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Molecular Analysis of Drug Resistance in Clinical Isolates of MDR *Salmonella enterica* Serovar Typhi in Faisalabad, Pakistan

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Abstract.- *Salmonella enterica* serovar Typhi (*S.* Typhi) is a strict human pathogen that causes a major disease, typhoid. In recent years, the emergence of MDR strains of *S.* Typhi has become a major problem. This study was designed to get an insight into local MDR *S.* Typhi by conventional methods and molecular techniques including detection of genes and integrons related to drug resistance. Nineteen *S.* Typhi isolates collected from typhoid patients in local hospitals were tested for susceptibility to commonly used antityphoidal drugs. All isolates were multi drug resistant as tested by disc diffusion method. Most commonly occurring drug resistance genes (mutated *gyrA*, *tet B, cat P, temβ - lactamase, sul 2,* and *blt* genes for ciprofloxacin, tetracycline, chloramphenicol, ampicillin, sulfonamide, and cephalosporins, respectively) were identified by PCR. PCR was also used for integron detection. Only one (5.26%) isolate showed resistance to ciprofloxacin and ceftriaxone. Drug resistance to cefixime, cefoparazone, and cephradine, ampicillin, tetracycline, trimethoprim-sulfomethoxazole and chloramphenicol was observed in 7 (37%), 3 (16%), 10 (16%), 17 (89%), 12 (63%), 17 (89%), and 17 (89%) isolates respectively. One (5%) isolate showed resistance to all 9 drugs and 8 (42%) to 6 drugs. The PCR results for resistant genes were generally in agreement with disc diffusion tests. As many as 17 isolates showed presence of Class 1 integrons. Class 2 and 3 integrons were not found. We conclude that multiple drug resistance is very common in local isolates of *S.* Typhi and Class 1 integrons are generally present.

Key words: Clinical isolate, molecular analysis, typhoid, MDR, *S.* Typhi.

INTRODUCTION

Typhoid fever, a potentially fatal illness is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi). It is one of the most serious epidemic enteric infections. Crump et al. estimated the global burden of typhoid in the year 2000 to be about 22 million new cases, 5% of which are fatal (Crump *et al*., 2004). The endemic occurrence of the disease in the developing countries may be ascribed to multiple causes including poor hygiene and injudicious use of antibiotics and improper efficacy of vaccines (Ling *et al*., 2000).

Toward the end of the 1980s and in 1990s, *S.* Typhi developed resistance simultaneously to all the drugs that were then used as first-line treatment in various parts of the world (chloramphenicol, trimethoprim, sulfamethoxazole, and ampicillin). The spread of antibiotic resistance results from the clonal dissemination of individual MDR *S.* Typhi strains or from the transfer of the plasmid to multiple *S.* Typhi strains. Resistance rarely emerges during the course of treatment (Thong *et al*., 2000). MDR has also been documented in Pakistan since 1988 and has rapidly attained alarming proportions (Shanahan *et al*., 2000).

Integrons form an important source for the spread of antibiotic resistance. Their ability to integrate gene cassettes and especially those encoding resistance to antimicrobial agents makes them prime tools for further dissemination of antibiotic resistance (Fluit and Schmitz, 2004). Acquisition and dissemination of these genes located within integron structure, results in an increase in antimicrobial resistance. At least six classes of integrons have been determined according to their *int1* gene. Classes I, II and III are the most studied and are largely implicated in the dissemination of antibiotic resistance. Integrons are strongly associated with multi drug resistance seen in gram negative bacilli in the hospital environment. It has been reported that 18 out of 25 isolates of *S.* Typhi were multi drug resistant and contained class 1 integrons (Poly *et al*., 2003).

Typhoid is a major disease in Pakistan and for its control it is important to check the emergence

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of MDR strains of *S.* Typhi. Knowledge about genes involved in drug resistance and their association with integrons from local isolates can be very helpful in this regard. This study is the first step in this direction.

MATERIALS AND METHODS

Bacterial strains

Nineteen clinical isolates of *S.* Typhi were taken from stock cultures which were obtained from blood of suspected typhoid patients admitted to Allied Hospital, Faisalabad. These bacterial strains were preserved in 10% dimethyl sulfoxide and were kept at -20°C till further use.

Isolation and purification of bacterial isolates

The stock cultures of *S.* Typhi isolates were revived by inoculation in 3 mL of Trypticase Soya Broth (TSB) followed by overnight incubation at 37ºC. A loopful from each tube was streaked on MacConkey agar plates and after incubation at 37ºC for 24 hours, colonies were picked for further studies.

PCR - based confirmation of Salmonella Typhi *isolates*

After identification by the biochemical tests, the *S.* Typhi isolates were confirmed by PCR. DNA from the bacterial cells was extracted by the conventional phenol/chloroform method, followed by treatment with RNase for the removal of contaminating RNA. Quantitative estimation of the isolated DNA was done by UV-double beam spectrophotometer (Carry 1C, Varian) at 260nm. The integrity and purity of DNA samples was checked by 1% agarose gel electrophoresis.

The *fliC* gene was amplified using primers ST1 5'TATGC CGCTACATATGATGAG3' and ST2 5' TTAACGCAGTAAAGAGAG3' which gives a product of 495 bps (Song *et al*., 1994) under conditions described by Haque *et al*. (2001). Briefly, 100 µl DNA amplification mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 pmol of each primer, 70 nmol of each dNTP, 2 U of *Taq* polymerase, 20 µl of template and distilled water to make up the volume. The reaction mixture was subjected to 25 cycles of 1 minute each at 94°C, 55°C and 72°C followed by heating at 72°C for 7 minutes.

Drug sensitivity testing

After confirmation of *S.* Typhi isolates, drug sensitivity was checked by using disc diffusion method according to the recommendation of National Committee for Clinical Laboratory Standards (1990). Among the 9 drugs used, there were four cephalosporins representing various generations (cefoparazone, ceftriaxone, cefixime, and cephradine), ampicillin, chloramphenicol, tetracycline, trimethoprim-sulfomethoxazole, and ciprofloxacin.

Identification of drug resistance genes

PCR was performed for the identification of most common drug resistance genes for each drug. These included *sul* 1 and *sul* 2 for sulfonamide/ sulfomethoxazole, *tem β-lactamase* for ampicillin, mutated *gyr* A for ciprofloxacin, *cat* P for chloramphenicol, *tet* B for tetracycline, and *blt* gene for cephalosporins. Respective primers are given in Table I.

PCR conditions

Generally, the 100 µL reaction mixture contained 10mMTris HCl (pH 8.3),50mM KCl, 1.5 mM $MgCl_2$, 95 nmol of each dNTP, 150 pmol of each primer, 2 U of *Taq* polymerase (Fermentas), 20 µl (0.1µg/µl) DNA template and TE buffer to make the volume. Initial denaturation for 5 min at 94°C was followed by 30 cycles of 1 min each at 94°C, 50°C, and 72°C. Finally extension was done at 72°C for 7 minutes. The annealing temperature for mutated *gyrA* gene was 60°C.

The DNA fragments amplified by PCR were fractionated electrophoretically on 2% agarose gel.

Identification of integrons

PCR was performed for the identification of three classes of Integrons in the *S.* Typhi isolates. Class I Integrons were identified by targeting 5' and 3' conserved segments. Class II and Class III Integrons were identified by targeting *Int I 2* and *IntI 3* genes respectively. The primers used are listed in Table II.

Table I.- Sequences of primers for PCR based detection of drug resistance genes.

Primer	Oligonucleotide sequence	Targeted gene	Drugs	Amplicon (bps)	References
Sul3	5′ TCA ACA TAA CCT CGG ACA GT 3	*sul II*	Sulfonamide	707	Chu *et al.* (2001)
Sul4	5′ GAT GAA GTC AGC TCC ACC T 3′				
A1	5′ GCA CGA GTG GGT TAC ATC GA 3′	*tem*	Ampicillin	311	Carlson *et al.* (1999)
A2	5′ GGT CCT CCG ATC GTT GTC AG 3′				
Am1	5′ ATG AGT ATT CAA CAT TTC CGTGT 3′	*tem β-lactamase*	Ampicillin	876	Chu *et al.* (2001)
Am2	5′ TTA CCA ATG CTT AAT CAG TGA CG 3′				
Cip1	5′ TAC CGT CAT AGT TAT CCA CGA 3′	mutated *gyr A*	Ciprofloxacin	342	Molbak (1999)
Cip2	5′ GTA CTT TAC GCC ATG AAC GT 3′				
Catp-F	5′ CCTGCCACTCATCGCAGT 3′	*catP*	Chloramphenicol	639	Guerra *et al.* (2001)
Catp-R	5′ CACCGTTGATATATCCC 3′				
TB-F	5′ CTC AGT ATT CCA AGC CTT TG 3′	*tet B*	Tetracycline	440	Bertrand *et al.* (1983)
TB-R	5′ CTA AGC ACT TGT CTC CTG TT 3′				
BLT-F	5′ CCCCTATTTGTTTATTTTTC 3′	*blt*	Cephalosporins	962	Gniadkowski *et al.* (1998)
BLT-R	5′ GACAGTTACCAATGCTTAAT 3′				

Table II.- Sequences of primers for PCR based detection of Integrons.

Primer	Oligonucleotide sequence	Targeted gene	Amplicon (bps)	References
IntI1 F	5′ ATC ATC GTC GTA GAG ACG TCG G 3'	*5′ CS*	Variable	Rosser and Young (1999)
IntI1 R	5′ GTC AAG GTT CTG GAC CAG TTG C 3'	*3′ CS*		
IntI2 F	5' GCA AAT GAA GTG CAA CGC 3'	*Int I 2*	467	Reyes *et al.* (2003)
IntI2 R	5' ACA CGC TTG CTA ACG ATG 3'			
IntI3 F	5' GCA GGG TGT GGA CGA ATA CG 3'	*Int I 3*	760	Senda *et al.* (1996)
IntI3 R	5' ACA GAC CGA GAA GGC TTA TG 3'			

The 100 µL reaction mixture contained 10mMTris HCl (pH 8.3),50mM KCl , 1.5 mM $MgCl_2$, 95 nmol of each dNTP, 150 pmol of each primer, 3 U of *Taq* polymerase (Fermentas), 20 µl (0.1µg/µl) DNA template and TE buffer to make the volume. Initial denaturation for 5 min at 94°C was followed by 30 cycles of 1 min each at 94°C, 54°C, and 72°C. Finally extension was done at 72°C for 7 minutes.

RESULTS

S. *Typhi isolates*

Nineteen *S.* Typhi isolates collected from typhoid patients in local hospitals were revived from stock cultures and reconfirmed by PCR targeting *fli C* gene (Haque *et al*., 2001).

Drug sensitivity

All isolates of *S.* Typhi were tested for susceptibility to commonly used drugs for the treatment of typhoid by disc diffusion methods. The drugs included cefoparazone, ceftriaxone, cefixime, cephradine, ampicillin, chloramphenicol, tetracycline, trimethoprim -sulfomethoxazole, and ciprofloxacin. All isolates were resistant to at least two drugs. Only one isolate showed resistance to ciprofloxacin and ceftriaxone. Antibiotic resistance to cephradine, cefixime, and cefoparazone was observed in 10 (53 %), 7(37 %) and 3(16 %) isolates respectively. As many as 17 isolates (89 %) were resistant to ampicillin, trimethoprim-sulfomethoxazole and chloramphenicol. Resistance to tetracycline was seen in 12 (63 %) isolates.

When drug resistance patterns were classified (Table III), one (5%) isolate showed resistance to all 9 drugs (pattern 1). All other isolates showed resistance to 6 drugs or less. Among those isolates which were resistant to 6 drugs (8; 42%), two different patterns were observed; pattern 1 was seen in 6 (31%) isolates, where as pattern 2 was observed in 2 (10%) isolates. Two isolates (10%) were

Table III.- Drug resistance patterns of MDR isolates by disc diffusion method.

Patterns	Drug resistance by disc diffusion	Genes detected	%
Isolates resistance to 9 drugs (n=1)	Amp, Cfm, Cfp, Chl, Cip, Cro, Rad, Sxt, Tet	mutated gyr A, *tet B, cat P, sul 2 temβ - lactamase, blt*	5
Isolates resistant to 6 drugs Pattern 1 (n = 6)	Amp,Chl, Cfm, Sxt, Tet, Rad	*tet B, cat P, sul 2 temβ - lactamase, blt*	32
Isolates resistant to 6 drugs Pattern 2 (n = 2)	Amp,Cfp, Chl, Sxt, Tet, Rad	*tet B, cat P, sul 2 temβ - lactamase*	11
Isolates resistant to 4 drugs Pattern 1 (n = 2)	Amp, Chl, Sxt, Tet	*tet B, sul 2*	11
Isolates resistant to 3 drugs Pattern 1 (n = 6)	Amp, Chl, Sxt,	*cat P, temβ - lactamase*	32
Isolates resistant to 2 drugs Pattern 1 (n = 1)	Sxt, Rad	*sul 2*	5
Isolates resistant to 2 drugs Pattern 1 (n = 1)	Sxt, Tet	*tet B, sul 2*	5

Amp, Ampicillin; Cfm, Cefixime; Cfp, Cefoperazone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Cro, Ceftriaxone; Rad, Cephradine; Sxt, trimethoprim-sulfomethoxazole; Tet, Tetracycline.

showed resistance to 3 drugs. Two (11%) isolates were resistant to only 2 drugs each showing a different pattern.

Drug resistance genes

The representative results are shown in Figure 1. One isolate showed amplification product of 342 bp for mutated *gyrA* gene that encodes resistance to ciprofloxacin. This isolate was resistant to all drugs. This result was consistent with disc diffusion results. Tetracycline resistance gene *tet B* was targeted and all 19 isolates showed amplification product of 440bp whereas 17 isolates showed amplification product of 630bp for *cat P* gene that encodes resistance to chloramphenicol. These results were compatible with disc diffusion test. Out of 19 isolates, *temβ - lactamase* gene was detectable in 15 isolates. All the 19 isolates showed an amplification product of 707 bp for *sul 2* gene encoding resistance to sulfonamide/ sulfomethoxazole. However by disc diffusion method, only 17 isolates showed resistance. For cephalosporins, only 10 isolates were resistant with disc diffusion while seven of these isolates showed an amplification product of 962 bp for *blt* gene. These results are summarized in Table III.

Integrons

S. Typhi isolates were checked for the presence of Class 1 integrons by targeting 5'conserved segment (CS) and 3'conserved segment (CS) of the entire integron. For Class 2 and Class 3 integrons *IntI 2* and *IntI 3* genes were targeted. Out of 19 *S.* Typhi isolates, 17 showed amplification products of various sizes with primers targeting Class 1 integrons. All *S.* Typhi isolates were negative for Class 2 and Class 3 integrons.

DISCUSSION

Typhoid fever is an important public health problem in many developing countries that have not yet achieved sufficient management of drinking water and sewage disposal (Ling *et al*., 2000). A major problem in recent years has been the emergence of drug resistance in strains of *S.* Typhi causing infections, in some recent instances to even second line antibiotics such as quinolones and third generation cephalosporins. It is recognized that drug resistant typhoid is frequently associated with increased morbidity and toxicity (Mehta and Ariya, 2002).

Integrons are genetic elements that behave as a gene expression vector that express resistance to different antibiotics. Integron mediated antibiotic resistance genes are common among clinical Enterobacteriaceae associated with disease in humans (Martinez and de la Cruz, 1999).

This study was aimed to assess the drug resistance status of local isolates of *S.* Typhi both by microbiological and molecular methods. The drug resistance was studied against 9 drugs by disc diffusion method, identification of relevant genes, and for presence of integrons. Three commonly used 3rd generation (cefoparazone, ceftriaxone, cefixime) and one first generation (cephradine) cephalosporin

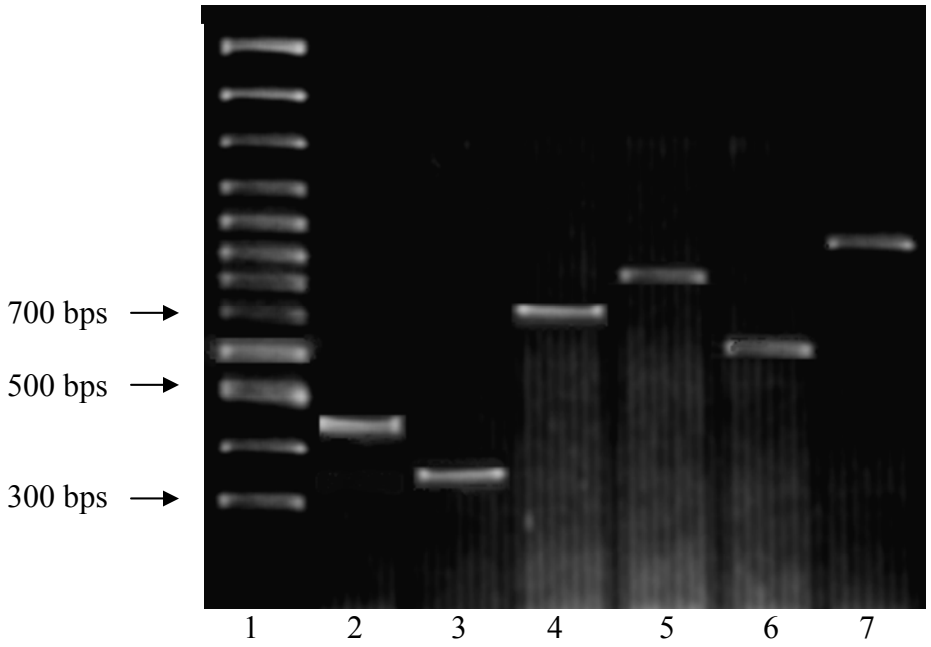

Fig. 1. Amplification of drug resistance related genes. Lane 1, Molecular weight marker (SM0323S) showing 3000, 2000, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 base pairs (bp) in descending order; lane 2, amplification product of 440 bps for *tetB* gene representing tetracycline resistance; lane 3, amplification product of 342 bps for mutated *gyrA* gene representing ciprofloxacin resistance; lane 4, amplification product of 707 bps for *sulII* gene representing sulphonamide resistance; lane 5, amplification product of 876 bps for *tem β-lactamase* gene representing ampicillin resistance; lane 6, amplification product of 639 bps for *catP* gene representing chloramphenicol resistance; lane 7, amplification product of 962 bps for *blt* gene representing cephalosporin resistance.

were included beside most relevant fluoroquinolone, ciprofloxacin and conventionally used antityphoidal drugs, chloramphenicol, tetracycline, trimethoprim-sulfomethoxazole, and ampicillin.

The results showed that these isolates were highly resistant to all the drugs except ciprofloxacin and third generation cephalosporins. Ciprofloxacin and ceftriaxone were most effective as only one isolate was resistant. Incidentally this isolate was resistant to all the 9 drugs. Other isolates were resistant to 6 or less drugs (Table III).

There may be multiple genes conferring resistance to a single antimicrobial drug and it may not be possible to explore all of them in a single study. Therefore, the results of phenotypic drug resistance (disc diffusion tests) and molecular detection of respective genes may not always tally (Srinivasan, 2007).

Our results of disc diffusion tests and PCR targeting related drug resistance genes were generally compatible. Some discrepancy was seen in results for ampicillin where one resistant isolate was PCR negative. This may be because though resistance to ampicillin is most often caused by the presence of β-lactamases, but mutation in penicillin binding proteins (PBPs) resulting in reduced affinity for β -lactam antibiotics may also be involved.

By disc diffusion test, 10 isolates were resistant to cephalosporins but only 7 were PCR positive for *blt* gene. The negative result in 3 cases may be because many other genes are related to cephalosporin resistance and it was not possible to cover all of them in our study.

Conversely, in case of sulphonamide resistance all isolates were PCR positive for *sul II* gene but only 17 showed resistance by disc diffusion. This is probably because we used discs of trimethoprim-sulfomethoxazole, not sulfonamide alone. The trimethoprim resistance is due to *dfr* genes (Chiu *et al*., 2004). We did not include these genes in our study because there are at least 20 subtypes and though dfr_{A1} is most common, it

accounts for only 77% cases of trimethoprim resistance (Guerra *et al*., 2003). In turn this gene has four subclasses which need different sets of primers (Lee *et al*., 2004). It was not possible to cover all of these variants by PCR. On the other hand, *sul2* gene that is responsible for sulfamethoxazole resistance is universally present in clinical isolates of *Salmonella* resistant to this drug (Aarestrup *et al*., 2003).

In our study, Class I integrons were detected in 17 (89.50%) isolates by targeting the 5' and 3' end conserved regions of the entire integron. All the isolates were negative for both class II and class III integrons.

Although the sample number was small in this study, it does provide an indication that multiple drug resistance is very common in local isolates of *S*. Typhi and is usually associated with Class I integrons. There is a distinct possibility that *S*. Typhi resistant to all drugs may emerge in near future so there is a need for more sophisticated studies with larger sample size.

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