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Original Article

Plasmid-Encoded Multidrug Resistance of *Salmonella typhi* and some Enteric Bacteria in and around Kolkata, India: A Preliminary Study

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Abstract:

The present study investigates the occurrence of R-plasmid in *Salmonella typhi* isolates from enteric fever cases in and around Kolkata (1991-2001), India following in vitro conjugation experiments, isolation of plasmid DNAs and agarose gel electrophoretic analysis. The multidrug resistant (MDR) *S. typhi* strains contained a transferable plasmid conferring resistance to ampicillin, chloramphenicol, cotrimoxazole and tetracycline. The plasmid encoding ACCoT-resistance of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris* were conjugative and co-migrated with the plasmid of MDR *S. typhi* isolates. The antibiotic sensitive *S. typhi* isolates did not contain any plasmid. Thus the findings of the present study confirmed the instability of the R-plasmid in *S. typhi*, and that the antibiotic sensitive *S. typhi* strains acquired the R-plasmid from other enteric bacteria such as *E. coli*, *K. pneumoniae* and *P. vulgaris* to undergo a suitable adaptation for survival in the changing antibiotic environment.
Key Words: *Salmonella typhi*, multidrug resistance, conjugative R-plasmid

Introduction

Salmonella typhi is noteworthy in the etiology of outbreaks and sporadic cases of typhoid fever, which remains as an important public health problem, causing 16 million cases of the disease and about 600,000 deaths, annually, all over the world.(1) There has been increasing concern about the prevalence of multidrug resistant (MDR) *S. typhi* strains in developing countries. Several reports indicated MDR *S. typhi* with plasmid-mediated resistance to conventional antityphoid antibiotics: chloramphenicol (C), ampicillin (A) and cotrimoxazole (Co) thriving in different parts of the world including India.(2-11) In the present study, in order to search the probable host bacteria of a plasmid conferring resistance to A, C, Co and tetracycline (T) among outbreak causing as well as sporadic isolates of *S. typhi* in and around Kolkata, India, we investigated the occurrence of R-plasmids among MDR isolates of *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella pneumoniae* from different clinical cases.

Materials and Methods

Bacterial Strains

Outbreak causing as well as the sporadic MDR blood culture isolates of *S. typhi* (resistance pattern ACCoT) obtained in between 1991-2001, and the other enteric bacteria (*E. coli*, *K. pneumoniae* and *P. vulgaris* from urinary tract infection cases) that had the common resistance pattern ACCoT during 1995-2001 were used to study the transferability of their multidrug resistance. The MDR *S. typhi* isolates showed either sensitivity or resistance to nalidixic acid (Nx). The antibiotic sensitive *S. typhi* and the *E. coli* C600 (F⁻Nx^R) strains were also included in the study, as the recipient strains. The *S. typhi* recipient strain was obtained by culturing blood from enteric fever patient attending the Calcutta School of Tropical Medicine for treatment. *E. coli* V517 strain was used as the molecular marker for plasmid.

In Vitro Transferability of Antibiotic Resistance

Transfer of antibiotic resistance of the MDR bacterial strains (having the common resistance pattern ACCoT): *S. typhi* (n=70), *E. coli* (n=42), *K. pneumoniae* (n=13) and *P. vulgaris* (n=6) was carried out by conjugation experiments following the standard protocols (12,13), with slight modification described elsewhere.(14) The minimum inhibitory concentrations (MICs) for antibiotics against which resistance was transferred from donors to the recipients were checked by agar dilution method.(15)

Isolation of Plasmid DNA and Agarose Gel Electrophoresis

The MDR isolates of *S. typhi* (n=70), *E. coli* (n=42), *K. pneumoniae* (n=13) and *P. vulgaris* (n=6) having the common resistance pattern ACCoT, the corresponding transconjugant strains acquiring ACCoT-resistance, and the antibiotic sensitive *S. typhi* isolates (n=20) were subjected for plasmid DNA isolation following the protocol of Birnboim and Doly (16), and Kado and Liu (17), with some modifications. According to the protocol of Birnboim and Doly, from

10 ml of bacterial culture (in nutrient broth, Hi-Media, India) plasmid DNAs were isolated using 0.48 ml, 1 ml and 0.8 ml of solution I, II and III, respectively. After the addition of solution III, the lysate was kept in ice for 30 minutes and centrifuged for 15 minutes. Phenol-chloroform (1:1) treatment was followed with the clear supernatant, plasmid DNA was precipitated with equal volume of chilled isopropyl alcohol, and DNA pellet was dissolved in 100 µl of TE buffer. In case of Kado and Liu method, we used chilled isopropyl alcohol to precipitate the plasmid DNA instead of diethyl ether.

Agarose gel electrophoresis of the isolated plasmid DNAs was carried out in tris-borate buffer system (18), using 0.8% agarose, for 4 h at 50v. The gel was stained with ethidium bromide and results were documented in gel-doc system. Electrophoretic separation of plasmid by molecular weight and subsequent size estimations were accomplished using reference strain of *E. coli* V517.

Results

Transfer of resistance from *S. typhi* to *E. coli* C600

The MDR *S. typhi* strains showing the resistance pattern ACCoT or ACCoTNx transferred ACCoT-resistance to *E. coli* C600 recipient strain, and thus the recipient strains acquired the resistance traits for A, C, Co and T. The transfer frequencies among the epidemic causing *S. typhi* strains, namely BS13, AS12, M54 obtained respectively from Bagnan, Asansol and Khardah in the year 1991, were 0.83×10^{-5} , 0.73×10^{-5} , 0.78×10^{-5} , respectively (Table 1). MDR *S. typhi* strains, viz. B2/92, isolated in the year 1992, also transferred ACCoT-resistance with a frequency of 0.89×10^{-5} . In the secondary transfer studies, the *E. coli* C600 transconjugants obtained from the primary conjugation experiments were used as the donors, and the antibiotic sensitive *S. typhi* was the recipient strain. Here also, the transfer of ACCoT-resistance was noticed (Table 1).

Table 1: Results of transfer experiments and minimum inhibitory concentrations (MICs) of antibiotics for donors and transconjugants

Set No.	Donor strain (Resistance Pattern)	Recipient strain (Resistance Pattern)	Transconjugant (Resistance Pattern)	Conjugation frequency	MICs (m g/ml) of Donor and Transconjugant
I	<i>S. typhi</i> BS13 (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pBS13] (ACCoTNx)	0.83×10^{-5}	A (5000) C (5000) Co (1200) T (500)
	<i>E. coli</i> C600 [pBS13] (ACCoTNx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pBS13] (ACCoT)	0.68×10^{-7}	A (5000) C (5000) Co (1200) T (500)
II	<i>S. typhi</i> AS12 (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pAS12] (ACCoTNx)	0.73×10^{-5}	A (5000) C (5000) Co (1200) T (500)
	<i>E. coli</i> C600 [pAS12] (ACCoTNx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pAS12] (ACCoT)	0.12×10^{-6}	A (5000) C (5000) Co (1200) T (500)
III	<i>S. typhi</i> M54 (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pM54] (ACCoTNx)	0.78×10^{-5}	A (2000) C (5000) Co (1200) T (800)
	<i>E. coli</i> C600 [pM54] (ACCoTNx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pM54] (ACCoT)	0.38×10^{-7}	A (2000) C (5000) Co (1200) T (800)
IV	<i>S. typhi</i> B2/92 (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pB2/92] (ACCoTNx)	0.89×10^{-5}	A (2000) C (2500) Co (500) T (450)
	<i>E. coli</i> C600 [pB2/92] (ACCoTNx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pB2/92] (ACCoT)	0.62×10^{-7}	A (2000) C (2500) Co (500) T (450)
V	<i>S. typhi</i> D1/01 (ACCoTNx)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pD1/01] (ACCoTNx)	0.39×10^{-5}	A (1800) C (1500) Co (250) T (250)
	<i>E. coli</i> C600 [pD1/01] (ACCoTNx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pD1/01] (ACCoT)	0.48×10^{-7}	A (1800) C (1500) Co (250) T (250)

A=ampicillin, C=chloramphenicol, Co=cotrimoxazole, T=tetracycline, Nx=nalidixic acid, p=plasmid

In another experiment, the *S. typhi* strain D1/01, having the resistance pattern of ACCoTNx, transferred the ACCoT-resistance to the antibiotic sensitive *S. typhi* strain through *E. coli* C600, indicating the non-transferability of Nx-resistance from D1/01 strain (Table 1).

Transfer of Resistance from *E. coli*, *K. pneumoniae* and *P. vulgaris* to *S. typhi*

In this study, the MDR *E. coli*, *K. pneumoniae* and *P. vulgaris* strains transferred ACCoT-resistance to the recipient strains (Table 2).

Table 2: Transfer of antibiotic resistance from donor to recipient strains and transfer frequencies

Set No.	Donor strain (Resistance Pattern)	Recipient strain (Resistance Pattern)	Transconjugant (Resistance Pattern)	Conjugation frequency
I	<i>E. coli</i> IEC3 (ACCoTNxCp)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pEC3] (ACCoT)	0.39×10^{-7}
	<i>S. typhi</i> B72 [pEC3] (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pEC3] (ACCoT Nx)	0.98×10^{-5}
II	<i>K. pneumoniae</i> K1 (ACCoT)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pK1] (ACCoT)	0.25×10^{-7}
	<i>S. typhi</i> B72 [pK1] (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pK1] (ACCoT Nx)	0.92×10^{-6}
III	<i>P. vulgaris</i> Prv2 (ACCoT)	<i>E. coli</i> C600 (Nx)	<i>E. coli</i> C600 [pPrv2] (ACCoT Nx)	0.98×10^{-6}
	<i>E. coli</i> C600 [pPrv2] (ACCoT Nx)	<i>S. typhi</i> B72 (Sensitive)	<i>S. typhi</i> B72 [pPrv2] (ACCoT)	0.12×10^{-7}

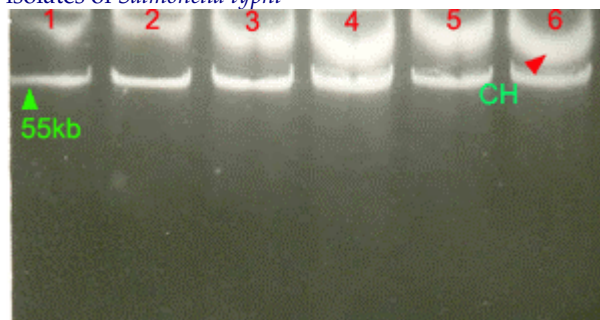
A=ampicillin, C=chloramphenicol, Co=cotrimoxazole, T=tetracycline, Nx=nalidixic acid, Cp=ciprofloxacin

The MDR *E. coli* showing resistance to A, C, Co, T, Nx, and ciprofloxacin (Cp) and *K. pneumoniae* (resistant to ACCoT) transferred ACCoT-resistance to the antibiotic sensitive *S. typhi*; Nx Cp-resistance was not transferred, and the transfer frequencies were 0.39×10^{-7} and 0.25×10^{-7} , respectively. In the secondary transfer studies, all types of transconjugants obtained from the primary conjugation studies were used as the donors that transferred ACCoT-resistance to *E. coli* C600 with transfer frequencies 0.98×10^{-5} and 0.92×10^{-6} , respectively. The donor *P. vulgaris* strain Prv2 transferred the complete resistance pattern of ACCoT to the antibiotic sensitive *S. typhi* strain through the primary recipient *E. coli* C600 with transfer frequencies 0.98×10^{-6} and 0.12×10^{-7} , respectively.

Plasmid profile

The MDR *S. typhi* isolates (resistance pattern ACCoT) obtained during 1991 enteric fever outbreak in several parts of West Bengal were screened for the presence of plasmid. The all *S. typhi* strains from the three different epidemic zones of West Bengal contained plasmids, which co-migrated with each other. Fig. 1 shows the plasmids of three different *S. typhi* strains BS13, AS12, and M54 collected from Bagnan, Asansol and Khardah, respectively.

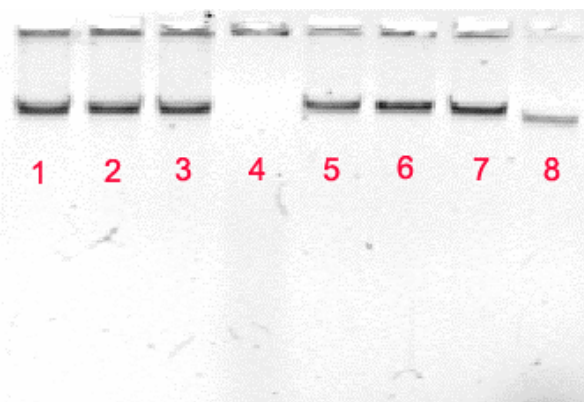
Figure 1: Agarose gel electrophoresis of plasmid DNAs from sporadic isolates and outbreak causing isolates of *Salmonella typhi*



Lane 1: *S. typhi* strain AS12 (ACCoT) of 1991; Lane 2: *S. typhi* strain BS13 (ACCoT) of 1991; Lane 3: *S. typhi* strain M54 (ACCoT) of 1991; Lane 4: *S. typhi* strain B2/92 (ACCoT) of 1992; Lane 5: *E. coli* C600 transconjugant (pB2/92); Lane 6: *S. typhi* strain D1/01 (NxACCoT) of the year 2000. CH, chromosome.

Recurrence of same resistance pattern (ACCoT) was noticed in *S. typhi* strains during 1992 and 2000 too. These strains contained plasmids, which co-migrated with plasmid DNA obtained from *S. typhi* isolates of 1991.

Figure 2: Agarose gel electrophoresis of the plasmid DNAs isolated from *Salmonella typhi* and the transconjugants



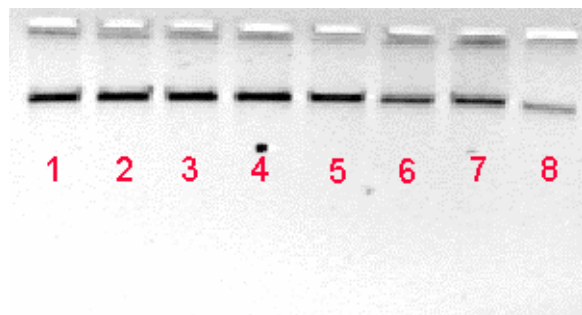
Lane 1: *S. typhi* (ACCoT) of 1991 (BS13); Lane 2: *E. coli* C600 primary transconjugant (pSTBS13); Lane 3: *S. typhi* B72 secondary transconjugant (pSTBS13); Lane 4: *S. typhi* B72 (sensitive to antibiotics); Lane 5: *S. typhi* (NxACCoT) of 2000 (BS225); Lane 6: *E. coli* C600 primary transconjugant (pSTBS225); Lane 7: *S. typhi* B72 secondary transconjugant (pSTBS225); Lane 8: plasmid size marker of 53.7 kb from *E. coli* V517.

Fig. 2 illustrates that the Plasmid DNAs isolated from the primary and secondary transconjugants co-migrated with the plasmid isolated from their corresponding donor strains, and are about 55 kb. The antibiotic sensitive strains of *S. typhi* did not show any plasmid band in the gel.

In search of the plasmid conferring multi drug resistance to A, C, Co, and T among MDR *S. typhi* isolates, we isolated plasmid DNA from MDR *E. coli*, *K. pneumoniae* and *P. vulgaris*, which showed ACCoT resistance pattern. The strains, *E. coli*, *K. pneumoniae*, and *P. vulgaris* as well as their transconjugants showed plasmid band co-migrated with the plasmid

of approximately 55 kb isolated from MDR *S. typhi* having ACCoT-resistance (Fig. 3).

Figure 3: Agarose gel electrophoresis of plasmid DNAs



Lane 1: *S. typhi* strain BS13 (ACCoT) of 1991; Lane 2: *E. coli* strain EC3 (NxCPACCoT); Lane 3: *S. typhi* B72 transconjugant (pEC3); Lane 4: *K. pneumoniae* strain K1 (ACCoT); Lane 5: *S. typhi* B72 transconjugant (pK1); Lane 6: *P. vulgaris* Prv2 (ACCoT); Lane 7: *S. typhi* B72 transconjugant (pPrv2); Lane 8: plasmid size marker of 53.7 kb from *E. coli* V517.

Discussion

S. typhi is noteworthy in the etiology of outbreaks and sporadic cases of typhoid fever, an endemic disease in India. Presently, enteric fever caused by MDR *S. typhi* continues to be a problem across the country. Such multidrug resistance in *S. typhi* has been reported to be plasmid mediated.(2-10) The plasmid mediated *en bloc* transfer of ACCoT-resistance of *S. typhi* isolates has been reported earlier from different parts of India.(19, 20) Karmakar et al. (11) reported 120 kb plasmid encoding resistance to A, C, T, and streptomycin (S) in *S. typhi* isolates from Kolkata during 1989-1990 enteric fever epidemic. Haldar et al. (21) reported a transferable plasmid carrying resistance genes for A, C, T and S in *S. typhi* isolates from Kolkata in the year 1995. Many other authors (5,9,10) from different parts of the world reported the plasmid-mediated resistance of A, C, Co and T in *S. typhi* isolates and that these plasmids are large (approximately 180 kb) and conjugative, and originated from Southeast Asia.

In the present study, results of the primary and secondary conjugation experiments revealed that resistance to A, C, Co and T of *S. typhi* isolates associated with enteric fever outbreaks in three different regions of West Bengal in 1991 was transferable. When *S. typhi* isolates, obtained in the years 1992 and 2000, having the common resistance pattern (ACCoT) were investigated, these also showed transferability of ACCoT-resistance. The

transconjugants were selected using C, but on antibiotic susceptibility testing we found the transconjugants exhibiting resistance to A, Co, T, in addition to C, with MICs similar to that of their corresponding donor strains (Table 1). This finding prompted us for an investigation of R-plasmid contained in them. Agarose gel electrophoretic analysis revealed the presence of a single plasmid of approximately 55 kb among the isolates associated with the 1991 enteric fever outbreak. The corresponding transconjugants also contained the similar plasmid. The same plasmid patterns of MDR isolates from three outbreak regions suggest the wide spread occurrence of plasmid mediating ACCoT-resistance of outbreak causing *S. typhi* in different parts of the state West Bengal in 1991. MDR *S. typhi* with similar antibiogram, which had been isolated in different years (1992 and 2000) of the present study, contained the same plasmid. This phenomenon indicated the existence of a plasmid carrying ACCoT-resistance in the bacterial population in this region.

In our studies, there was co-existence of antibiotic-sensitive and MDR strains of *S. typhi* (22), but unlike MDR strains the sensitive strains did not contain any plasmid. The presence of conjugative R-plasmid in MDR *S. typhi* and absence of any plasmid in the sensitive strain has been reported earlier by researchers from Kolkata, India.(11) R-factors originally found in community isolated *E. coli* strains, in Mexico, transferred by *in vitro* conjugation experiments to *S. typhi*, in which the R-factor found unstable after 100 generations in liquid culture.(23) Thus, in *S. typhi* the R-plasmid is an unstable plasmid that may appear or disappear at any time resulting in the emergence of drug resistant or drug sensitive isolates. The selection exerted by antibiotic treatment of enteric fever may be the cause of acquisition of R-plasmid.(24) Through the acquisition of a plasmid conferring multidrug resistance, the strain undergoes the necessary and appropriate adaptation for survival in the changing antibiotic environment. Thus, it appears that the already existing sensitive strain, by the acquisition of a R-plasmid, has emerged as a resistant strain within the *S. typhi* bacterial population in and around Kolkata, and has been able to adapt the challenge of antibiotics as they are introduced into clinical medicine. But what might be the reservoir host of the R-plasmid from which the sensitive *S. typhi* strain could acquire it (R-plasmid) during adverse situation?

The prevalence of *S. typhi* harbouring the plasmid encoding ACCoT-resistance in Kolkata has not yet been studied. We suspected that the resistance plasmid might have been transferred from other

enteric bacteria in human, because human carriers or patients are the source of *S. typhi* infection. We, therefore, were interested in searching for the probable origin of the R-plasmid conferring ACCoT-resistance in *S. typhi*. We explained, performing *in vitro* conjugation experiments, the possibility of acquisition of R-factor from various intestinal flora like *E. coli*, *P. vulgaris*, *K. pneumoniae* isolated from different clinical cases. *In vitro* and *in vivo* acquisitions of R-plasmids from common bacterial flora of intestine by *S. typhi* have been reported earlier. The phenomenon of acquisition of R-plasmid can be explained based on the facts described by Datta et al. (24), who before antibiotic therapy isolated plasmid-less antibiotic sensitive *S. typhi* strain from enteric fever case. The strain, however, acquired, after antibiotic therapy, the ACCo-resistance in the bowel of a patient from *Klebsiella aerogenes*, which was originally resistant to A, C, Co. Both *S. typhi* and *K. aerogenes* were found to contain similar plasmids. Ridley (25) also reported about the acquisition of R-plasmids by *S. typhi*, in the bowel of patient with enteric fever. Schwalbe et al (26), by studying the plasmid profile, demonstrated the transfer of drug resistance from the intestinal flora, viz, *E. coli* and *K. pneumoniae* to *S. typhi*. *In vitro* intergenetic conjugation experiments demonstrated transfer of drug resistance between intestinal MDR *E. coli* and *S. typhi* strains.(27) Thus, one or more *S. typhi* clones in the patients' intestine acquired resistance plasmids by transfer from the resistant commensal bacteria, and the resistant clone replaced the original sensitive *S. typhi* as a result of the antibacterial therapy. Two factors play role in the fact of acquisition of R-plasmids: the capacity of the antibiotic resistance transposon to spread between plasmids (28), and the selection exerted antibiotic treatment of enteric fever.(24, 29)

In this study, it was considered that the commensal bacteria like *E. coli*, *P. vulgaris*, *K. pneumoniae* might be the source of dissemination of the plasmid conferring resistance to A, C, Co and T in *S. typhi*, based on the fact that most of these isolates (*E. coli*, *P. vulgaris*, *K. pneumoniae*) in between 1995 and 2001 showed a common resistance pattern (ACCoT), and that the resistance pattern was transferred to the drug sensitive *S. typhi*, obtained in between 1992 and 2001 (22), with the same degree of resistance. In addition, plasmids contained in the *E. coli*, *P. vulgaris*, *K. pneumoniae* strains as well as in their corresponding transconjugants comigrated with that contained in *S. typhi* isolates having ACCoT-resistance pattern. Thus the present findings suggest that the MDR *S. typhi* may arise from sensitive isolates by acquisition of multidrug resistance plasmid from antibiotic-resistant enteric bacteria. However, additional investigations

are needed to ascertain the role of plasmid in the mediation of multidrug resistance among enteric bacteria.

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