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Characterization of multi-drug resistant *Salmonella typhi* isolated from Pakistan

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SUMMARY

Thirty-nine strains of *Salmonella typhi*, isolated in 1995 from four Districts in Pakistan, Rawalpindi, Islamabad, Kharian and Jehlem, were catalogued and examined. Chromosomal DNA from each isolate was digested with *Xba*I restriction endonuclease and subjected to pulsed-field gel electrophoresis. Three clonal variants comprising of 17–19 DNA fragments were identified. Antibiotic susceptibility testing identified that 37 of the *S. typhi* were resistant to chloramphenicol, trimethoprim and ampicillin. These antibiotic resistance genes were found to be located on one of four plasmids belonging to incompatibility group IncHI1 and ranging in size from 150–175 Kb. The genes responsible for this resistance in each case were the chloramphenicol acetyltransferase (CAT) type I, the dihydrofolate reductase (DHFR) type VII and the β-lactamase TEM-1 respectively.

INTRODUCTION

Typhoid fever continues to represent a major public health challenge with an estimated 16·6 million cases and 600000 deaths annually [1]. It is a disease predominantly associated with the developing world and, indeed, Indonesia and Papua New Guinea both report $>$ 1000 cases per 100000 inhabitants [2]. While *Salmonella typhi*, the causative organism of typhoid fever, may present at all ages in endemic areas such as Pakistan, the highest incidence of infection is in pre-school and school-aged children [3]. Since 1948 chloramphenicol has been the drug of choice to eradicate this infection. In many parts of the world, however, resistance has emerged to this and other clinically useful antimicrobial agents utilized, particularly ampicillin and trimethoprim [4–9]. In Pakistan, chloramphenicol resistance in *S. typhi* was first identified in 1987 with multi-drug resistant (MDR) strains, resistant to each of the first line antimicrobials being reported shortly afterwards [9]. A number of studies have reported that antimicrobial resistance in *S. typhi* is plasmid-encoded [6, 9, 10]. This has been shown to be the case in Pakistan [9]. Furthermore, the MDR plasmids in *S. typhi* frequently belong to the IncHI incompatibility group [6].

In contrast to the view that *S. typhi* represents a single clone, a number of studies have recently demonstrated the existence of strain variation amongst *S. typhi* in endemic regions [11–14]. Liu and Sanderson have shown that major genomic rearrangements have occurred within the *S. typhi* genome providing molecular evidence for this diversity [15].

We have recently analysed MDR *S. typhi* isolated in Vellore, India [16]. In the current study we have extended our investigations of MDR *S. typhi* to Pakistan. The genes associated with antimicrobial

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resistance are identified and each MDR isolate is examined for strain variation.

METHODS

S. typhi isolates

A total of 39 *S. typhi* strains were included in the study of which 25 were isolated from the Rawalpindi District, 5 were isolated from the Islamabad District, 8 were isolated from the Kharian District and 1 was isolated from the Jehlem District. All of the *S. typhi* were obtained in 1995.

Antimicrobial sensitivity testing

The minimum inhibitory concentrations (MIC) of chloramphenicol (Boehringer Mannheim, East Sussex, UK), amoxycillin and amoxycillin plus clavulanic acid (both from SmithKline Beecham Pharmaceuticals, Surrey, UK), trimethoprim and ceftazidime (both from GlaxoWellcome, Crewe, UK), tetracycline (Lederle Laboratories, Gosport, UK), streptomycin (Sigma Chemical Co. Ltd, Poole), cefotaxime (Roussel Laboratories Ltd, Uxbridge, UK) and ciprofloxacin (Bayer, Newbury, UK) were determined for each isolate as recommended in the British Society for Antimicrobial Chemotherapy (BSAC) guidelines [17]. All plates were incubated at 37 °C for 18 h and the MIC was defined as the lowest concentration on which there was no visible growth. The control strains employed were *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 10418.

Pulsed-field gel electrophoresis

Genomic DNA was prepared as described by Butler and colleagues [18]. DNA, restricted with *Xba*I (TCTAGA) was separated on a 1% agarose gel by PFGE employing a CHEF DR II system (Bio-Rad) at 14 °C for 22 h at 200 V with a pulse time from 1–60 sec.

Conjugation studies

All the isolates were tested for the ability to transfer their resistance determinants by the method of Amyes and Gould [19]. The conjugative experiments employed the rifampicin-resistant *E. coli* K-12 J62-2 as the recipient and were conducted at 28 °C.

Plasmid analysis

Plasmid DNA was extracted from each transconjugant by a modification of the method described by Takahashi and Nagano [20]. The plasmid DNA was digested with 10 units *Eco*RI restriction endonuclease according to the manufacturer's instructions (Gibco BRL, UK) for 2 h at 37 °C. Resultant DNA fragments were analysed by electrophoresis on a 0·8% agarose gel at 60 V for 17 h, stained with ethidium bromide (50 mg/l), visualized and photographed under ultraviolet light.

DNA hybridizations

Dot blots of the plasmid DNA from the transconjugants were prepared on transfer membrane (Hybond N+) following the manufacturer's instructions (Amersham International plc, UK). Control plasmids were the DHFR type Ia, type Ib, type V and type VII genes as detailed by Adrian and colleagues [21], the CAT variants types I, II and III (all provided by Dr Kevin Towner, University of Nottingham, UK) and plasmids R1 [22] and R1010 [23] encoding TEM-1 and SHV-1 genes respectively. Oligonucleotide probes for distinguishing between different DHFR genes were employed [21]. A 30 base oligonucleotide probe as follows 5′-TATGTGTAGAAACTGCCGGAAATCGTCGTG specific for the CAT I gene was utilized. Hybridizations were carried out with an ECL 3′ oligo labelling and detection kit according to the manufacturer's recommendations (Amersham International plc, UK). Stringency washes were performed as described previously [24].

β-Lactamase analysis

β-Lactamases were extracted from the transconjugants and investigated by isoelectric focusing (IEF) as described previously [25]. The identity of the plasmid encoded β-lactamases was confirmed by screening transconjugants by PCR employing the TEM primers 5′-TGGGTGCACGAGTGGGTTAC-3′ and 5′-TTATCCGCCTCCATCCAGTC-3′. The

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final volume in the tubes for amplification was 100 μl and consisted of 10 × Taq PCR buffer, 2·5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, 0·1 μg DNA and 2 units of Taq polymerase. The amplification reaction, conducted in a Techne thermocycler, consisted of 1 cycle at 94 °C for 5 min followed 30 cycles at 94 °C for 2 min, 57 °C for 1 min and 72 °C for 2 min. A final extension step ran at 72 °C for 10 min. Controls consisted of plasmid DNA encoding other β-lactamases.

PCR amplification for incompatibility testing

A 365 bp region of the RepHI1A replicon was amplified from plasmid DNA by use of a Taq polymerase kit obtained from Gibco BRL, UK. The final volume in the tubes for amplification was 100 μl and consisted of 10 × Taq PCR buffer, 2·5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer (5′-GGTCC-AACCCATTGCTTTAC and 5′-CACGGAAAGAA-ATCACAAC as recommended by Gabant [26] and purchased from Oswel DNA Service, University of Southampton), 0·1 μg DNA and 2 units of Taq polymerase. The amplification reaction, conducted in a Techne thermocycler, consisted of 30 cycles at 94 °C for 0·5 min, 55 °C for 0·5 min and 72 °C for 0·5 min. A final extension step ran at 72 °C for 10 min. Plasmids of incompatibility groups P and W were included in the PCR experiments as negative controls while plasmid R27, incompatibility group IncHI1, was included as a positive control.

RESULTS

Antimicrobial sensitivity testing

Of the 39 *S. typhi* isolates, 37 were resistant to chloramphenicol (MIC > 128 mg/l), amoxycillin (MIC > 128 mg/l) and trimethoprim (MIC > 128 mg/l). Two of the isolates were sensitive to each of these agents. All of the *S. typhi* isolates were sensitive to the extended-spectrum cephalosporin, ceftazidime and the fluoroquinolone, ciprofloxacin (Table 1).

Pulsed-field gel electrophoresis

Digestion of chromosomal DNA with the restriction endonuclease *Xba*I produced clearly resolvable restriction endonuclease analysis (REA) patterns after PFGE. Three very similar REA patterns were identified amongst the 39 *S. typhi* isolates consisting of 17–19 DNA fragments (Fig. 1, Table 1). pattern A was by far the most prevalent PFGE profile; 29 of the *S. typhi* strains exhibited this pattern which consisted of 18 DNA fragments. Furthermore, this pattern was the most widely distributed and was identified in *S. typhi* strains isolated from each of the four different districts. Pattern B consisted of 19 DNA bands and was identified in 8 of the *S. typhi* isolates. This pattern was identical to pattern A except for a single additional DNA fragment. *S. typhi* of this PFGE profile were isolated from both Rawalpindi and Islamabad districts. Finally, pattern C was represented by the two antibiotic-sensitive *S. typhi* which had been isolated in Rawalpindi District. The 17 bands in this PFGE profile were identical to 17 of the DNA bands identified in patterns A and B. The relatedness of the different PFGE profiles was scored by the coefficient of similarity where an F value of 1·0 indicates that two isolates have identical PFGE patterns. The following F values were calculated: patterns A and B = 0·93; patterns A and C = 0·95; patterns B and C = 0·92. On the basis of the determined F values, patterns A, B and C were classified as clonal variants of each other.

Transfer of antimicrobial resistance determinants and antimicrobial sensitivity testing

Amongst the 39 *S. typhi* isolates, 37 were able to transfer amoxycillin resistance into *E. coli* J62-2 at 28 °C (Table 1). Each of the 37 transconjugants were found to confer resistance to chloramphenicol (MIC > 128 mg/l) amoxycillin (MIC > 128 mg/l) and trimethoprim (MIC > 128 mg/l). In addition, each of these plasmids mediated resistance to tetracycline (MIC > 128 mg/ml) and streptomcyin (MIC 64 mg/l). As before, no resistance was identified to either ceftazidime or ciprofloxacin (Table 1).

Plasmid analysis

On the basis of identical restriction digest patterns, each plasmid was allocated to one of four groups (Fig. 2, Table 1). Group a was the largest, comprising 28 plasmids, each calculated as being 160 kb in size. Group b contained 7 plasmids which were calculated as 150 kb. Both group c and group d each contained only one plasmid, sized at 170 kb and 175 kb respectively. All the groups a and d plasmids originated in *S. typhi* isolates with a PFGE pattern A except the

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Table 1. *Antimicrobial sensitivity testing of the* S. typhi *isolates and transconjugants and the allocation of each to PFGE pattern groups and plasmid groups*

S. typhi no.	*S. Typhi* MIC			Transconjugant antibiogram	PFGE group	Plasmid group
	Cm	Tp	Ap			
ST 55	4	0·0625	1		C	
ST 56	> 128	> 128	> 128	CmTpApSmTc	B	c
ST 57	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 58	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 59	4	0·0625	1		C	
ST 60	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 61	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 62	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 63	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 64	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 65	> 128	> 128	> 128	CmTpApSmTc	A	d
ST 66	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 67	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 68	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 69	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 70	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 71	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 72	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 73	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 74	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 75	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 76	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 77	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 78	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 79	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 80	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 81	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 82	> 128	> 128	> 128	CmTpApSmTc	B	a
ST 83	> 128	> 128	> 128	CmTpApSmTc	B	b
ST 84	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 85	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 86	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 87	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 88	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 89	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 90	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 91	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 92	> 128	> 128	> 128	CmTpApSmTc	A	a
ST 93	> 128	> 128	> 128	CmTpApSmTc	A	a

plasmid isolated from ST 82 (PFGE pattern B). Groups b and c plasmids originated in *S. typhi* isolates with a PFGE pattern B.

Gene detection

Each plasmid, regardless of the endonuclease restriction pattern, produced a positive hybridization result with the *dhfr* VII and the *cat* 1 oligonucleotide probes. The β-lactamases responsible for mediating ampicillin resistance were examined by isoelectric focusing. Each β-lactamase was found to co-focus with the TEM-1 control at a pI value of 5·4. The molecular identity of the β-lactamase genes were confirmed by PCR with universal TEM primers. As none of these enzymes conferred extended-spectrum activity, they were allocated to the TEM-1 group of enzymes.

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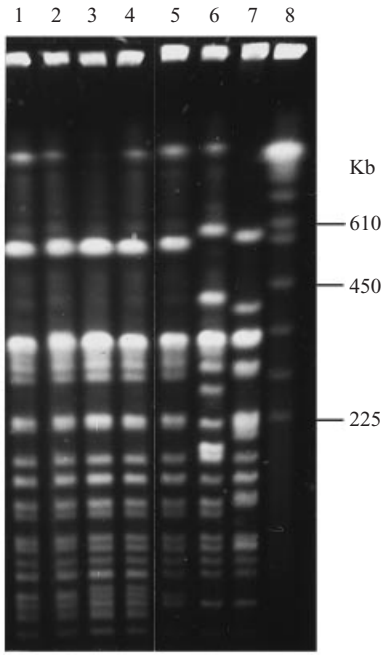

Fig. 1. PFGE generated endonuclease restriction fragment length polymorphisms of three clonal variants amongst the *S. typhi*. Lanes 1, 2, pattern A; lanes 3, 4, pattern B; lane 5, pattern C; lanes 6, 7, unrelated *S. typhi* for comparison; lane 8, *S. cerevisiae* standard.

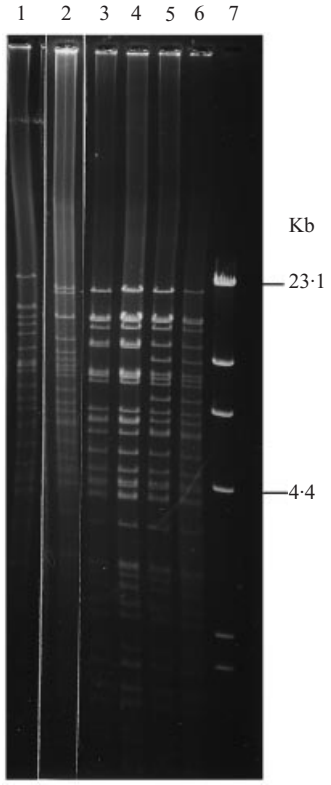

Fig. 2. Endonuclease restriction patterns of representative group plasmids in the transconjugants. Lane 1, group c; lane 2, group d; lanes 3, 4, group b; lanes 5, 6, group a; lane 7, λ pre-cut with *Hin*dIII.

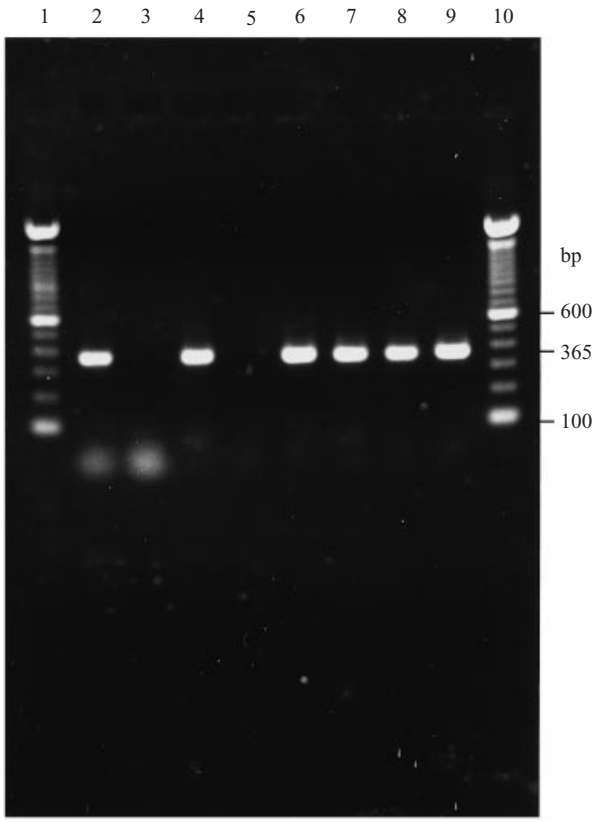

Fig. 3. PCR amplified 365 bp region of the RepHI1A replicon in each of the representative group plasmids in the transconjugants. Lane 1, 100 bp DNA ladder; lane 2, plasmid R27; lane 3, plasmid Inc P; lane 4, plasmid R27; lane 5, plasmid Inc W; lane 6, plasmid group a; lane 7, plasmid group b; lane 8, plasmid group c; lane 9, plasmid group d; lane 10, 100 bp DNA ladder.

Incompatibility testing by PCR amplification

Each transconjugant contained a plasmid belonging to incompatibility group IncHI1. No amplification of the negative controls occurred (Fig. 3).

DISCUSSION

S. typhi continue to cause significant morbidity and mortality. Infection with this organism was complicated in many parts of the world particularly in the late 1980s and early 1990s by the emergence of strains resistant to chloramphenicol, trimethoprim-sulphamethoxazole and ampicillin [3, 4, 9]. Unfortunately little data has been available on the mechanisms and genetics of resistance in *S. typhi* in contrast to other organisms. In this study multi-drug resistant isolates of *S. typhi* from different regions of Pakistan have been

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characterized in order to determine how resistance may have developed and spread. In this study, of the 39 *S. typhi* isolated in Pakistan, 37 conferred resistance to each of these three first-line antimicrobial agents suggesting that in 1995 these MDR isolates were still prevalent. The MIC of each of these drugs was > 128 mg/l. Only a few other studies have employed the more accurate MIC method rather than disk sensitivity in screening *S. typhi* isolates [4, 6].

Amongst the 39 transconjugants, restriction endonuclease fingerprinting identified four different plasmid types with one plasmid type, group a predominating. The few studies that have investigated the carriage of plasmids in *S. typhi* in any detail have reported the presence of single large plasmids amongst MDR *S. typhi* [5, 6, 9] suggesting the presence of epidemic plasmids amongst these strains. The plasmids in the current study were found to belong to the incompatibility complex IncHI1. While previous studies have reported plasmids in *S. typhi* belonging to Inc groups I, B and N, the most recent data suggest that MDR plasmids in *S. typhi* are frequently of the IncHI group [6]. Furthermore, we have investigated an epidemic plasmid from MDR *S. typhi* in India. This plasmid not only belongs to the IncHI incompatibility complex but exhibits a very similar restriction endonuclease pattern to the predominant plasmid in the current study [16]. To date, detailed analysis of these plasmids has not been performed but it has been suggested that MDR plasmids in *S. typhi* may originate in commensal flora known to harbour large self-transmissible plasmids encoding antibiotic resistance genes [27].

The spread of antibiotic resistance genes is usually associated with either the clonal spread of an epidemic strain or through the independent acquisition of the resistance genes on plasmids, transposons or integrons. In the current study, three clonal variants of an epidemic strain were detected, 37 of which were found to harbour a large transmissible MDR plasmid encoding a range of antibiotic resistance genes. This information would suggest that a specific strain of *S. typhi* was prevalent in 1995 which was able to host a specific plasmid type. It is of interest that the PFGE profile (pattern C) of the two sensitive isolates lacked two small DNA fragments, present in the prevalent MDR isolates represented by pattern A. It may be speculated that this lack of banding may reflect the absence of antibiotic resistance plasmids in these isolates. These findings are in contrast to those in India, where the antibiotic-sensitive *S. typhi* population exhibited a distinct PFGE profile from the antibiotic-resistant population [16].

Until now, however, there have been few investigations to analyse the genetic and biochemical basis of the spread and development of antimicrobial resistance in *S. typhi*. Such detailed investigations are crucial in order to understand and control the dissemination of the antibiotic resistance genes. The identification of TEM-1 as the determinant of β-lactam resistance amongst the *S. typhi* was perhaps not surprising as this β-lactamase accounts for approx. 80% of all plasmid-mediated β-lactamases amongst clinical isolates [28]. This particular β-lactamase is extremely efficient in binding to and hydrolysing both amoxycillin and ampicillin, two of the most widely utilized agents throughout the world. TEM-1 is recognized as being the progenitor of many extended-spectrum β-lactamases and inhibitor-resistant β-lactamases which may be selected for in the presence of cephalosporin agents and β-lactam/β-lactamase inhibitor combinations respectively [28]. Although no resistance to either of these groups of antimicrobials was identified amongst the *S. typhi* in this study, it would be prudent to control their administration in order to prevent such β-lactamases from emerging.

The gene responsible for trimethoprim resistance amongst the *S. typhi* was the type VII DHFR. As with the identification of TEM-1, this finding was not surprising as this enzyme has been reported to be geographically widespread [29]. It is of interest that in a recent study examining the distribution of trimethoprim resistance genes amongst Enterobacteriaceae isolated from healthy volunteers in South Africa, that the type VII DHFR was predominantly located on the chromosome and not a plasmid [30].

In this study, we have reported for the first time, the genes associated with plasmid-mediated antibiotic resistance in MDR strains of *S. typhi* in Pakistan. MDR plasmids in Pakistan appear to be associated with one of three clonal variants of an *S. typhi* strain. These findings suggests that the pattern and acquisition of resistance determinants in *S. typhi* in Pakistan is the same as in India.

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