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MOLECULAR AND EPIDEMIOLOGICAL ANALYSIS OF MULTIDRUG-RESISTANT *SALMONELLA PARATYPHI* A OBTAINED FROM DIFFERENT REGIONS OF PAKISTAN

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Abstract

Incidence of paratyphoid infections caused by *Salmonella paratyphi* A is significantly mounting high in the country. Indiscriminate use of antibiotics which often results in the development of drug-resistant strains, is making the treatment difficult. This situation becomes worst when organism acquire multidrug resistance (MDR). In the present study, various isolates of MDR *S. paratyphi* A, obtained from different regions of Pakistan, were analyzed for plasmid profile and multiplex PCR to determine the resistance gene associated with plasmid and chromosomal DNA of *S. paratyphi* A.

Multi-drug resistant isolates of *S. paratyphi* A obtained during the year 2001-2006 from different regions of Pakistan have been included in this study. These isolates showed resistance to chloramphenical (30µg), ampicillin (10µg), trimethoprim-sulfamethoxazole (23.5µg), and tetracycline (30µg). Some strains were also found resistant to ofloxacin and nalidixic acid. The plasmids from bacterial strains were found to be approximately 220 kbp, harboring antibiotic resistance genes. A multiplex PCR was used to identify any variation in antibiotic resistance genes encoding the MDR phenotypes in clinical isolates of *S. paratyphi* A. A homogenous pattern of multiplex PCR product revealed that MDR isolates of *S. paratyphi* A harbored the same resistance genes. This study will help taking effective measures for controlling disease in the region, and the data could be used for future medical reference.

Introduction

Typhoid is one of the oldest recorded threatening infectious diseases of human. Outbreaks of typhoid fever caused by *S. typhi* and *S. paratyphi* A are still a serious health problem worldwide. The incident rate of this disease in Asia-pacific region is estimated at more than 100 cases/100,000 population per year. The highest burden of disease has been observed in children (Bhutta & Hendricks, 1996). In Pakistan, the incidence of enteric fever in children is estimated at 170 per 100,000 of the population, while serology based incidence is estimated at 710 per 100,000 of the population (Siddiqui *et al*., 2006).

Typhoid is a sporadic disease in developed countries that occurs mainly in returning travelers, with occasional point source epidemics (Ackers *et al*., 2000). In early 19th century typhoid fever was an important cause of illness in the United States and Europe but the provision of clean water and good sewerage systems decreased the incidence in these regions (Osler, 1912).

Today most of the burden of the disease occurs in the developing world, where sanitary conditions are poor. Many hospitals lack facilities for blood-culture and most of the patients with typhoid are treated as outpatients.

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The emergence of drug-resistant strains of *S. paratyphi* A is another serious concern. Morbidity and mortality of such infection has significantly increased in the recent years. Sometimes second-line antibiotics, such as quinolones and third-generation cephalosporin were also found ineffective.

Typhoid fever caused by *S. paratyphi* A is similar to *Salmonella typhi* but it is less severe. Studies on *S. paratyphi* A strains in defined epidemiological setting are very few (Crump *et al*., 2004). The sensitivity of blood-culture for *S. typhi* is well described but there is very little data about *S. paratyphi* A. MDR in *S. paratyphi* A strains are rapidly increasing due to antimicrobial pressure, therefore, resistant to antibiotics, whether present on plasmid or chromosome of these resistant strains needs to be analyzed for the management of disease. To achieve this aim, in the present study, molecular techniques such as multiplex PCR and plasmid analysis was carried out to screen the resistance genes associated with the paratyphoid infection.

To control the present situation it is timely to understand the epidemiology and basic molecular aspects of the disease during the current epidemics, and these isolates of *S. paratyphi* A could be helpful in determining effective treatment and the development of effective vaccines.

Material and Method

About 117 strains of MDR *S. paratyphi* A were isolated during the year 2001-2006 from different regions of Pakistan. Resistance patterns to ampicillin, chloramphenicol, tetracycline, trimethoprim and sulfonamide were determined by disc-diffusion method (Bauer *et al*., 1966). Five susceptible isolates were also included in the study.

a. Extraction of plasmid: A colony of *S. paratyphi* A was inoculated in 5 ml of LB broth and incubated overnight at 37^{o}C in shaking water bath. The cells were centrifuged at 5000 rpm at room temperature for 10 minutes. Plasmids were then extracted by the alkaline lysis method as described by Wang *et al*., (1994).

b. Electrophoresis: Approximately 8 µl of the plasmid DNA was loaded to 1% agarose gel in TAE buffer system with *Escherichia coli* 39R standard plasmid DNA as a molecular weight marker. The electrophorised gel was stained with ethidium bromide (1.5 µg/ml) and analyzed on a Bio-Rad Geldoc-2000 gel-documentation system equipped with a Quantity 01 Software (Bio-Rad Laboratories, Richmond, California).

c. Bacterial conjugation: Conjugation experiments were performed by inoculating 7-8 ml of Brain Heart infusion broth with approximately 10^5-10^6 cfu of donors *S. paratyphi* A organisms and an equal number of *E. coli* (azide-resistant) recipients to see transferability of plasmid. After incubation at 37^{o}C, transconjugants were selected by plating the cells onto Mueller-Hinton agar containing azide and antibiotics nalidixic acid (32µg/ml) or streptomycin (32µg/ml).

d. Individual PCR and multiplex PCR for identification of resistance genes: For the detection of antibiotic resistant genes, a set of primers were used to identify the resistant genes present in *S. paratyphi* A strains, isolated from different regions of Pakistan.

The resistance genes *sul-2* (sulfonamide), *cat* (chloramphenicol), *dfrA*14 (trimethoprim), and *tetA* (tetracycline) and the gene for *tem* β-lactamase (ampicillin) were amplified by targeting *S. paratyphi* A DNA. PCR Primers were used at a final concentration of 1µM in a 50ml reaction mixture containing 1.5mM $MgCl_2$; 200µM each of dATP, dCTP, DGTP and dTTP; 1 X reaction buffer and 1 µl of DNA (Wain *et al*., 2003).

PCR conditions were used for the amplification of DNA as described by Wain *et al*., (2003): Initial denaturation at 95°C for 5 min followed by 28 cycles of 95°C for 30s, 57.5°C for 1 min, and 72°C for 2 min.

Primers sequence:

Primer name	Sequence 5' - 3'	Tm	Product (bp)	Reference sequence
CAT-F	TCC CAA TGG CAT CGT AAA GAA C	58.4	293	Transposon Tn9 from *E. coli*
CAT-R	TCG TGG TAT TCA CTC SAG AGC G	62.1		Accession number:JO1841
DHFR-F2	TTT GAT GTC CAA CCT GAG CGG G	60.6	189	*E. coli* dhf1b (dfrA14) gene
DHFR-R2	TGC GAA AGC GAA AAA CGG CG	62.7		Accession number Z50804
DHFR7-F	GTGTCGAGGAAAGGAATTTCAAGCTC	59.6	191	*E. coli* dhftVII gene Tn5086
DHFR7-R	TCACCTTCAACCTCAACGTGAACAG	59.1		Accession number 43090
DPS-F	TCCA AGG CAG ATG GCA TTC CC	59.4	156	Su/II gene from *Pasteurella multicoda* plasmid plGI.
DPS-R	CGACGAGTT GG CAG ATGATT TC	60.6		Accession number: U57647.1
DPSI-F	GGATGGGATTTTTGAGACCCCGC	66.8	308	Transposon Tn21 from *E. coli*
DPSI-R	ATCTAACCCTCGGTCTCTGGCGTCG	64.3		Accession number AF071413
TEM-F	TTT TCG TGT CGC CCT TAT TCC	57.9	798	General beta lactamase (TEM-1)
TEM-R	CGT TCA TCC ATA GTT GCC TGA CTC	62.7		*N. meningitidis* plasmid pAB6 Accession number: AF 126482
TET-F	GCA CTT GTC TCC TGT TTA CTC CCC	64.4	687	*Shigella flexneri* transposon Tn10
TET-R	CCT TGT GGT TAT GTT TTG GTT CCG	61.0		Accession number: AF162223.1

Results

The prevalence of multidrug resistance (MDR) *S. paratyphi* A obtained during the year 2001-2006, from different regions of Pakistan, were evaluated. One hundred and seventeen isolates of *S. paratyphi* A were studied for drug-sensitivity pattern. The isolates were found resistant to chloramphenicol, ampicillin, tetracycline, and trimethoprim-sulfamethoxazole. In addition some of the strains were resistant to ofloxacin and nalidixic acid as well (Table 1).

Seven different groups of *S. paratyphi* A were identified on the basis of drug sensitivity pattern (Table 1). Representatives from each group were selected for plasmid analysis; and detection of resistant genes by PCR. The analysis of plasmid in MDR isolates revealed the presence of large plasmid with an approximate molecular mass of 220 kbp. No significant difference was however noted in the size of the plasmids in any of the isolates (Fig. 1).

Conjugation transfer of plasmid: Conjugation was done using *E. coli* azide-resistant recipient to see if the plasmid was transferable. The resistant isolates of *S. paratyphi* A were able to transfer amoxicillin resistance into *E. coli* at 28°C. Each of the transconjugants was found to confer resistance to chloramphenicol, ampicillin, trimethoprim, sulfomethoxazole, tetracycline and streptomycin.

Molecular analysis of antimicrobial resistance gene in MDR *S. paratyphi* A using multiplex PCR: Transconjugates were found to possess a 220 kbp plasmid encoding resistance to ampicillin, chloramphenicol, sulphonamides, trimethoprim and tetracyclines. The β-*lactamase* gene responsible for mediating ampicillin-resistance was confirmed by using universal *tem* primers. The chloramphenicol acetyletransferase gene was confirmed by universal *cat* primers. The gene responsible for tetracycline resistance was confirmed by *tet* universal primer, sulfonamides with *sul-2* primer and trimethoprim resistance by *dfrA14* primers.

Table 1. Groups of *S. paratyphi* A isolates obtained during 2001-2006.

Groups	Number of isolates of *S. paratyphi* A
I (sensitive to all antibiotics)	50
II (NA resistant)	13
III (AMP, C, SXT, TET resistant)	39
IV (AMP, C, SXT & NAL resistant)	2
V (AMP & SXT resistant)	4
VI (AMP & C resistant)	0
VII (only C resistant)	9
Total	**117**

Key: Sulfamethoxazole-Trimethoprim SXT (23.5µg); Naladixic acid NA (5µg) Ampicillin AMP (10µg); Chloramphenicol C (30µg); Tetracycline TET (30µg)

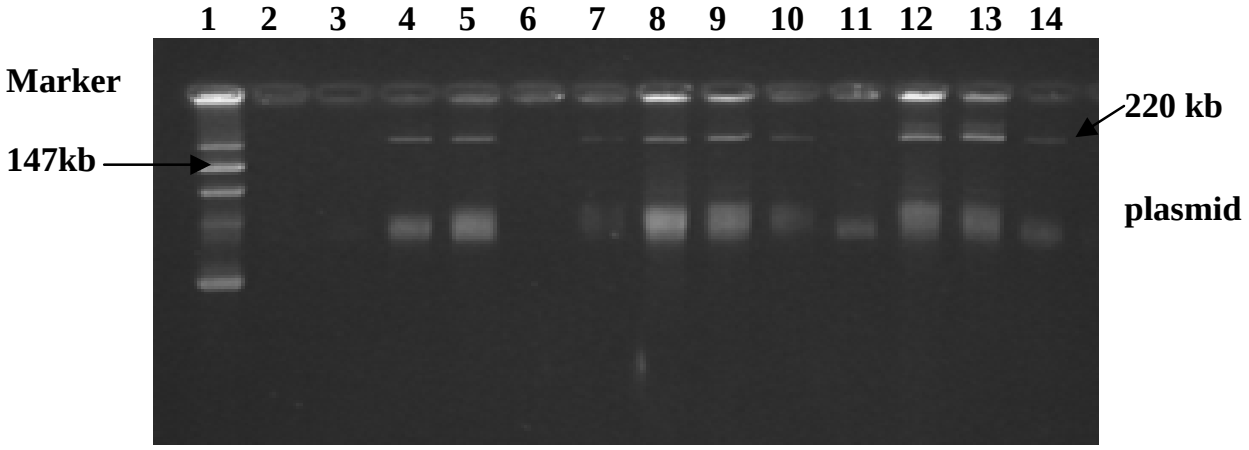

Fig. 1. Plasmids of MDR *S. paratyphi* A: Lane 1 contain marker; lane 2, 3 and 11 antibiotic sensitive *S. paratyphi* A; lane 4-5 MDR *S. paratyphi* A isolated during 2001-2002; lane 7-10, 12-14, MDR *S. paratyphi* A isolated during 2003-2006.

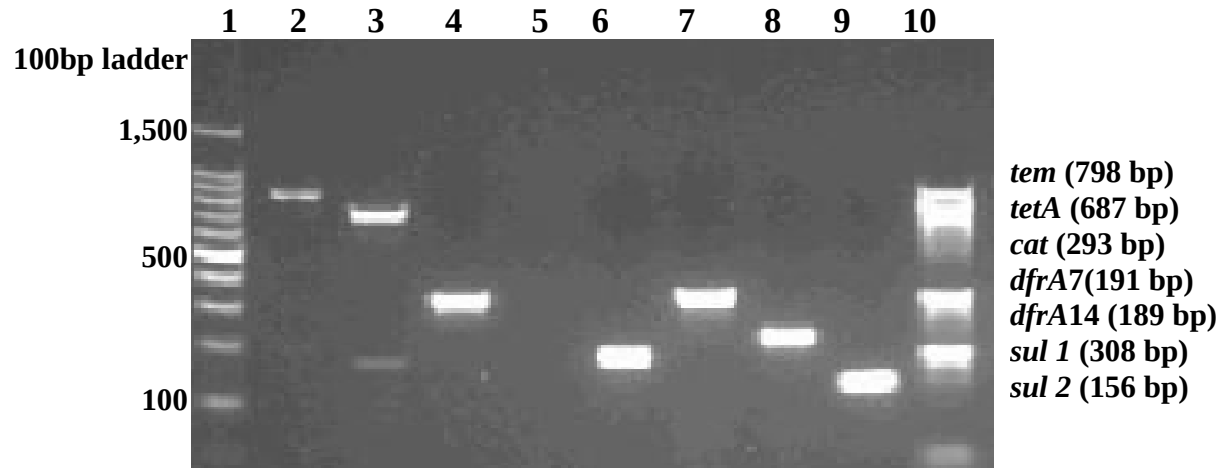

Fig. 2a. Multiplex and individual PCR for the detection of resistance genes of *S. paratyphi* A. Lane 1 contains 100bp marker; 2-4 and 6-9 is individual PCR; lane 10 represents multiplex PCR of *S. paratyphi* A

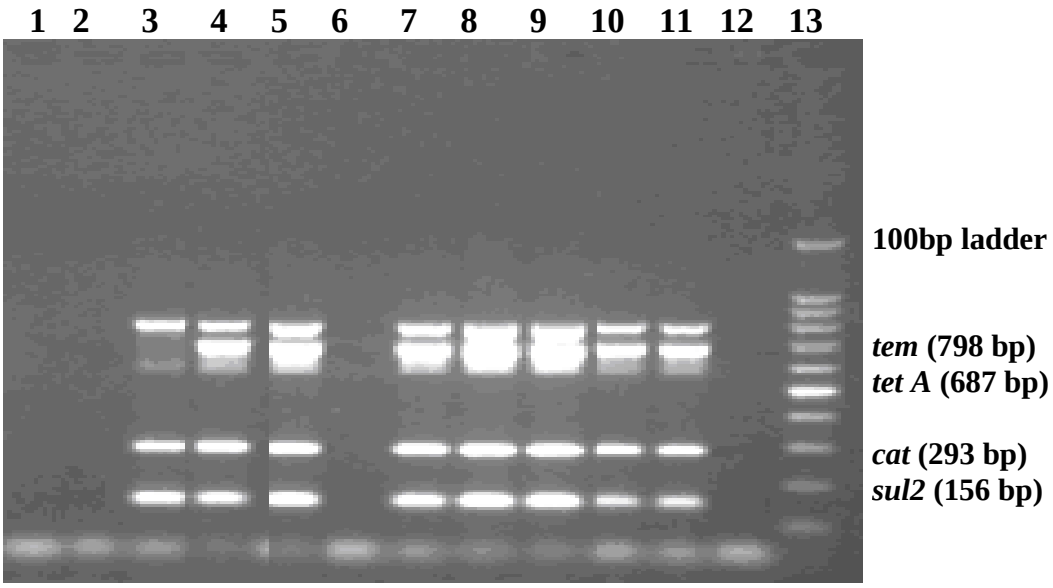

Fig. 2b. Multiplex PCR for the detection of resistance genes from representative *S. paratyphi* A isolates of different year. Lane 13, standard marker; lane 1, 2, contain antibiotic sensitive *S. paratyphi* A of 2004, 2005; lane 6 contains sensitive *S. paratyphi* A of 2003 and lane 12 contains sensitive *S. paratyphi* A of 2002; lane 4, 3 represents MDR *S. paratyphi* A of 2005, 2006 lane 5 of 2004, 7-8 of 2003, lane 9-10 of 2002, and lane 11 of 2001.

Multiplex PCR of isolates obtained during 2001-2006 revealed the amplification of *tem, tet, cat,* and *sul2* genes (Fig. 2b) in almost all the strains with an exception of one isolate of 2006 (lane 3, Fig. 2b) which revealed the amplification of only three genes *tem, cat* and *sul2* genes.

Discussion

Paratyphoid infection, caused by *S. paratyphi* A, is rising in the country due to the non-availability of safe drinking water and other predisposing factors. Studies on *S. paratyphi* A strains in defined epidemiological settings has been found very rare. Since 1999 more number of *S. paratyphi* A than *S. typhi* strains were isolated from different regions of the world including China, India and Pakistan (Crump *et al*., 2004). Clinical feature of paratyphoid fever are almost similar to typhoid but the symptoms are relatively milder with a shorter incubation period. In a recent surveillance study, it was reported that there were 285 *S. typhi* episodes and 84 episodes of *S. paratyphi* A in the four sites (Indonesia, 14%; Pakistan 15%; India and China 24% and 64%) (Ochiai *et al*. 2005).

The improper medication particularly inappropriate antibiotic therapy is making the treatment difficult and contributing to the development of multidrug resistant strains (MDR). Drug resistance could be developed either by mutation or by acquiring plasmids from other resistant strains. Resistant plasmids may harbor several drug resistant markers. These markers are generally recognized by disc-diffusion method and by PCR (Bauer *et al.*, 1966; Wain *et al*., 2003).

In the present study 117 strains of *S. paratyphi* A isolated from different regions of Pakistan were analyzed for the plasmid profile and to determine the size of these plasmid. Multiplex PCR was performed to determine the presence of resistance conferring gene associated whether with the plasmids or with the chromosomal DNA of *S. paratyphi* A. The development of drug-resistance in microbial strains affects the mortality, treatment, control

and spread of disease. Development of multidrug resistant strains is making treatment of the disease difficult and expensive (Hampton *et al*., 1998, Mirza *et al*., 1993).

Plasmid profile analysis is a useful DNA-based technique applied in epidemiological studies. Plasmids are extrachromosomal genetic elements which are autonomously replicating double stranded DNA molecules distinct from the cellular chromosome.

Multiplex PCR is a variant of PCR which enables amplification of many targets of interest in a single reaction by using more than one pair of primers. Since its first description in 1988 by Chamberlain & Caskey (1988), this method has been applied in many areas of DNA testing, including analyses of deletion mutations, polymorphisms or quantitative assays and reverse transcription PCR. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required. We identified 7 different groups of *S. paratyphi* A on the basis of drug-sensitivity patterns. Among 117 isolates of *S. paratyphi* A, 50 were found sensitive, while 13 were nalidixic acid resistant; 39 were resistant to AMP, C, SXT and TET; 2 were resistant to AMP, C, SXT & NAL; 3 to AMP & SXT; and 9 were resistant to only chloramphenicol.

In 1990, a single transferable plasmid of 98MDa conferring the MDR phenotype in over 90% of the isolates was reported in Pakistan (Mirza & Hart 1993). Most of the cases of MDR in *S. typhi* were due to an IncH11 plasmid worldwide (Hampton *et al*., 1998). In the present study, we tried to evaluate the present situation for *S. paratyphi* A with respect to drug resistance in Pakistan.

Multi-drug resistant isolates of *S. paratyphi* A obtained during the year 2001-2006 from different regions of Pakistan have been characterized to determine the basis of resistance. Around 117 isolates of *S. paratyphi* A were screened against chloramphenical, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, ofloxacin and nalidixic acid. Most of the isolates were found to harbor large conjugation transmissible plasmids encoding a range of antibiotic resistant genes.

Conclusion

In the present study, the antibiotics resistant genes were associated with the plasmids in MDR isolates of *S. paratyphi* A. All drug-sensitive isolates were plasmid-free, whereas MDR isolates were characterized by a transferable plasmid of approximately 220 kbp size. The plasmid encoded resistance to ampicillin, chloramphenicol, sulphonamides, tetracyclines, and trimethoprim. Multiplex PCR has been successfully applied for the detection of resistance genes by using universal primers. This study could be of some help in determining effective treatment measure for controlling the disease in this region, and the data could also be used for future medical references.

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