Original Article

Antibiotic resistance among *Salmonella enterica* serovars Typhi and Paratyphi A in Pakistan (2001-2006)

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Abstract

Objectives: To compare antimicrobial resistance in *S*. Typhi and *S.* Paratyphi A isolates from Pakistan.

Methods: Blood samples were collected through > 175 laboratory collection points in major cities and towns across the country. The study included 3,671 *S*. Typhi and 1,475 *S.* Paratyphi A isolates (2001-2006). Multidrug resistance (MDR) was defined as resistance to first-line agents co-trimoxazole, chloramphenicol and ampicillin.

Results: In total, 79.3% *S*. Typhi and 59.9% *S*. Paratyphi A were isolated from patients under 15 years of age. During the study period, the MDR rate increased in *S*. Typhi (34.2 to 48.5% p<0.001), but decreased in *S*. Paratyphi A (44.5 to 8.6% p<0.001). Quinolone resistance (MIC>1μg/ml) increased in both *S*. Typhi (1.6 to 64.1% p<0.001) and *S*. Paratyphi A (0 to 47% p<0.001). The increase in the proportion of strains showing high level quinolone resistance (MIC >4μg/ml) was greater in S. Paratyphi A when compared to *S*. Typhi. Resistance to first-line drugs was higher in those <15 years of age whereas quinolone resistance was higher in older patients.

Conclusion: Differences between *S*. Typhi and *S*. Paratyphi A, in terms of evolution of resistance to first-line agents and to quinolones, are evident in this population. The rapid increase in quinolone resistance in S. Paratyphi A when compared to S. Typhi is concerning and requires further study.

Key Words: *Salmonella*, Antibiotic resistance, Typhi, Paratyphi, Pakistan

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Introduction

Enteric fever is an acute, life-threatening, febrile illness caused by *Salmonella enterica* serovars Typhi, Paratyphi A, B and C, and is estimated to be responsible for 21 million illnesses and 200,000 deaths worldwide annually. It is further associated with significant economic consequences in developing countries [1]. The incidence rate of enteric fever in central and south Asia is estimated at more than 100 cases/100,000 population per year with the highest burden of disease seen in children [1-5].

Emergence of antimicrobial resistance, in particular multidrug resistance (MDR, resistance to chloramphenicol, ampicillin and co-trimoxazole) has greatly complicated disease management [6,7]. MDR *S*. Typhi first reported from Bangladesh in 1986 and from Pakistan in 1988 is currently endemic in South Asia [8-10]. Resistance in *S*. Paratyphi A is also emerging with a number of reports documenting prevalence of MDR *S*. Paratyphi A in this region [11-12].

Fluoroquinolones are regarded as the treatment of choice for enteric fever in adults. However, limited access to reliable clinical microbiology facilities and the ready availability and excessive use of low-cost substandard generics of fluoroquinolone has led to the emergence of fluoroquinolone resistance [13]. Clinical failures and inadequate responses to therapy are documented in patients infected with *Salmonella* strains with reduced susceptibility to fluoroquinolones (MIC > 0.125 ug/ml) [14-17]. Point mutations in chromosomal genes (*gyrA*, *gyrB*, *parC*, and *parE*) resulting in strains with reduced susceptibility as well as plasmid-encoded transferable resistance to fluoroquinolones have been reported from many countries [18-24].

In Pakistan, the incidence of culture-proven enteric fever in children is estimated at 170 per 100,000 of the population, whereas serology

based incidence is estimated at 710 per 100,000 of the population [25]. Published reports suggest that the MDR rate amongst *S*. Typhi in the country decreased from 50% in 1995 to 20% in 2001 [6] while the rate in *S*. Paratyphi A increased from 14% in 1996 to 44% in 2003 [26]. The aim of this study was to explore the current level of resistance to first-line agents and to quinolones amongst *S*. *enterica* serovars Typhi and Paratyphi A in Pakistan. We further aimed to study the change in antimicrobial resistance among these organisms over a six-year period, 2001-2006.

Material and Methods

S. *enterica* serovars Typhi and Paratyphi A isolated from blood cultures at the Aga Khan University's Clinical Microbiology Laboratory were included in the study. Aga Khan University Hospital is a 550-bed, tertiary care centre located in Karachi, Pakistan. Its clinical laboratory receives samples from both in-patients and outpatients. The latter are collected through more than 175 collection units located in all major cities and towns across the country.

From January 2001 to December 2006, a total of 175,987 blood specimens were submitted to the clinical laboratory. Blood specimens were processed in the BACTEC system (Becton and Dickenson, USA). Positive blood samples were further cultured on sheep blood, chocolate and McConkey agar plates and incubated at 35^0-36^0C for 24 to 48 hours. Suggestive non-lactose-fermenter colonies were identified on the basis of standard biochemical tests using API20E and agglutination with *Salmonella* 09, Vi-specific and Hd antisera (*S*. Typhi), and with 02 and Ha antisera (*S*. Paratyphi A) (Difco laboratories). Antimicrobial susceptibility tests were performed using the Kirby Bauer disc diffusion method in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines. Discs used included chloramphenicol (30µg), ampicillin (10µg), co-trimoxazole (1.25/23.75µg), nalidixic acid (30µg) and ofloxacin (5µg) [27]. Isolates resistant to chloramphenicol, ampicillin, and co-trimoxazole were termed multi-drug resistant (MDR).

Strains resistant to nalidixic acid were further confirmed as being fluoroquinolone resistant using minimum inhibitory concentration (MIC). MIC was determined by the standard agar dilution method using Muller Hinton agar as described in CLSI using *Escherichia coli* ATCC 25922 as a control 27. A breakpoint of 0.125µg/ml was used as reduced susceptibility, and MIC of >1.0µg/ml as resistance to quinolones [28].

The data was extracted from the computerised hospital information system and transferred to SPSS 15.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. In descriptive analysis, means and standard deviations of the continuous variables and percentages of the categorical variables were computed. In inferential analysis, comparisons between resistant and sensitive strains by gender, age and location were conducted through chi square or Fisher's exact test where appropriate. Year-wise trends were assessed through a chi square test for trend. A p-value of less than or equal to 5% was considered as statistically significant.

Results

A total of 3,671 *Salmonella* Typhi and 1,475 *Salmonella* Paratyphi A strains isolated between 2001 and 2006 were studied. In both *S*. Typhi and *S*. Paratyphi A, the majority of isolates were from children under the age of 15 years. However, as shown in Table 1, there was a greater representation of *S*. Typhi (79.3%) in the under 15 group as compared to *S*. Paratyphi A (59.9%) p<0.001. No gender related difference in isolation rates of *S*. Typhi and *S*. Paratyphi A were detected.

Table 1. Descriptive characteristics of patients positive for *S. enterica* serovar Typhi and Paratyphi A (2001-2006).

Characteristics	*S. Typhi* n (%)	*S. para A* n (%)	p value
Age in years			
<15 Years	2910 (79.3)	895 (59.9)	
15 & above	735 (20.0)	592 (39.8)	<0.001*
Missing information	26 (0.7)	8 (0.5)	
Gender			
Male	2125 (57.9)	842 (56.3)	
Female	1544 (42.0)	651 (43.5)	0.316
Missing information	2 (0.1)	2 (0.1)	
Total Lumber	3671	1495	

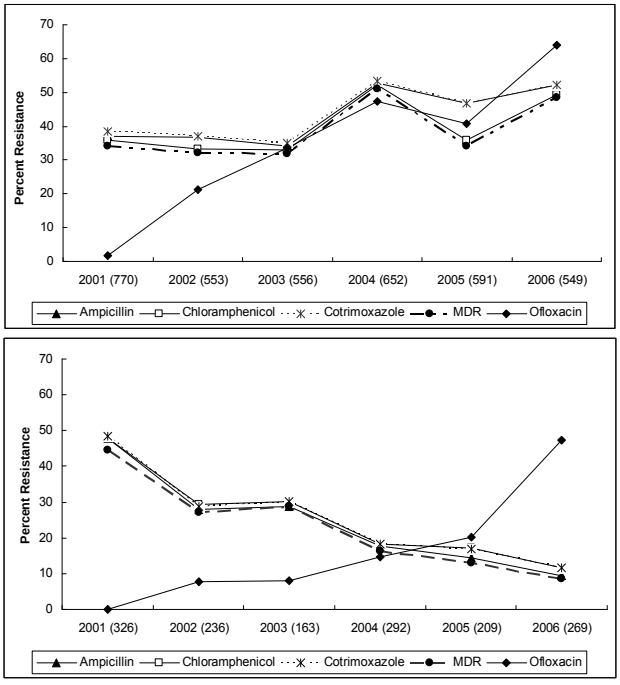
p-value calculated through chi squared test excluding the missing values, significance (*) p<0.05.

During the 6-year study period, resistance to co-trimoxazole, ampicillin and chloramphenicol showed a steady increase in *S*. Typhi (p<0.001). Conversely, the trend in *S*. Paratyphi A saw the MDR rate fall from 44.5% in 2001 to 8.6% in 2006

(p<0.001). During the same period, an increase in fluoroquinolone resistance was noted in both the groups (p<0.001) (Figures 1A & 1B).

Figure 1. A (upper): Antimicrobial resistance; *S. enterica* serovar Typhi (2001-2006). B (lower): Antimicrobial resistance; *S. enterica* serovar Paratyphi A (2001-2006).

Resistance is expressed as a percentage of the total strains isolated per year. The number of strains tested per year is depicted; (n value).
Resistance to individual first-line agents as well as MDR increased in *S*. Typhi (p< 0.001), but decreased in *S*. Paratyphi (p<0.001).
Resistance to Ofloxacin increased significantly in both organisms studied (p<0.001).

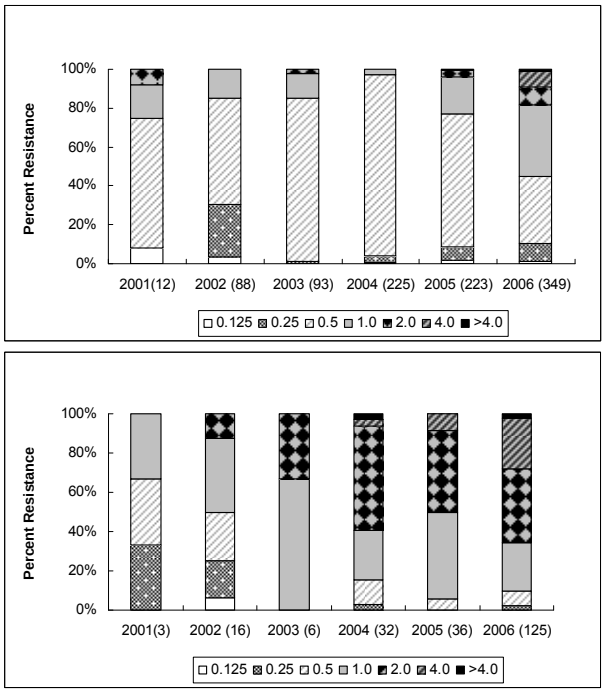


Comparison of the cumulative data (2001-2006) for *S*. Typhi and *S*. Paratyphi A suggests a significant association between MDR and ofloxacin resistance in *S*. Typhi; 59.8% of MDR S. Typhi isolates were resistant to ofloxacin compared to only 16.5% of sensitive S. Typhi isolates (p<0.001). This trend, however, did not hold true for *S*. Paratyphi A where a negative association between MDR and quinolone resistance was apparent: 5.4% of MDR *S*. Paratyphi A isolates were resistant to ofloxacin as opposed to 19.6% of sensitive isolates (p<0.001).

Analysis of MIC levels to ofloxacin in both the study organisms showed that the level of quinolone resistance in terms of MIC values was increasing in both *S*. Typhi and *S*. Paratyphi A over the 6-year study period. The increase in percentage of strains with ofloxacin MIC levels (>4mg/ml) was, however, markedly greater in *S*. Paratyphi A 0% (2001) to 28% (2006) as compared to *S*. Typhi 0% (2001 to 9%(2006) (Figures 2A & 2B).

Figure 2. A: MIC to Ofloxacin; *S. enterica* serovar Typhi; B: MIC to Ofloxacin; *S. enterica* serovar Paratyphi A.

Minimum Inhibitory Concentration (MIC) values for *S*. Typhi and paratyphi A that were naladixic acid resistant on initial screening (2001-2006).
The number of strains tested per year is shown (n value).
MIC values (range 0.125 to >4.0 μg/ml) are as follows as depicted in figure legend.



Isolates from younger patients (<15years) were more likely to be resistant to ampicillin (p=0.017) and co-trimoxazole (p=0.016) in the case of *S.* Typhi, and to ampicillin (p=0.002), chloramphenicol (p<0.001) and co-trimoxazole (p<0.001) in cases of *S*. Paratyphi A. Resistance to fluoroquinolones in both organisms, however, was more likely in the older age groups (p<0.001) (Table 2).

Table 2. Distribution of antibiotic sensitivity in *S. enterica* serovars Typhi and Paratyphi isolates according to age (2001-2006).

Feature	Age (y)	n	Amp	Chloram	Cotrim	MDR	Oflox
			%-R	%-R	%-R	%-R	%-R
ST (n:3671)	<15	2909	44.2	40.5	44.8	39.3	31.2
	≥15	735	39.4	38.2	39.9	36.1	41.4
	P		0.017*	0.270	0.016*	0.107	<0.001*

SPT (n:1495)	<15	895	27.9	29.5	29.9	26.6	13.5
	≥15	592	20.7	21.3	20.3	19.1	20.8
	P		0.002*	<0.001*	<0.001*	0.001*	<0.001*

ST= *S.* Typhi; SPT= *S.* Paraty-phi A; Amp = Ampicillin; Chloram = Chloramphenicol; Cotrim = Co-trimoxzole; MDR = Multi drug resistant = Resistant to Ampicillin, Chloramphenicol and Co-trimoxazole; Oflox = Ofloxacin. Age analysis was not conducted for 27 S. Typhi and 8 S. Paratyphi A patients due to missing information. p-value calculated through chi squared test excluding the missing values, significance (*) p<0.05.

Discussion

We compared resistance trends of *S*. *enterica* serovars Typhi and *S*. Paratyphi A isolated between 2001 and 2006. During that period, the isolation rate of S. Typhi was significantly greater than that of *S.* Paratyphi A. This finding is in agreement with published data indicating typhoid fever as more prevalent than paratyphoid in this region [12,25,29].

A higher frequency of both Typhi and Paratyphi A in the younger age group, reported in this study, also concurs with published literature showing a greater burden of enteric fever in children as compared to adults [2-5,25]. Comparison of strain distribution between adults and children showed that children bore 79.3% of the *S*. Typhi burden as compared to 59.9% of *S*. Paratyphi A. This difference, while similar to that reported from India [29,30], is difficult to explain. It is possible to hypothesise that, when compared to *S*. Typhi, the lower overall prevalence of *S*. Paratyphi A in the community reduces chances of exposure and hence likelihood of disease in the younger age group.

The increase in resistance amongst *S*. Typhi to the first-line agents co-trimoxazole, chloramphenicol and ampicillin is concerning and likely to reflect persistence and dissemination of resistant *S*. Typhi strains within our community. In contrast, the decrease in resistance to the first-line agents in *S*. Paratyphi A is encouraging but surprising in view of earlier reports of upward resistance trends among *S*. Paratyphi A in the country [26]. A similar decrease in resistance among *S*. Paratyphi A has also been documented in a recent report from India [31]. It is possible to link this downward trend in resistance among *S*. Paratyphi A to the fact that third-generation cephalosporins have, to a large extent, replaced co-trimoxazole, chloramphenicol and ampicillin as the treatment of choice for enteric fever in children. Our data further suggests, however, that the resistant strains of *S*. Paratyphi A have not succeeded in establishing themselves in the community. It has been suggested that antimicrobial resistance in *Salmonella* may be associated with a fitness cost [32]. Whether the resistance in *S*. Paratyphi A has an associated fitness cost needs to be investigated. *S*. Paratyphi A has been shown to harbour resistance plasmids [33]. It is possible that in the absence of antibiotic pressure, these plasmids are not retained but may be re-acquired when antibiotic pressure is reimposed [34].

Fluoroquinolone resistance in *S*. Typhi as well as in *S*. Paratyphi A increased during the 6-year study period, both in terms of frequency and MIC levels. Fluoroquinolones available over the counter in the country are used to treat a number of infections including urinary and respiratory tract infections. Resistance is most likely a consequence of their widespread use in the community. Such increase in resistance to fluoroquinolones amongst *S*. enterica has been widely reported [14-17]. However, the sharp rise in *S*. Paratyphi A MIC levels as compared to *S*. Typhi is concerning. This observation, suggesting that *S*. Paratyphi A is more prone to developing fluoroquinolone resistance, requires further exploration.

The difference in resistance between the younger and older age groups noted in this study is difficult to explain. Moreover, our finding is in contrast to data from Delhi reporting no difference in antimicrobial resistance across age groups in their patients [30]. Our data of higher resistance to co-trimoxazole, chloramphenicol and ampicillin among isolates from the younger age group and greater fluoroquinolone resistance in strains from older patients is consistent with antimicrobial usage in these two patient groups. The difference between our study and that from Delhi may well be related to the fact that, in the Delhi study, patients with history of antimicrobial therapy were excluded. The finding of age-related difference in antimicrobial resistance is also surprising given that risk factors for typhoid reportedly include a recent case in the family [35,36]. However, typhoid fever in endemic settings has been linked with high-dose exposure from multiple sources and therefore the possibility of an extra-familial source cannot be excluded in such cases [37]. Prior antimicrobial usage is reported to be a risk factor for typhoid [37,38]. However, the impact of such antimicrobials on the resistance pattern of the infecting organisms needs to be considered. The

role of commensal flora within host intestinal flora harbouring resistance plasmids and contributing to development of resistance *S*. *enterica* serovars is also a distinct possibility requiring exploration.

In conclusion, our data suggests that there appear to be differences between *S. enterica* serovar Typhi and *S*. Paratyphi A in terms of resistance trends to first-line agents, co-trimoxazole, chloramphenicol and ampicillin. These differences need to be explored further. Increasing resistance to fluoroquinolones is alarming and of particular concern is the rapid rise in MIC levels among *S*. Paratyphi A. Similarly, differences in resistance patterns between younger and older age groups need to be further studied.

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