IV gene has not been identified in other parts of the world. This may be as the result of: (1) the mechanism is restricted to this part of India; (2) failure to detect the low level of

resistance demonstrated by this mechanism under normal sensitivity testing conditions; and (3) failure to detect transconjugants bearing the *dhfr*IV, again because of the low level of resistance demonstrated when ISTA is used. However, the unique induction mechanism of the DHFR type IV means that the actual MIC may be much higher when the enzyme has been induced prior to the sensitivity test [37].

The continued persistence of this resistance mechanism, despite the presence of ostensibly more effective resistance mechanisms, the DHFR types Ia and V, suggests some level of success for the *dhfr* gene for the type IV in this region. The evolution of this inducible DHFR may be a result of the conditions influencing antibiotic resistance in this region, i.e. high use and 'self-dosing' with antimicrobials available without prescription in any amount affordable to the customer [13].

Thus high rates of resistance to trimethoprim in the normal gut flora of this population are due to mechanisms previously identified in pathogenic strains. Resistance is plasmid- and transposon-mediated and integron activity seems to be associated with the *dhfr*V genes identified in the plasmids responsible for trimethoprim resistance. High rates of resistance have been reported in Vellore in *S. flexneri* and *S. shigae*, 84% and 88% resistance respectively, carrying transferable resistance to trimethoprim [53]. The genes identified here are capable of transfer to pathogenic strains and will constitute a reservoir of resistance which may threaten the use of trimethoprim in this area, especially in the treatment of shigellosis, typhoid and paratyphoid that are endemic in this region.

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