INTRODUCTION

Salmonella infection causes significant mortality worldwide. Infections with Salmonellae can result in various clinical presentations like enteric fever, gastroenteritis, septicaemia with or without suppurative lesion and carrier state. Typhoid fever is endemic in developing countries, more so in Indian subcontinent.\(^1\) Salmonella infections, especially those involving bloodstream, have high mortality (about 30%). This can be reduced to about 1% with appropriate use of antibiotics.\(^2\,^3\) Salmonella enterica serotype Typhi and paratyphi A, B and C cause typhoid fever, while non-typhoidal Salmonellae (NTS) that have more than 2500 serotypes, cause gastroenteritis and invasive infections like meningitis and osteomyelitis in immunocompromised patients and children.\(^4\) Salmonella enterica is mostly acquired directly or indirectly through human feces by faecal-oral route from the diseased person or a carrier. In Pakistan, where these infections are endemic, they are a major cause of morbidity and mortality.

Threat of growing resistance to antibiotics is of grave concern to human health. Resistant strains also lead to prolonged illness and more rate of complications.\(^5\) Resistance of Salmonella Typhi to chloramphenicol, cotrimoxazole and ampicillin developed in the 1980s. This led to an increasing use of fluoroquinolones. Gradually resistance also developed to fluoroquinolones. High prevalence of antibiotic resistant Salmonellae pose public health concern since they lead to treatment failure. Multi-drug resistant (MDR) strains (resistant to chloramphenicol, cotrimoxazole and ampicillin) developed in the 1980s. This led to an increasing use of fluoroquinolones. Gradually resistance also developed to fluoroquinolones.

Outbreaks of MDR Salmonella (S.) Typhi may be difficult to manage, especially in developing countries where resources are already limited. Outbreaks have been reported throughout the world especially in south-east Asia, Indian subcontinent, Africa and South America.\(^6\,^7\) An outbreak of MDR S. Typhi in late 1990s in Tajikistan caused more than 24,000 infections.\(^8\) Hence, there is dire need to explore new avenues for treatment of resistant Salmonellae.

The aim of this study was to analyse in vitro effect of therapeutic agents, not commonly used for treatment of Salmonella infections.
METHODOLOGY

It was a cross-sectional, observational research carried out at Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi. Research was conducted from June 2011 to May 2013. Blood cultures from different wards of the hospital, received in the laboratory, were incubated in BACTEC 9050 system. Other specimens like stool, pus and urine were also collected according to the standard protocols. Positive blood culture bottles were sub-cultured on blood, Mac Conkey and XLD agars (Oxoid, UK) after gram stain. For stool specimens, enrichment was done in selenite broth. Non-lactose fermenting colonies, growing on Mac Conkey agar or red/transparent colonies on XLD agar, were identified by standard biochemical and serological tests. All Salmonellae isolates from clinical specimens, isolated from June 2011 to June 2013, were included in the study. Only one isolate per patient (e.g. first strain after admission) was included in the study. Duplicate samples from same subject or those whose diagnosis could not be confirmed were excluded. The isolates were identified using phenotypic colony characteristics and API 10S; and in case of any doubt, confirmed by biochemical reactions with API 20 E (BioMerieux SA, Marcy l’Etoile, France) and serotyping with specific antisera using polyclonal and monoclonal O and H antisera (Bio-Rad, Marnes-la-Coquette, France) according to the Kauffmann-White classification scheme. Salmonella enterica serotype Typhi (Salmonella Typhi) was suspected when the isolate was non-lactose fermenter with little hydrogen sulphide production. It was confirmed by agglutination with serogroup D, somatic antigens 09 and flagellar antigen Hд. Polysaccharide Vi antigen was also tested.

The disks of antimicrobial agents used were chloramphenicol (30 µg), co-trimoxazole (1.25/23.75 µg), ampicillin (10 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), doripenem (10 µg), imipenem (10 µg), ertapenem (10 µg), aztreonam (30 µg), moxifloxacin (5 µg), cefpirome (30 µg), cefepime (30 µg), and gatifloxacin (5 µg). All disks were of Oxoid Company, Hampshire, UK. The inoculated agar plates containing the suitable antibiotic discs were incubated aerobically for 16-18 hours at 36°C and zone diameters were measured. All isolates that were MDR or were intermediate or resistant to ciprofloxacin on disk diffusion were subjected to MIC test using E-test strip (AB Biodisk, Solna, Sweden). The E-test was first validated with broth dilution MIC using cation adjusted Muller-Hinton broth. The same 0.5 McFarland organism suspension of the isolates was used with Mueller-Hinton agar (Oxoid, Hampshire, UK) and incubated under similar conditions, and according to the manufacturer's instructions. The antibiotics tested for MICs were imipenem, cefpirome, aztreonam, cefpodoxime, azithromycin and tigecycline. Escherichia coli ATCC 25922 was used as control for the disk diffusion and MIC testing. The results were interpreted following criteria for Salmonella or Enterobacteriaceae in Clinical Laboratory Standards Institutes guidelines. Isolates that were resistant to ampicillin, cotrimoxazole and chloramphenicol were declared MDR (multi-drug resistant) isolates. Verification studies of E-strips were carried out as and when required.

Based on recommendations of EUCAST-European committee on antimicrobial susceptibility (www.eucast.org) and a previous study, the cut-off for azithromycin was taken to be ≥ 32 µg/ml. Isolates were preserved at -45 / -60°C in nutrient agar with glycerol. To check the relationship between zone diameter in mm and MIC in mg/L, the two variables in our study, we used the Statistical Package for Social Sciences (SPSS) version 15 (IBM Chicago, Illinois, USA). Relationship was checked using Pearson's correlation.

RESULTS

A total of 128 isolates were recovered from 2230 specimens. Out of 128 isolates, 76 (59.3%) isolates were from blood culture, 26 (20.3%) isolates from stool, 16 (12.5%) isolates from pus, 9 (7.0%) from urine, and 1 (1.3%) was from fluids. Median age of the patients was 22 years ranging from 1 to 77 years. The male to female ratio was 9:1. They belonged to 12 different districts, all from north/north-west of the country. Highest number of culture positive cases 85 (51%) were between 8 and 17 years. Total number of cases less than 8 years of age were 9 (8.3%), while total cases of more than 17 years age were 52 (41%).

Resistance percentage of Salmonellae to various antibiotics by Disk diffusion method is given in Table I. For azithromycin, breakpoints were available from BSAC for Salmonella enterica serovar Typhi (S. Typhi) only.

All isolates that were either MDR or were ciprofloxacin intermediate or resistant on disk diffusion technique were subjected to MIC test for the antibiotics for which E-strips were available.

Table I: Resistance percentage of Salmonellae to various antibiotics by disk diffusion method.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>S. Typhi</th>
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<tr>
<td></td>
<td>I</td>
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<tr>
<td>Ampicillin 30µg</td>
<td>12%</td>
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<tr>
<td>Chloramphenicol 30µg</td>
<td>0%</td>
</tr>
<tr>
<td>Cotrimoxazole 30µg</td>
<td>1.2%</td>
</tr>
<tr>
<td>Ceftriaxone 30µg</td>
<td>0%</td>
</tr>
<tr>
<td>Ciprofloxacin 5µg</td>
<td>66%</td>
</tr>
<tr>
<td>Cefpodoxime 10µg</td>
<td>7%</td>
</tr>
<tr>
<td>Doripenem 10µg</td>
<td>1.2%</td>
</tr>
<tr>
<td>Imipenem 10µg</td>
<td>13%</td>
</tr>
<tr>
<td>ertapenem 10µg</td>
<td>3.5%</td>
</tr>
<tr>
<td>Aztreonam 30µg</td>
<td>8.2%</td>
</tr>
<tr>
<td>Gatifloxacin 10µg</td>
<td>14.1%</td>
</tr>
<tr>
<td>Cefepime 30µg</td>
<td>0%</td>
</tr>
<tr>
<td>Gatifloxacin 5µg</td>
<td>0%</td>
</tr>
</tbody>
</table>

For cefpirome and tigecycline, no MIC breakpoints were available in CLSI. Interpretation of tigecycline MIC results was determined according to the recommendations of the United States Food and Drug Administration (US FDA) given in the package insert for treating Enterobacteriaceae (susceptible = 2 µg/ml; 4 = intermediate, resistant = 8 µg/ml) and those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (susceptible = 1 µg/ml; resistant, 2 µg/ml). According to both criteria, all the isolates were sensitive.

For cefpirome, no previous breakpoints could be found. The range of MIC was from 0.047 to 0.75. MIC for antibiotics is given in detail in Table II. The Pearson’s correlation between the two variables was found to be significant (-0.79, p-value < 0.001) and showed a negative correlation between the zone size and MIC.

**DISCUSSION**

In this study, it was attempted to find solutions to the emerging problem of resistance in *Salmonella* Typhi. Azithromycin is oral administered and can be given safely to children. MIC$_{90}$ for azithromycin in *Salmonella* Typhi in these isolates was 16 µg/ml while the earlier studies have reported MIC for it to be in the range of 4 - 16 µg/ml (15 - 17), and MIC$_{90}$ in the study from Karachi was 8 µg/gram/ml.¹⁵ Breakpoints of azithromycin versus Enterobacteriaceae were not available in CLSI till 2014 and is still investigational in 2015. EUCAST has mentioned the MIC for *Salmonella* Typhi as 16 µg/ml. In BSAC (British Society for Antimicrobial Chemotherapy) breakpoints are given for *S.* Typhi but these isolates, according to BSAC criteria (18 ≤ resistant), were mostly resistant in vitro. It has, however, been shown to have high cure rate and lesser defervescence time as compared to quinolones.¹⁶ In this study none of the isolates of *S.* Typhi showed a high azithromycin MIC (64 µg/ml). Hence this drug has a potential for therapeutic use, being oral antibiotic as well. This study would pave the way for breakpoint determination for azithromycin after clinical correlation.

Ciprofloxacin resistant strains have risen sharply in recent past. In 1999, 37 isolates of *Salmonella* were examined and all were sensitive to ciprofloxacin.¹⁸ In this study, only 26% were sensitive. It may be due to excessive use of ciprofloxacin to treat typhoid fevers. Since the authors used revised breakpoints for ciprofloxacin sensitivity of *Salmonella* isolates, hence nalidixic acid was not used as a surrogate marker.

For imipenem and tigecycline, the difference in MIC$_{90}$ and MIC$_{50}$ was minimal. Tigecycline had the lowest MIC$_{90}$ and MIC$_{50}$ levels. In an earlier study, no resistance to ceftriaxone was noted in *Salmonella* Typhi as well as *Salmonella paratyphi*.²⁰ However, resistance to ceftriaxone has been reported due to plasmid mediated cephalosporinases and extended spectrum beta-lactamases.²¹ Hence, testing *Salmonella* isolates to ceftriaxone remains mandatory. In a study by CDC, ceftriaxone resistance was absent in *Salmonella* Typhi and *paratyphi*.²²

In this study, MICs of various antibiotics were obtained against *Salmonella* Typhi. It is expected that these
MICs would be utilized by renowned antimicrobial susceptibility testing agencies like CLSI and EUCAST in establishing breakpoints for these antibiotics against Salmonella isolates.

Except azithromycin, the other tested anti-microbials are not available in oral form. Since imipenem, cefpirome and tigecycline are effective against anaerobes also, they can be used successfully in mixed anaerobe and Salmonella infections like intra-abdominal infections. Unlike this, in a previous study from Karachi, none of the isolates were resistant to ceftriaxone.15 This shows emerging resistance to cephalosporins and needs, to explore for alternatives. High cost, requirement of parenteral administration and poor intracellular penetration makes ceftriaxone a difficult therapeutic option.

In Pakistan, MDR cases were 70% in 1996,23 and 30% in 2010.15 In this study, MDR cases were 39% in Salmonella Typhi. Molecular analysis is required, in future studies, to throw light on this aspect. However, the acquisition of ESBL in MDR cases would be a disaster, compromising the utility of third generation cephalosporins in these cases. Already there is one report of ESBL in Salmonella Typhi from Philippines.24 ESBL genes being plasmid mediated have potential of spreading quickly.

Studies comparing association of MICs of new medicines in cases with treatment failures are required, before these agents are marketed for clinical use in typhoid cases. It would be required to establish MIC breakpoints for these anti-microbials as well. Tissue concentration achieved; side effects and intracellular penetration would be the main deciding factors in therapeutic response. Economical and affordable antibiotics are required in developing countries.

CONCLUSION

Imipenem, azithromycin, tygycycline, aztreonam, cefpodoxime and cefpirome are potential therapeutic agents for the resistant of Salmonella Typhi infections. Azithromycin should be used with caution as MICs are higher in vitro. However, intracellular increased concentration in vivo, may prove to be a good therapeutic option.

REFERENCES


