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Antibiogram Profile of *Salmonella enterica* Serovar Typhi in India – A Two Year Study

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Abstrak: Demam kepialu masih merupakan suatu masalah kesihatan yang serius di negara-negara membangun, dan kemunculan strain rintang-multidrug (MDR) telah mengurangkan pilihan terapeutik untuk rawatan penyakit ini. *National Salmonella and Escherichia Centre* di Kasauli, India telah menerima sebanyak 128 pencilan *Salmonella* Typhi pada tahun 2008–2009. Pencilan-pencilan ini telah dinilai untuk rintangan antimikrob*, prevalent resistotypes* dan perkadaran strain MDR, menggunakan kaedah standard untuk 11 antimikrob. Suatu penurunan yang mendadak dalam perkadaran strain MDR telah diperhatikan. Hanya 4.7% daripada pencilan didapati MDR dengan *resistotypes* chloramphenicol-ampicillin-streptomisin-asid nalidiksik-trimethoprim (C-A-S-Na-Tr) dan chloramphenicol-ampicillin-asid nalidiksik-trimethoprim (C-A-Na-Tr), yang nilainya lebih rendah jika dibandingkan dengan kajian-kajian lain di India. Rintangan asid nalidiksik didapati wujud dalam 93.8% pencilan. Lebih-lebih lagi, perbezaan dalam min *minimum inhibitory concentration* (MIC) bagi ciprofloxacin untuk strain rintangan asid nalidiksik dan strain peka asid nalidiksik didapati signifikan (p<0.001), yang membawa perhatian kepada penggunaan lanjutan ciprofloxacin dalam rawatan demam kepialu kerana potensi kegagalan rawatan. Kadar rendah strain MDR meningkatkan kebarangkalian *first-line drugs* untuk rawatan demam kepialu.

Kata kunci: Rintangan Multi-drug, *Salmonella* Typhi, Rintangan Antimikrob

Abstract: Typhoid fever continues to remain a major health problem in the developing world, and the emergence of multidrug-resistant (MDR) strains has further reduced therapeutic options for treatment of the disease. The National Salmonella and Escherichia Centre in Kasauli, India received 128 *Salmonella* Typhi isolates during 2008–2009. These were evaluated for antimicrobial resistance, prevalent resistotypes and the proportion of MDR strains, using standard methods for 11 antimicrobials. An abrupt decrease in the proportion of MDR strains was observed. Only 4.7% of the isolates were found to be MDR with resistotypes chloramphenicol-ampicillin-streptomycin-nalidixic acid-trimethoprim (C-A-S-Na-Tr) and chloramphenicol-ampicillin-nalidixic acid-trimethoprim (C-A-Na-Tr), which is very low compared to other studies from India. Nalidixic acid resistance was found to be present in 93.8% of the isolates. Moreover, the difference in the mean minimum inhibitory concentration (MIC) of ciprofloxacin for nalidixic acid-resistant and nalidixic acid-sensitive strains was found to be statistically significant (p<0.001), which calls into question the further use of ciprofloxacin for the treatment of typhoid fever because of potential treatment failures. The low proportion of MDR strains increases the possibility of first-line drugs for the treatment of typhoid fever.

Keywords: Multidrug Resistance, *Salmonella* Typhi, Antimicrobial Resistance

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INTRODUCTION

Enteric fever continues to be a major health problem despite the use of antibiotics and the development of novel antibiotics. The highest proportion of reported cases and deaths have been reported in Asia, followed by Africa and Latin America (WHO 1996). Approximately 22 million new cases of enteric fever have been reported to occur annually, resulting in 200,000 deaths (Crump *et al.* 2004). *Salmonella enterica* serovars Typhi and Paratyphi A are the predominant aetiological agents responsible for enteric fever in India (Jesudason & John 1992).

Since 1948, chloramphenicol has effectively reduced the morbidity and mortality associated with typhoid fever (Woodward *et al.* 1948); however, the emergence of chloramphenicol-resistant strains has rendered it less effective (Paniker & Vimala 1972). Treatment has been further complicated by the emergence of antimicrobial resistance to ampicillin and cotrimoxazole. In India, antibiotic resistance in *S*. Typhi strains has been reported since 1960, and the first outbreak of multidrug-resistant (MDR) *S*. Typhi was reported in Calicut (Agarwal 1962).

Since then, MDR *S.* Typhi (MDRST) strains have been reported in India (Jesudason & John 1990), Pakistan, the Arab Gulf (Rowe *et al.* 1990) and Vietnam (Rowe *et al.* 1997). MDR strains have subsequently developed resistance to fluoroquinolones (Rowe *et al.* 1995). Moreover, an epidemic of quinolone-resistant MDRST has also been reported in Tajikistan (Murdoch *et al.* 1998). A decrease in the incidence of MDRST has been reported in several studies from India (Chande *et al.* 2002; Saha *et al.* 2002; Sanghavi *et al.* 1999). A decline in MDR isolates with no corresponding increase in sensitive strains has been reported in Bangladesh (Rahman *et al.* 2002); in contrast, a resurgence of resistant strains has been reported in Ludhiana, India (Kumar *et al.* 2002).

Therefore, we evaluated the status of MDR strains of *S*. Typhi in the country to clarify the present status of MDR among typhoid bacillus.

MATERIALS AND METHODS

Test Strains

The study included 128 *S*. Typhi isolates received during the years 2008–2009 at the National Salmonella and Escherichia Centre (the National Reference Laboratory for serotyping of *Salmonella* and *Escherichia coli*), Central Research Institute, Kasauli, India. The isolates were obtained from blood samples (received from different parts of India), and the ages of the patients ranged from 1.5 months to 69 years.

Biochemical Identification

Biochemical identification of the isolates was performed as described by Holt *et al.* (1994). Briefly, the sample was inoculated on a MacConkey agar plate and incubated at 37°C overnight. Using a well-isolated colony from the MacConkey

agar plate, various biochemical media were inoculated and incubated overnight at 37°C. Subsequently, the results were observed and interpreted.

Serological Identification

The serological identification of the isolates was performed as described by Popoff & Minor (1992). Briefly, the isolate was inoculated on a nutrient agar slant and incubated at 37°C overnight. A small amount of the culture was picked up from the slant and emulsified in normal saline on a glass slide. The emulsified culture was then mixed separately with various antisera [(Anti-O9, Anti-O12, Anti-Vi and Anti-Hd) Becton-Dickinson, USA] and observed for agglutination.

Disc Diffusion Susceptibility Testing

The antibiotic susceptibility patterns of the isolates were determined by the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and interpretative criteria [National Committee for Clinical Laboratory Standards (NCCLS) 2000] using antibiotic discs (Hi Media Lab. Pvt. Ltd., Mumbai, India), viz., cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg) ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg) and trimethoprim (5 µg). Briefly, a well-isolated single colony was inoculated in peptone water and incubated for 4–6 hrs. The growth was adjusted to match 0.5 MacFarland standards (NCCLS 2000). The adjusted inoculum was applied to dried Mueller-Hinton agar. Antimicrobial discs were applied to the inoculated plates and incubated overnight at 37°C. Subsequently, the plates were read and the results were interpreted as sensitive, intermediate or resistant. *Escherichia coli* strain ATCC 25922 was used as the control.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) were determined by the agar dilution method (CLSI, 2006) using purified antibiotic powders (Hi Media Laboratories, Pvt. Ltd., Mumbai, India). Briefly, several dilutions of the antibiotics were prepared in the plates by adding the required amount of the antibiotic in the molten Mueller-Hinton agar. The test strains were inoculated onto the Mueller-Hinton agar plates containing different dilutions of antibiotic and incubated at 37°C overnight. The plates were observed and the MIC was the highest dilution of antibiotic inhibiting the growth of the test strain.

Statistical Analysis

The mean MICs of ciprofloxacin for the nalidixic acid-resistant and nalidixic acid-sensitive strains were analysed using Student's t-test.

RESULTS

Of the 128 isolates, only 6 MDR isolates were encountered (Table 1). Susceptibility to ceftazidime, ciprofloxacin and gentamicin was observed for 100% of the isolates in the year 2008, whereas in the year 2009, the

susceptibility remained the same for ciprofloxacin and ceftazidime but decreased to 97.44% for gentamicin. The highest resistances were to nalidixic acid in both years. No resistance was observed for cefotaxime, ceftazidime, ciprofloxacin, tetracycline, gentamicin, and kanamycin during the study period. In total, 10% of the strains were found to be resistant for chloramphenicol, ampicillin, streptomycin and trimethoprim in the year 2008, whereas a decreased proportion (1.28%) was found to be resistant for the same antibiotics in the year 2009. Susceptibility to all tested drugs was found in 6.25% of the isolates. Of the three resistotypes, two MDR resistotypes were observed (Table 2), and most of the isolates were found to be resistant to nalidixic acid. The difference in the mean MIC values of ciprofloxacin for nalidixic acid-resistant *S.* Typhi (NARST) and nalidixic acid-sensitive *S.* Typhi (NASST) was found to be statistically significant (p<0.001).

The MIC range for the nalidixic acid-resistant strains was 480–960 µg/ml, and those for chloramphenicol, ampicillin, streptomycin and trimethoprim were 32–64 µg/ml, 32–512 µg/ml, 32–64 µg/ml and 20–40 µg/ml, respectively (Table 3).

Table 1: Antibiogram pattern of *S. enterica* serovar Typhi strains.

S. no.	Antibiotic	N (%) of isolates (2008)			N (%) of Isolates (2009)		
		Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
1.	Cephotaxime	49 (98)	1 (2)	0 (0)	72 (92.31)	6 (7.69)	0 (0)
2.	Chloramphenicol	45 (90)	0 (0)	5 (10)	77 (98.72)	0 (0)	1 (1.28)
3.	Ceftazidime	50 (100)	0 (0)	0 (0)	78 (100)	0 (0)	0 (0)
4.	Ciprofloxacin	50 (100)	0 (0)	0 (0)	78 (100)	0 (0)	0 (0)
5.	Tetracycline	49 (98)	1 (2)	0 (0)	72 (92.31)	6 (7.69)	0 (0)
6.	Ampicillin	45 (90)	0 (0)	5 (10)	77 (98.72)	0 (0)	1 (1.28)
7.	Streptomycin	30 (60)	16 (32)	4 (8)	75 (96.15)	2 (2.56)	1 (1.28)
8.	Gentamicin	50 (100)	0 (0)	0 (0)	76 (97.44)	2 (2.56)	0 (0)
9.	Kanamycin	42 (84)	8 (16)	0 (0)	68 (87.18)	10 (12.82)	0 (0)
10.	Nalidixic acid	3 (6)	0 (0)	47 (94)	5 (6.41)	0 (0)	73 (93.59)
11.	Trimethoprim	45 (90)	0 (0)	5 (10)	77 (98.7)	0 (0)	1 (1.28)

Table 2: Resistotypes observed during the study period.

Resistotypes	Percentage of isolates*	
	2008	2009
C-A-S-Na-Tr	8	1.3
C-A-Na-Tr	2	–
Na	96	93.6

Notes: C-chloramphenicol; A-ampicillin; S-streptomycin; Na-nalidixic acid; Tr-trimethoprim

* $\frac{\text{No. of isolates with a particular resistotype in the year}}{\text{Total no. of isolates in the year}}$

Table 3: MICs among *S. enterica* serovar Typhi isolates during 2008–2009.

S. no.	Antibiotic	MIC (µg/ml)		
		Sensitive	Intermediate	Resistant
1.	Cephotaxime	0.014–1.875	20	–
2.	Chloramphenicol	0.9375–7.5	20	32–64
3.	Ceftazidime	0.059–3.75	–	–
4.	Ciprofloxacin	0.0097–0.78*	–	–
5.	Tetracycline	0.237–7.5	10	–
6.	Ampicillin	0.3125–5.0	–	32–512
7.	Streptomycin	2.5–5.0	10	32–64
8.	Gentamicin	0.029–2.5	10	–
9.	Kanamycin	1.875–7.5	30	–
10.	Nalidixic acid	3.75–7.5	–	480–960
11.	Trimethoprim	0.015–0.625	–	20–40

Note: *Difference between mean MIC of ciprofloxacin for NARST and NASST was statistically significant, compared by Student's t-test.

DISCUSSION

Since 1948, chloramphenicol had been the treatment of choice for typhoid fever until the emergence of chloramphenicol-resistant *S*. Typhi in the early 1970s, which caused outbreaks in Mexico, South India, Vietnam, Korea and Thailand (Srivastava & Aggarwal 1994). Chloramphenicol has been reported to reduce mortality from approximately 20% to 1%, along with a decrease in the duration of the fever from 14–28 days to 3–5 days (Mandal *et al.* 2004). After the emergence of chloramphenicol-resistant strains, ampicillin and cotrimoxazole were found to be effective alternative drugs; however, the explosive emergence of strains of *S*. Typhi with resistance to trimethoprim and ampicillin has caused many problems in developing countries.

Since 1989, outbreaks caused by *S*. Typhi resistant to streptomycin, sulphonamides and tetracycline have been reported in many countries, especially Pakistan and India (Mandal 1990). Such strains have been termed MDR. MDR strains have also caused outbreaks in Bangladesh (Albert *et al.* 1991), several countries in Southeast Asia (Tinya-Superable *et al.* 1995) and South Africa (Mourad *et al.* 1993). Outbreaks associated with immigrant workers from the Indian subcontinent have also been reported in the Arabian Gulf (Wallace *et al.* 1993).

MDR strains are a major therapeutic concern for physicians in developing countries. Contributing factors may include drug misuse and inappropriate prescribing practices as well as intrinsic plasmid-mediated factors. MDRST has been reported to cause severe illness resulting in serious complications and high

mortality (Pang *et al.* 1995). The increased virulence of MDR strains may be due to the presence of other virulence genes on its R-plasmid.

Of the 128 isolates of the *S.* Typhi strain, 122 (95.3%) were found to be susceptible for chloramphenicol, ampicillin and trimethoprim (Table 1). In the present study, the re-emergence of susceptibility to historically used drugs in strains of *S.* Typhi was observed in higher proportions than those reported from New Delhi and Kolkata (Raveendran *et al.* 2008; Sen *et al.* 2007; Achla *et al.* 2005), with the exception of one study from northern India which reported 96% sensitivity to chloramphenicol (Madhulika *et al.* 2004). The results of the present study are in agreement with other studies from the central west part of India, which also reported sensitivities to chloramphenicol, ampicillin and trimethoprim (Nagshetty *et al.* 2010; Krishnan *et al.* 2009) except for Nagur that reported 100% resistance to these drugs (Tankhiwale *et al.* 2003).

The re-emergence of susceptibility to these drugs may be a result of the emergence of *de novo* susceptible strains or the loss of high molecular weight self-transmissible plasmids (Misra *et al.* 2005). However, resistance may develop again if MDR strains are able to transfer their R-plasmids, which encode resistance determinants, to the strains sensitive to these drugs.

These findings may be of immense importance to health authorities in order to rationalise the policy of empirical treatment of typhoid fever. Furthermore, a return to the use of these drugs includes advantages such as their availability in the developing world, lower cost and well-established clinical efficacy (Kumar *et al.* 2009). It should be noted that the risk of relapse and the development of a carrier state have been found to be higher among patients treated with ampicillin than those treated with chloramphenicol (Sood *et al.* 1999). The values of the MICs for sensitive, intermediate and resistant isolates for different antibiotics used in the study are well within the ranges specified by CLSI (2006), except for streptomycin, for which there is no clear consensus regarding the interpretation of susceptibility test results. In the present study, the MIC values of streptomycin ranged from 2.5–5 µg/ml and 32–64 µg/ml for sensitive and resistant isolates respectively whereas it was found to be 10 µg/ml for the isolates having intermediate susceptibilities (Table 3).

Although streptomycin has not used to treat *Salmonella* infections, testing for susceptibility to streptomycin is widely used as an epidemiological marker (Doran *et al.* 2006). For example, resistance to streptomycin is part of the characteristic phenotype of *S.* Typhi resistance to chloramphenicol, ampicillin, streptomycin and trimethoprim.

External quality assessment exercises involving laboratories across Europe that report susceptibility test results for isolates of *Salmonella* have found that agreement between laboratories, although quite high overall, was least satisfactory for streptomycin (Threlfall *et al.* 1999). One reason for this poor agreement is the absence of widely accepted criteria for the interpretation of susceptibility testing for streptomycin.

The CLSI specifies a disk content (10 µg), a method and interpretative criteria (susceptible, ≥15 mm; intermediate, 12–14 mm; resistant, ≤11 mm) for the disk diffusion susceptibility testing of streptomycin against Enterobacteriaceae

(CLSI 2006). Unlike most antimicrobial agents, however, there are no corresponding CLSI interpretative criteria for dilution susceptibility testing.

Although several individual studies have been conducted to assess the interpretative criteria for dilution susceptibility testing of streptomycin (Bywater *et al.* 2004; Madsen *et al.* 2000), the re-evaluation of interpretative criteria for susceptibility testing of streptomycin against Enterobacteriaceae has received a low priority due to little clinical interest.

In the present study, a significant decrease in the proportion of MDR strains (Table 2) was found compared to earlier reports that indicated multidrug resistance of up to 64.2% (Nagshetty *et al.* 2010; Krishnan *et al.* 2009; Madhulika *et al.* 2004; Tankhiwale *et al.* 2003). Surprisingly, MDR strains did not replace or gain a survival advantage over susceptible strains, as both susceptible and resistant strains were isolated. However, a significant decrease in the proportion of MDR strains was observed, indicating that the MDR typhoid epidemic in the country is waning. Similar observations have also been reported from other studies in the country (Nagshetty *et al.* 2010; Krishnan *et al.* 2009; Madhulika *et al.* 2004).

Moreover, contrary to earlier studies (Rahman *et al.* 2002; Jesudason *et al.* 1996), the proportion of isolates susceptible to all antimicrobials except nalidixic acid was found to be relatively high, indicating the emergence of susceptible strains of *S*. Typhi. This may be due to the much lower proportion of MDRST, thereby reducing the possibility of horizontal transfer of the R-plasmid to other strains.

Continuing previous work from our laboratory (Kumar *et al.* 2009), isolation of high proportions of NARST were still observed in 2009, although they were lower than in 2008. Resistance to nalidixic acid is a marker for low-level resistance to ciprofloxacin, which may result in treatment failures (Walia *et al.* 2005). The present study shows that in 2009 the NARST isolates showed higher MIC values of ciprofloxacin compared to those in NASST [$p<0.001$]. These values are similar to the study of the isolates in 2008 (Kumar *et al.* 2009). Therefore, the high proportion of NARST raises doubts concerning the efficacy of ciprofloxacin for the treatment of typhoid fever.

Considering the higher MIC values for ciprofloxacin in *S*. Typhi, the low proportion of MDRST indicates that reintroduction of cheaper first-line antibiotics may be effective. However, continued surveillance on antimicrobial susceptibility patterns and the study of MDR is necessary before a definitive statement can be made.

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