African Journal of Microbiology Research Vol. 6(4), pp. 793-797, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1055
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Emergence of extended-spectrum β-lactamase producing *Salmonella typhi* in Pakistan

Khalid Mahmood[1], Mateen Izhar[1], Nakhshab Choudhry[2], Ghulam Mujtaba[3] and Naeem Rashid[4]*

[1]Department of Microbiology, Sheikh Zayed Hospital, Lahore, Pakistan.
[2]Department of Biochemistry, Sheikh Zayed Hospital, Lahore, Pakistan.
[3]Institute of Nuclear Medicine and Oncology (INMOL), Lahore, Pakistan.
[4]School of Biological Sciences, University of the Punjab, Lahore, Pakistan.

Accepted 29 December, 2011

Enteric fever caused by *Salmonella typhi*, is an increasing health problem affecting the major population in tropical and subtropical regions of the world. Development of multi drug resistance in *S. typhi* strains has further increased the severity of the problem. In Pakistan and neighboring countries, more than 80% *S. typhi* strains have been reported as multi drug resistant. A total of 4200 isolates collected during four years study period starting from Jan. 2006 to Dec. 2009, were initially screened using the first-line anti typhoid drugs. Out of them 408 were resistant to all the first-line antityphoid drugs. It was confirmed by polymerase chain reaction that all these isolates contained *fliC, sul, catP, tem* and *gyrA* genes. Only three of them (0.7%) had shown extended-spectrum β-lactamases production by double disk synergy test. Infection control surveillance, better hygiene along with controlled use of anti microbials would minimize the impact of extended-spectrum β-lactamases and their spread in hospital and intensive care unit patients.

Key words: Extended-spectrum β-lactamase, multi drug resistance, s*almonella typhi*, polymerase chain reaction, Pakistan.

INTRODUCTION

Multi–drug resistant (MDR) *Salmonella typhi*, causing enteric fever continues to be a major public health problem in tropics and subtropics of the world, affecting both local population and travelers to the endemic area. Frequent out breaks have been reported in South Asian countries (Le et al., 2005; Mulvey et al., 2003). In Pakistan, Iran, Nepal, Bangladesh and India more than 80% MDR *S. typhi* have been reported during the past two decades (Mohanty et al., 2006; Menezes et al., 2011). Treatment scenario has changed from first line anti typhoid such as chloremphenicol (C), ampicillin (AMP), and trimethoprim-sulphamethoxazol (SXT) to fluoroquinolones and cephalosporins (Parry, 2003; Zaki and Karande, 2011). Extended spectrum β-lactamases (ESBL) are enzymes that can hydrolyze oxyimino-beta lactams causing resistance to third generation cephalosporins, resulting in treatment failure and association with higher morbidity and mortality among immuno-compromised patients (Rasheed et al., 2000; Bush, 2001). Nosocomial infection caused by ESBL producing *S. typhi* has been reported from Latin America, France, Senegal, Africa, Asia, and Europe (Winokur et al., 2001; Weill, 2004a, b; Gniadkowski, 2001; Su et al., 2005). The emergence of ESBL in MDR *S. typhi,* constitutes a new challenge and has become a matter of concern especially in under developed countries.

S. typhi has been found to produce a wide variety of ESBL types including TEM, SHV, PER and CTXM enzyme (Batchelor et al., 2005; Paterson, 2006; Tzouvelekis et al., 2003). Phenotypic detection methods

*Corresponding author. E-mail: naeemrashid37@hotmail.com or naeem.ff.sbs@pu.edu.pk. Tel: +92 42 99231534. Fax: +92 42 99230980.

Table 1. Primer sequences used in this study for PCR amplification.

Primer	Sequence (5'-3')	Gene	Size (bp)
fliC F	ACTGCTAAAACC ACTACT	*fliC*	363
fliC R	TGGAGACTTCGGTCGCGTAG		
sul2 F	TCAACATAACCTCGGACAGT	*sul2*	707
sul2 R	GATGAAGTCAGCTCCACCT		
catP F	CCTGCCACTCATCGCAGT	*catP*	623
catP R	CCACCGTTGATATATCCC		
tem F	GCACGAGTGGGTTACATCGA	*tem*	311
tem R	GGTCCTCCGATCGTTGTCAG		
gyrA F	TACCGTCATAGTTATCCACGA	*gyrA*	313
gyrA R	GTACTTTACGCCATGAACGT		

perform poorly in bacterial isolates harbouring AmpC gene (Robberts et al., 2009). AmpC β-lactamases are included in class C and are not inhibited by clavulanic acid or other β-lactamase inhibitors. Thus if an ESBL confirmatory test using clavulanic acid is not performed, many AmpC producing strains may be presumed to be ESBL producing strains (Fred et al., 1999; Hague, 2011). Detection of microorganisms with multiple β-lactamases that may interfere with the phenotypic confirmatory tests can only be accomplished by iso-electric focusing or DNA sequencing methods that are usually not available in clinical labs (Goussard and Courvalin, 1999; Pfaller et al., 2001).

The primary objective of this study was to assess the incidence of ESBL producing MDR *S. typhi* and their antimicrobial sensitivity pattern in Pakistani population using double disc synergy test (DDST) from blood samples of the patients suffering from enteric fever who visited out patients departments or were admitted in Sheikh Zayed Hospital Lahore, Pakistan.

METHODS

This prospective and cross sectional study was conducted from January 2006 to December 2009 at tertiary care, university teaching Federal Post Graduate Sheikh Zayed Medical Complex (referral hospital with 450 beds) in Lahore, Pakistan. *S. typhi* were isolated from the blood of the patients admitted in the Hospital. Blood sample from the patients were collected in blood culture bottles and incubated at 37°C for 24 h. Sample from blood culture bottle were inoculated on MacConkey and S.S agar. Plates were then incubated at 37°C for 24 h. Identification of the micro-organisms was carried out by using API 20 E (Bio Meraux, Hazelwood, Durham NC, USA), polyvalent "O"Anti sera (Bio-Rad Laboratories CA, USA) and subsequently specific (*fliC)* gene was isolated by polymerase chain reaction (PCR). Antimicrobial susceptibility tests were performed by using standard disc diffusion method following Clinical Laboratory Standards Institute (CLSI) recommendations. The panel of antibiotics tested includes AMP, C, SXT, ciprofloxacin (CIP), ofloxacin (OFX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), imipenem (IPM), and meropenem (MEM). *Escherichia coli (*ATCC 25922) was used as a control. *S. typhi* isolates were labeled MDR when showing resistance to all the first line antityphoid drugs.

Genomic DNA from MDR samples was extracted by standard phenol/chloroform method (Sambrook et al., 1989) and subsequently parts of the genes responsible for resistance to these drugs were amplified by PCR using the primers given in Table 1.

All the MDR *S. typhi* isolates were screened for ESBL production using CAZ and CTX indicator discs (Oxoid, Cambridge, UK). The isolates showing the zones of inhibition (ZOI) ≤ 22 mm and 27 mm for CAZ and CTX respectively were further tested in combination with amoxicillin/clavulanic (AMC). The isolates showing an increase in ZOI by greater than or equal to 5 mm when evaluated in combination with AMC were phenotypically considered as ESBL producers.

DDST was performed by inoculating Mueller-Hinton (MH) agar with test isolates to give a semi confluent growth. CAZ 30 µg and AMC 20 µg discs were then placed strategically 25-30 mm apart (centre to centre) following over night incubation aerobically at 37°C. ESBL production was inferred when the zone of inhibition around the indicator disc was expanded by AMC.

RESULTS

During the four years study period from January 2006 to December 2009, a total of 4200 *S. typhi* isolates were collected from blood culture samples of typhoid patients. The ratio of *S. typhi* ranged between 1372 (32.6%) isolates during 2006, 1050 (25%) in 2007, 1018 (24.2%) in 2008 and 760 (18.09%) during 2009. The results indicated that the isolation of *S. typhi* was significantly decreased to 18.09% in 2009 from 32.6% in 2006 (Table 2).

Out of total 4200 *S. typhi* specimen 408 (9.7%) were resistant to all the first-line anti-typhoid drugs (C, AMP and SXT), therefore were labeled as MDR *S. typhi.* Isolation rate of MDR *S. typhi* reduced to 5% (38 isolates) during 2009 from 13% (180 isolates) during 2006

Table 2. Isolation of *S. typhi* during the study period (n=4200).

Year	No. of isolates	%age	MDR positive	%age	ESBL Producer
2006	1372	32.6	180	13.1	1
2007	1050	25	110	10.4	1
2008	1018	24.2	80	7.8	1
2009	760	18.09	38	5	0
Total	4200	100	408	36.3	3 (0.73 %)

Table 3. Antimicrobial resistant pattern of *S. typhi* isolates during 2006-2009.

Drug	Year 2006 (n = 1372)		Year 2007 (n = 1050)		Year 2008 (n = 1018)		Year 2009 (n = 760)	
	n	%age	n	%age	n	%age	n	%age
Chlorompjenicol	632	46.06	278	27.3	195	19.1	101	13.2
Ampicillin	962	70.1	685	49.9	236	23.1	161	21.1
Co-trimoxazole	878	63.9	602	43.8	248	24.3	183	24.0
Ciprofloxacin	220	16.1	380	36.1	498	48.9	410	53.9
Nalidixic acid	1236	90.08	742	70.6	1206	87.9	312	95.6
Ceftriaxone	196	14.2	160	15.2	162	15.9	118	13.5
Cefotaxime	248	18.0	171	16.2	132	12.9	108	14.2
Ceftazidime	226	16.4	158	15.0	145	14.2	90	11.8
Sparfloxacin	208	14.8	127	12.0	108	10.6	96	12.6
Gentacin	248	18.0	168	16.0	140	13.7	120	15.7
Imipenem	36	2.0	26	2.4	31	3.0	18	2.3
Meropenem	42	3.1	31	2.9	31	3.0	17	2.2

n = number of isolates.

(P< 0.001) as given in Table 2**.** Among the first line anti typhoid drugs C showed decreasing resistance patterns from 46.06% in 2006, 27.3% in 2007, 19.1% in 2008 and 13.2% in 2009. AMP also showed decreasing resistance patterns from 70.1% in 2006, 49.9% in 2007, 23.1% in 2008, and 21.1% in 2009. Resistant patterns for SXT were also decreased from 63.9% in 2006, 43.8% in 2007, 24.3% in 2008, and 24% in 2009. On the other hand CIP showed year wise increased resistant patterns from 16.1% in 2006, 36.1% in 2007, 48.9% in 2008, and 53.9% in 2009 (Table 3).

ESBL production was suspected in isolates showing reduced susceptibility to CRO, CAZ or CTX and was tested for ESBL production using DDST. Only three isolates, one in each year from 2006-2008 out of 408 (0.7%) were found to be ESBL producer. No incidence of ESBL production was observed in 2009.

All the 408 MDR isolates were further examined for the presence of *fliC* gene by PCR by using fliC F and fliC R primers as priming strands and genomic DNA of MDR isolates resulted in the amplification of 363 bp DNA fragments indicating that all the isolates were *S. typhi.* Three of the representative isolates are shown in (Figure 1A). We also performed PCR by using gyrA F and gyrA R primers which resulted in the amplification of a DNA fragment of 313 bp in all the isolates (Figure 1B).

When we performed PCR by using sul2 F and sul2 R primers, a 707 bp DNA fragment was amplified in all the cases reflecting the presence sul2 gene which was responsible for resistance against SXT (Figure 1C). The presence of *tem* gene, responsible for resistance against AMP, was demonstrated by the amplification of 311 bp DNA fragments (Figure 1D) by using tem F and tem R primers. Presence of catP gene is one of the factors responsible for resistance against C in *S. typhi* isolates. When we performed PCR by using catP F and catP R primers, a DNA fragment of 623 bp was amplified (Figure 1E) reflecting the presence of catP gene in all the 408 isolates.

DISCUSSION

MDR in *S. typhi* is a major therapeutic concern and now ESBL emergence in these isolates has constituted a new challenge for physicians treating typhoid fever in developing countries where typhoid fever is endemic. In the present study, decreasing isolation rate, both in *S. typhi* and MDR *S. typhi* has been observed. Decreased isolation of *S. typhi* is assumed to be due to over all improvements in environmental, water and sanitary conditions, public awareness and personal hygiene. It

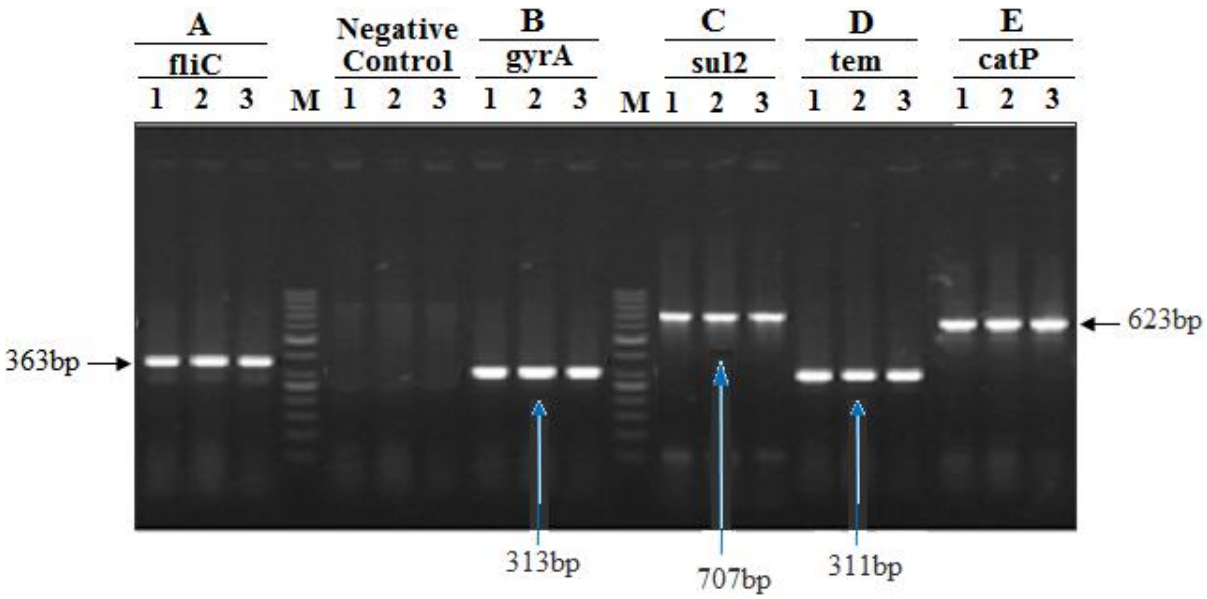

Figure 1. Ethidium bromide stained 1% agarose gel demonstrating the PCR amplification of fliC gene, for identification of *S. typhi*, and various genes responsible for antimicrobial drug resistance.

can also be attributed to withdrawal of first-line anti-typhoid drugs (C, AMP and SXT) for empirical therapy, preventing risk factors for acquiring MDR which includes isolation of patients, short hospital stay, continuous surveillance, controlled and judicial antibiotic use in hospital and ICU (Cassettari et al., 2009). Similar decreasing pattern has been observed in studies conducted in India, Nepal, Kuwait, and USA (Bradford, 2001; Pokharel et al., 2006; Rotimi et al., 2008). Prevalence of MDR *S. typhi* varies from 0 to 61 % in different parts of the world (Kariuki et al., 2010; Threlfall et al., 2003). Our study demonstrated the presence of 0.7% ESBL producers among MDR *S. typhi* isolates which is comparable to the studies conducted in Canada, Poland, England, and France where 0-3% ESBL production has been reported in MDR *S. typhi* (Batchelor et al., 2005; Weill et al., 2004; Mohanty et al., 2006). Since ESBL genes are usually located on mobile genetic elements, the emergence of an ESBL *S. typhi* may be attributed to exchange of mobile genetic elements.

Increased CIP resistance from 16.1 to 53.9% in MDR *S. typhi* isolates (Table 3), observed in this study, suggests that efficacy of CIP is unreliable against MDR *S. typhi* isolates. This reflects the wide spread use or misuse of CIP. Self prescription by patients and incomplete courses of treatment are features contributing to development of resistance to CIP in MDR *S. typhi*.

REFERENCES

Batchelor M, Hopkins K, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, Liebana E (2005). Bla (CTXM) genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrob. Agents Chemother. 49: 1319-1322.

Bradford PA (2001). Extended-spectrum b β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933–951.

Bush K (2001). New β-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin. Infect. Dis., 32: 1085-1089.

Cassettari VC, Da Selveria IR, Dropa M, Lincopan N, Mamizuka EM, Matté MH, Matté GR, Menezes PR (2009). Risk factors for colonization of newborn infants during an outbreak of extended-spectrum β-lactamase producing Klebsiella pneumonia in an intermediate-risk neonatal unit. J. Hosp. Infect., 71: 340-347.

Fred CT, Mohammad MJ, Gorton TS, Dembek ZF (1999). Detection and Reporting of Organisms Producing Extended-Spectrum β-lactamases: Survey of Laboratories in Connecticut. J. Clin. Micriobiol., 37: 4065-4070.

Gniadkowski M (2001). Evolution and epidemiology of extended spectrum ß-lactamases (ESBL) and ESBL producing microorganisms. Clin. Microbiol. Infect., 7: 597-608.

Goussard S, Courvalin P (1999). Updated sequence information for *tem* beta-lactamases genes. Antimicrob. Agents Chemother., 43: 367-370.

Hague R (2011). What is the threat from extended spectrum β-lactamase-producing organisms in children? Arch. Dis. Child., 96: 325-327.

Kariuki S, Revathi G, Kiiru J, Mengo DM, Mwituria J, Muyodi J, Munyalo A, Teo YY, Holt KE, Robert A, Dougan KG (2010). Typhoid in Kenya Is Associated with a Dominant Multidrug-Resistant *Salmonella enterica* Serovar Typhi Haplotype That Is Also Widespread in Southeast Asia. J. Clin. Microbiol., 48: 2171-2176.

Le TA, Lejay-Collin M, Grimont PA, Hoang TL, Nguyen TV, Grimont F, Scavizzi MR (2005). Endemic, epidemic clone of *Salmonella enterica* serovar typhi harboring a single multidrug-resistant plasmid in Vietnam between 1995 and 2002. J. Clin. Microbiol. 43: 3594-3599.

Menezes GA, Harish BN, Khan MA, Goessens WHF, Hays JP (2011). Antimicrobial resistance trends in blood culture positive *Salmonella typhi* isolates from Pondicherry, India, 2005–2009. Clin. Microbiol. Infect. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1469-0691.2011.03546.x.

Mohanty S, Renuka K, Sood S, Das BK, Kapil A (2006). Antibiogram pattern and seasonality of *Salmonella* serotypes in a North Indian tertiary care hospital. Epidemiol. Infect., 134: 961-966.

Mulvey MR, Soule G, Boyd D, Demczuk W, Ahmed R (2003).

Characterization of the first extended-spectrum β-lactamase-producing *Salmonella* isolate identified in Canada. J. Clin. Microbiol. 41: 460–462.

Parry C (2003). Antimicrobial drug resistance in *Salmonella enterica*. Curr. Op. Infect. Dis., 16: 467-472.

Paterson DL (2006). Resistance in Gram-negative bacteria: enterobacteriaceae. Am. J. Infect. Control, 34: S20-28.

Pfaller MA, Acar J, Jones RN, Verhoef J, Turnidge J, Sader HS (2001). Integration of molecular characterization of microorganisms in a global antimicrobial resistance surveillance program. Clin. Infect. Dis., 32: S156-167.

Pokharel BM, Koirala J, Dahal RK, Mishra SK, Khadga PK, Tuladhar NR (2006). Multidrug-resistant and extended-spectrum β-lactamase (ESBL)–producing *Salmonella enterica* (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: surveillance of resistance and a search for newer alternatives. Int. J. Infect. Dis., 10: 434–438.

Rasheed JK, AndersonJGJ, Yigit H, Queenan AM, Doménech-Sánchez A, Swenson JM, Biddle JW, Ferraro MJ, Jacoby GA, Tenover FC (2000). Characterization of the extended spectrum β-lactamase reference strain, klebsiella pneumonia K6 (ATCC 700603), which produces the novel enzyme SHV-18. Antimicrob. Agents Chemother., 44: 2382-2388.

Robberts FJL, PC Kohner PC, Patel R (2009). Unreliable Extended-spectrum B-lactamase Detection in the Presence of Plasmid-Mediated Ampc in *Escherichia coli* Clinical Isolates. J. Clin. Microbiol., 47: 358-361.

Rotimi VO, Jamal W, Pal T, Sovenned A, Albert MJ (2008). Emergence of CTX-M-15 type extended-spectrum beta-lactamaseproducing *Salmonella* spp. in Kuwait and the United Arab Emirates. J. Med. Microbiol., 57: 881–886.

Sambrook J, Fritsch E F, Maniatis T (1989). In: Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, NY, USA.

Su LH, Wu TL, Chia JH, Chu C, Kuo AJ, Chiu CH (2005). Increasing ceftriaxone resistance in *Salmonella* isolates from a university hospital in Taiwan. J. Antimicrob. Chemother., 55: 846-852.

Threlfall EJ, Fisher IST, Berghold C, Gerner-Smidt P, Tschape H, Cormican M, Luzzi I, Schnieder F, Wannet W, Machado J, Edwards G (2003). Trends in antimicrobial drug resistance in *Salmonella enterica* serotypes Typhi and Paratyphi A isolated in Europe, 1999-2001. Int. J. Antimicrob. Agents., 22: 487-491.

Tzouvelekis LS, Lukova V, Tassios PT, Fluit AC, Jones RN, Legakis NJ (2003). Resistance to ß-lactams among blood isolates of Salmonella spp. in European hospitals: results from the SENTRY Antimicrobial Surveillance Program 1997-98. Clin. Microbiol. Infect., 9: 149-152.

Weill F X, Demartin M, Tande D, Espie E, Rakotoarivony I, Grimont PA (2004a). SHV-12-like extended-spectrum-beta-lactamase-producing strains of *Salmonella enterica* serotypes Babelsberg and Enteritidis isolated in France among infants adopted from Mali. J. Clin. Microbiol., 42: 2432-2437.

Weill FX, Perrier-Gros-Claude JD, Demartin M, Coignard S, Grimont PA (2004b). Characterization of extended-spectrum β-lactamase (CTX-M-15)-producing strains of Salmonella enterica isolated in France and Senegal. FEMS. Microbiol. Lett., 238: 353–358.

Winokur PL, Canton R, Casellas JM, Legakis NJ (2001). Variations in the prevalence of strains expressing an extended spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas and the Western Pacific region. Clin. Infect. Dis., 32: S94–103.

Zaki SA, Karande S (2011). Multidrug-resistant typhoid fever: a review. J. Infect. Dev. Ctries, 5: 324-337.