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Multidrug Resistant Gene(S) Harboring On A 20 Kb Plasmid In *Salmonella typhi* That Causes Typhoid-Enteric Fever

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Abstract: Ten multidrug resistant *Salmonella typhi* were isolated from the blood and stool samples of clinically suspected patients suffering from typhoid fever. To see whether the drug resistance phenomenon was plasmid mediated, one of these resistant isolates was used for the plasmid analysis and a single 20 kb plasmid was found. This 20 kb plasmid was transferred to a sensitive *E. coli* LE 392 and after the plasmid transfer experiment *E. coli* LE 392 acquired resistance against those previously used antibiotics. Plasmid analysis of the transformed *E. coli* LE 392 further exhibited that it harbored a single 20 kb plasmid corresponding to that of the wild *S. typhi* which further confirmed that the 20 kb plasmid was carrying the gene(s) responsible for the multidrug resistance in *Salmonella typhi.*

Key words: Plasmid, *Salmonella typhi,* Typhoid, Multidrug resistant

Introduction

Typhoid fever, an acute systemic illness, is a major public health problem in the Indian subcontinent including Bangladesh (Pandey *et al*.,1990; Alam *et al*., 1992). About twelve million cases of typhoid fever occurs annually in the developing countries with more than seven million cases in Asia alone (Edelman and Levine, 1986). Recently this problem has further been complicated due to the large scale emergence of multiple drug resistant strains of *Salmonellae typhi,* causative organism of typhoid fever. Resistance of *Salmonellae typhi* to multiple drugs is not very common (Agarwal *et al*., 1981). Since the first report of chloramphenicol resistance from England (Colquhoun and Weetch, 1950) sporadic cases were reported from all parts of the world and before 1970 it was a rare phenomenon (Anderson and Smith, 1972). In 1972 a year long epidemic of enteric fever occurred in Mexico with multidrug resistant *Salmonella typhi* (Olarte and Galindo, 1973). In 1989 transferable drug resistance in *Salmonellae typhi* was reported from an outbreak of enteric fever in and around Calcutta Metropolis. In that study *Salmonellae typhi* strains resistant to ampicillin, amoxycilin, tetracycline, cotrimoxazole, streptomycin and chloramphenicol were reported (Halder *et al*., 1992). Morshed *et al*. (1986) first time reported about the appearance of multidrug resistant *Salmonellae typhi* in Bangladesh. Mishra *et al.* (1996) reported about isolation of *Salmonellae typhi* in India substantially resistant against ceftriaxone (21%) and cefotaxime (12%). While biomedical scientists are discovering newer and more potent anti-microbial drugs, the pathogenic bacteria with their demonstration to survive, are gaining resistance against them in a curious way. In a sensitive bacterial population, there may be a small number of drug resistant bacteria which develop resistance spontaneously as a result of mutation. More frequently resistance is due to the presence of additional gene (s) as plasmid DNA has been reported (Choudhury, 1988). These plamids carry gene (s) which endow drug resistance phenomenon to the host organism. In this work *Salmonellae typhi* strains resistant to multiple drugs were isolated from blood and stool samples of clinically suspected patients suffering from typhoid fever from the Rajshahi Medical College Hospital and from the local diagnostic centers at the Rajshahi town. In this paper we report on the isolation of a single 20kb plasmid from multidrug resistant *Salmonella typhi*. Evidence is presented that this 20 kb plasmid is carrying the gene(s) responsible for the multi-drug resistance in *Salmonellae typhi*.

Materials and Methods

Bacterial Strains: A total of 50 bacterial samples were isolated from blood and stool samples of clinically suspected patients suffering from typhoid fever. Most of the samples were collected from the pathology laboratory of the Microbiology Department of Rajshahi Medical College Hospital and some were collected from the local diagnostic centers at Rajshahi town. *E. coli* LE 392 strain used in the transformation experiment was supplied by the Department of Biochemistry and Molecular Biology, Yamaguchi University, Japan.

Media and culture conditions: LB broth was used as a complete medium. Nutrient agar was used as a solid medium through out the work. MacConkey agar (Gibco, USA) plates were used for the isolation and identification of the suspected bacteria and the bacteria were cultured at 37°C.

Antibiotic susceptibility testing: Bacterial strains resistant to two first generation cephalosporins were isolated from the fifty collected bacterial samples by disc diffusion method (Baur *et al*., 1966) using cephalexin (30 µg/disc) and cephradine (30 µ/disc). Resistant strains isolated by disc diffusion method were further confirmed for their drug sensitivity by antibiotic spread plate method using 0.6, 1.2, 1.8, 3.0, 3.6, 4.2, 4.8, 6.0, 7.2, 8.4 and 9.6 ml of both the cephalexin (0.25 mg/ml) and cephradine (0.25 mg/ml) solution in media.

Identifying the bacteria: To identify the isolated cephalosporin (cephalexin and cephradine) resistant bacterial strains, different cultural and biochemical test specific for *Salmonellae typhi* were performed according to conventional laboratory procedures (Chcesbrough, 1991).

Susceptibility test against the commonly used drugs: All the identified cephalosporin resistant strains were further tested against nine conventional antibiotics (Ampicillin 10 µg/disc, Amoxycillin 25 µg/disc, Cotrimoxazole 25 µg/disc, Doxycycline 30 µg/disc, Cloxacillin 5 µg/disc, Tetracycline 30 µg/disc, Penicillin G. 10 units/disc, Nalidixic acid 30 µg/disc, chloramphenicol 30 µg/disc for their sensitivity by disc diffusion method.

Plasmid DNA extraction and agarose gel electrophoresis: A Single

colony of the isolated multidrug resistant *Salmonellae typhi* was inoculated into 100 ml LB broth containing 0.25 mg/ml of cephalexin solution in 250 ml comical flask and incubated at 37°C over night with constant shaking. This culture was then subjected for the extraction of plasmid DNA according to Holmes and Quigley (1981). The extracted plasmid DNA was then purified with polyethylene glycol (PEG) according to Sambrook *et al*. (1989). The purified plasmid DNA was then subjected to electrophoresis by using 0.8% agarose according to Meyers *et al*. (1976). Plasmid transfer to a sensitive *E. coli* LE 392: Competent cells were prepared by calcium chloride procedure modified from Cohen *et al*. (1972). An *E. coli* LE 392 strain sensitive to cephalexin, cephradine and chloramphenicol was inoculated in a 20 ml LB broth and grown for 8 hours at 37°C with slow shaking for the experiment. Transformation of the isolated plasmid DNA to the *E. coli* LE 392 was carried out according to Cohen *et al*. (1972).

Extraction of transformed plasmid DNA from E. Coli. LE 392: After the transformation experiment, plasmid DNA was extracted from the transformed *E. Coli* LE 392 according to Holmes and Quigley (1981). The extracted plasmid DNA was purified and subjected to agarose gel electrophoresis.

Results

Isolation of cephalosporin resistant strain: Each of the fifty collected bacterial samples were tested for cephalosporin sensitivity by disc diffusion method using cephalexin (30 μg/disc) and cephradine (30 μg/disc). It was revealed that 12(25%) strains were completely resistant to both drugs, 13(26%) were almost resistant to both drugs, 20(40%) were sensitive to both drugs and the remaining 5 (10%) were sensitive to only a single drug. All the cephalosporin resistant strains obtained through disc diffusion method were again tested for their sensitivity by antibiotic spread plate method using cephalexin and cephradine in different selective concentrations (10, 20, 30, 50 and 120 μg/ml). It was found that all the twenty five strains were resistant up to 30 μg/ml of both cephalexin and cephradine. In the case of cephalexin it was also found that 19 (76%) strains were resistant up to 50 μg/ml, 15 (60%) were up to 100 μg/ml, 9 (36%) were up to 120 μg/ml and in the case of cephradine it was found that 13(52%) were resistant upto 50 μg/ml, 10 (40%) were up to 100 μg/ml and 5 (20%) were upto 120 μg/ml.

Identification of the bacteria: All the twenty-five cephalosporin resistant bacterial strains were subjected to different cultural and biochemical tests specific for *Salmonellae typhi* according to the conventional laboratory procedures (Chcesbrough, 1991). It was observed that out of twenty five cephalosporin resistant strains only 10 (40%) strains were identified as *Salmonellae typhi*.

Susceptibility profile of *Salmonellae typhi* against the commonly used drugs: The isolated ten cephalosporin resistant *Salmonellae typhi* strains were further tested for their sensitivity to nine conventional and commonly used drugs like Ampicilln (10 μg/disc), Amoxycillin (25 μg/disc), Cotrimoxazole (25 mg/disc), Doxycycline (30 μg/disc), Cloxacillin (5 mg/disc), Tetracycline (30 μg/disc), Penicillin G (10 units/Disc), Nalidixic acid (30 μg/disc), Chloramphenicol (30 μg/disc). It was found that all the ten cephalosporin resistant *Salmonellae typhi* strains exhibited resistance against these commonly used drugs. From the overall resistance pattern it was found that all the ten cephalosporin resistant *Salmonellae typhi* were resistant to Ampicillin (90%), Amoxycillin (90%), Cotrimoxazole (90%), Cloxacillin (75%), Tetracycline (80%), Doxycycline (40%), Chloramphenicol (60%), Penicillin G (80%) and Nalidixic acid (20%).

Plasmid analysis: A single colony of S_5 (Sample No. 5) from the ten multidrug resistant isolates of *Salmonellae typhi* were further cultured for the plasmid analysis. Plasmid DNA was extracted by the method of Holmes and Quigley (1981) and the purified with polyethylene glycol (PEG) according to Sambrook *et al*. (1989) and subjected to agarose gel electrophoresis, which revealed that this strain harbored a single plasmid. On the basis of electrophoretical mobility a molecular mass of about 20 kb was calculated (Table 1).

Transformation to the E. coli LE 392: To prove whether the 20 kb plasmid was carrying the gene (s) responsible for the multidrug resistance, the transformation experiment was carried out in a sensitive *E. coli* LE 392. Competent cells were prepared by calcium chloride procedure modified from Cohen *et al*. (1972). Transformation experiment in *E. coli* LE 392 was carried out as described by Cohen *et al*. (1972). After the transformation experiment, when control and experimental plates were compared, bacterial growth was observed on experimental plates containing 30 μg/ml, 50 μg/ml and 70 μg/ml of cephalexin (0.25 mg/l), cephradine (0.25 mg/ml) and chloramphenicol (0.25 mg/ml). But no growth was found on any of the control plates containing the antibiotics in the some concentrations. From the successful transformation of the 20 kb plasmid DNA to the *E. coli.* LE 392 clearly indicated that the 20 kb plasmid was harboring the gene (5) responsible for the multidrug resistance in *Salmonellae typhi*.

Analysis of the transformed plasmid DNA: After transfer of the 20 kb plasmid to the *E. coli* LE 392, the plasmid DNA was isolated again from the transformed *E. coli* LE 392 and subjected to agarose gel electrophoresis. From the electrophoretical mobility a single plasmid of about 20 kb in molecular mass was calculated which corresponds to that of the donor *S. typhi* in molecular mass. The analysis of the transformed plasmid DNA further confirmed that the 20 kb plasmid DNA isolated from *Salmonellae typhi* might contain the gene (s) responsible for the multi drug resistance in *Salmonellae typhi*.

Discussion

Fifty bacterial samples were isolated from blood and stool samples of clinically suspected patients suffering from typhoid fever. Most of the samples were collected from the pathology lab of the Microbiology Department of Rajshahi Medical College Hospital and some were collected from the local diagnostic centers at Rajshahi town.

The fifty collected bacterial samples were subjected to antibiotic susceptibility test with two first generation cephalosporin (cephalexin and cephradine) by disc diffusion method and the cephalosporin resistant strains were isolated. It was found that out of fifty collected bacterial samples twenty-five samples were identified as cephalosporin resistant. For further confirmation these 25 primarily isolated bacterial samples were again tested for their cephalosporin sensitivity by antibiotic spread plate method using were marked. It was found that twelve bacterial strains out of twenty-five can tolerate 100-120 μg/ml of both these antibiotics and out of these twelve bacterial strains five strains can tolerate upto 120 μg/ml of both cephalexin and cephradine. When the morphological studies and biochemical investigation were performed to all these 25 cephalosporin resistant samples, it was found that out of 25 samples only 10 were *Salmonellae typhi*. After the isolation of cephalosporin resistant *Salmonellae typhi* strains all the 10 strains were subjected to susceptibility testing against nine conventional and commonly used drugs in order to isolate multidrug resistant *Salmonellae typhi*. From the result of the resistance pattern of the 10 isolates it was found that the isolates were resistant to Ampicillin (90%) Amoxycillin (90%).

Table 1: Results of various biochemical tests for the identification of the cephalosporin resistant strains of *Salmonella typhi*

Strain No.	Fermentation Tests					MIU medium				KIA medium				
	Lac	Man	Glu	Suc	Ox	Cit	Mot	Ind	Urea	Slope	Butt	H_2S	Gas	Comments
S_1	-	+	+	-	-	-	+	-	-	R	Y	Weak +	-	*S. typhi*
S_2	-	+	-	-	-	+	+	-	-	R	Y	+	+	Not
S_4	-	+	-	-	+	-	+	+	-	Y	R	+	-	Not
S_5	-	+	+	-	-	-	+	-	-	R	Y	+	-	*S. typhi*
S_6	-	+	-	-	+	-	-	+	+	R	Y	+	-	Not
S_9	-	-	-	-	-	-	+	-	+	R	Y	+	-	Not
S_{13}	-	+	+	-	-	-	+	-	-	R	Y	+	-	*S. typhi*
S_{14}	-	+	-	-	+	-	-	+	+	Y	R	-	+	Not
S_{15}	-	+	+	-	-	-	+	-	-	R	Y	+	-	*S. typhi*
S_{17}	-	+	-	-	-	-	+	-	+	R	Y	+	-	Not
S_{18}	-	+	-	-	-	-	+	-	-	R	Y	+	-	Not
S_{19}	-	+	-	-	+	-	+	-	-	R	Y	+	-	*S. typhi*
S_{20}	-	-	-	-	-	+	-	-	-	R	Y	+	-	Not
S_{21}	-	+	+	-	-	-	+	-	+	R	Y	+	+	Not
S_{23}	-	+	-	-	-	-	+	+	+	R	Y	Weak +	+	Not
S_{24}	-	-	-	-	-	-	+	-	-	R	Y	+	-	*S. typhi*
S_{25}	-	-	-	-	+	-	+	+	-	Y	R	-	+	Not
S_{32}	-	-	-	-	-	-	+	-	-	R	Y	+	-	Not
S_{35}	+	+	-	+	-	-	+	+	+	R	Y	+	+	*S. typhi*
S_{38}	+	+	+	-	-	-	+	-	-	R	Y	+	-	*S. typhi*
S_{44}	-	-	+	-	+	-	-	-	+	Y	R	-	-	S. *typhi*
S_{45}	-	+	+	-	-	-	+	-	-	R	Y	+	-	Not
S_{46}	-	+	+	-	-	-	+	-	-	R	Y	+	-	S. *typhi*
S_{47}	-	+	+	-	+	+	+	-	-	R	Y	+	+	Not
S_{48}	-	+	+	-	-	+	+	-	-	R	Y	+	+	Not

Symbols: + = Positive test, - = Negative test, R = Red-pink (alkaline reaction), Y = Yellow (acid reaction)

Key: Lac = Lactose fermentation, Suc = Sucrose fermentation, Ox = Oxidase test, Mot = Motility test, Man = Mannose fermentation, Glu = Glucose fermentation, Cit = Citrate utilization test, Ind = Indole test, Urea = Urease test

Cotrimoxazole (90%) Cloxacillin (75%), Chloramphenicol (60%) Tetracycline (80%) Doxycyclin (40%), Penicillin G (80%) Nalidixic acid (20%) and Ciprofloxacin (10%). Nalidixic acid and Ciprofloxacin here found to be effective against typhoid fever caused by multi drug resistant *Salmonellae typhi.* Singh (1991) and Mandal (1990) also recommended that the third generation cephalosporins (cetotaxime and ceftriaxone) and ciprofloxacin are highly effective for the therapy of multidrug resistant typhoid fever in children (Fig. 1).

In order to understand whether or not the drug resistance phenomenon in *Salmonellae typhi* was plasmid mediated, a strain S_5 (sample No. 5) from the ten multidrug resistant *Salmonellae typhi* had been selected for isolation of plasmid DNA. From the electrophoretical mobility a single plasmid of 20 kb in molecular mass was calculated. This 20 kb plasmid DNA was then subjected to transformation to a sensitive *E. coli.* LE 392 strain using by the method described by Cohen *et al.* (1972). From this experiment, it was found that *E. coli* LE 392 which was sensitive to cephalexin and cephradine before transformation became resistant to those antibiotics after transformation. The transformed strains were further tested by antibiotic spread plate method using 30 μg/ml, 50 μg/ml, 70 μg/ml of cephalexin and cephradine plates. In case of cephalexin 20,17 and 12 drug resistant colonies and in case of cephradine 18, 12 and 7 drug resistant colonies were appeared on the respective plates. But no drug resistant colonies were appeared on any of the control plates. This result strongly indicated that multidrug resistance phenomenon was plasmid mediated and the 20 kb plasmid which was transferred to *E. coli* LE 392, was carrying the gene (s) responsible for the multidrug resistance in *S. typhi*. For further confirmation, plasmid DNA from the transformed *E. coli* L E 392 was extracted purified and subjected to agarose gel electrophoresis. From the electrophoretical mobility a single 20 kb plasmid was calculated again which corresponds with that of the donor *Salmonellae typhi* (S_5) in molecular mass. This result further confirmed that the

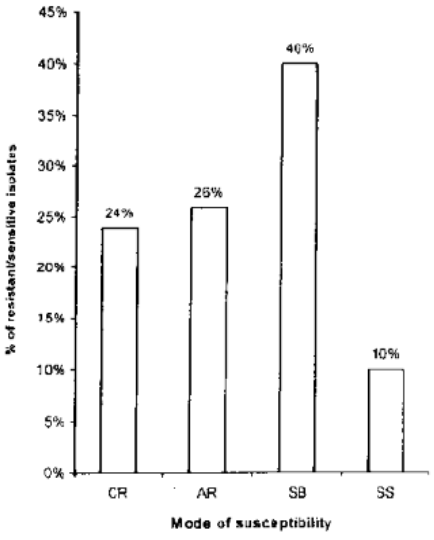

Fig. 1: Graphical representation of cephalosporin susceptibility test by disc diffusion method. Here CR = Completely resistant to cephalexin and cephradine, AR = Almost resistant to cephalexin and cephradine, SB = Sensitive to Both Drug and SS = Sensitive to a single Drug

20 kb plasmid DNA isolated from the *Salmonellae typhi* was carrying the gene (s) responsible for the multidrug resistance and the plasmid is transferable (Fig. 2).

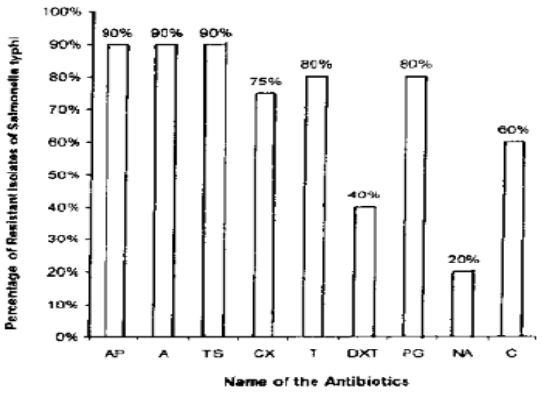

Fig. 2: Overall susceptibility pattern of ten cephalosporin resistant isolates of Salmonella typhi. Here AP = Ampicillin, A = Amoxycillin, TS = Cotrimoxazole, T = Tetracycline, DXT = Doxycycline, PG = Penicillin G, NA = Nalidixic Acid and C = Chloramphenicol.

Further work is required to make a complete restriction map of the 20 kb plasmid isolated from *Salmonellae typhi* and the specific gene (s) and the gene products should be identified and sequenced so that the gene function can be tempered to make the *Salmonellae typhi*, a causative organism for typhoid fever, a less vulnerable one.

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