IN VITRO EFFECT OF NEW ANTIBIOTICS AGAINST CLINICAL ISOLATES OF SALMONELLA PARATYPHI A

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ABSTRACT

Objective: The objective of this study was to determine in vitro MIC patterns of various therapeutic alternatives for the treatment of Salmonella Paratyphi A.

Study Design: Cross-sectional study.

Place and Duration of Study: Armed Forces Institute of Pathology Rawalpindi, from Jun 2012 to May 2014.

Material and Methods: Clinical samples were collected from suspected cases of salmonella infections. Culture was applied on Bactec 9050 special and/or standard media. Suspected Salmonella Paratyphi colonies were tested by API 20E and confirmed by serology. The isolates were also tested for resistance to ampicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, doripenem, imipenem, ertapenem, aztreonam, moxifloxacin, cefpirome, cefepime, gatifloxacin, and chloramphenicol by Kirby-Bauer disc diffusion method. MIC (Minimum Inhibitory concentration) was done on MDR and ciprofloxacin intermediate or resistant cases by E-strips.

Results: One hundred and eleven isolates of Salmonella Paratyphi were recovered from 2230 specimens. Resistance by disk diffusion technique noted in Salmonella Paratyphi A was ampicillin 60%, chloramphenicol 40%, cotrimoxazole 38%, ceftriaxone 7.9%, ciprofloxacin 8%, cefpodoxime 7.9%, imipenem and ertapenem 2.6%, aztreonam 1.3%, moxifloxacin 6.6%, and gatifloxacin 1.3%. No resistance was noted for doripenem and cefepime. MIC50 was 0.094 for Cefpirome, 0.125 Aztreonam, 0.25 imipenem and tigecycline, 2 cefpodoxime and 8 for azithromycin.

Conclusion: Azithromycin, Aztreonam, Imipenem, tygecycline, cefpodoxime and cefpirome are potential therapeutic agents for resistant Salmonella Paratyphi A infections.

Keywords: Antibiotics, Resistance, Salmonella Paratyphi A.

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INTRODUCTION

Salmonella infection causes significant mortality worldwide. Infections with Salmonella Paratyphi can result in various clinical presentations like enteric fever, gastroenteritis, septicemia with or without suppurative lesion and carrier state[1]. Salmonella infections especially those involving blood stream have high mortality, around 30%[1]. Paratyphoid fever by Salmonella Paratyphi especially Paratyphi A, is considered emerging disease because its incidence has increased dramatically during last two decades, causing more asymptomatic infections than Salmonella Typhi[2]. Salmonella enterica is mostly acquired directly or indirectly through human feces by faeco-oral route from the diseased person or a carrier.

Before starting clinical trials of new antibiotics, their in vitro efficacy should be measured against the disease causing bugs. Next step is the measurement of breakpoints with the help of clinical correlations of the in vitro efficacy. The in vitro effects are then categorized into Sensitive, Intermediate and Susceptible. In USA it is done by FDA or CLSI. In Europe the task is traditionally with EUCAST. This study will be the first step towards establishment of breakpoints for Salmonella Paratyphi A for several new drugs.

After the development of quinolone resistance, third generation cephalosporins is becoming popular as treatment but sporadic cases of resistance to them have also been

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reported[3]. Strains previously resistant to first-line drugs (co-trimoxazole, chloramphenicol, and ampicillin) are now showing decreasing resistance[4-6]. The probable reason is the withdrawal of selective pressure[5].

Azithromycin is said to be effective in nalidixic acid resistant and multidrug resistant cases but is still under evaluation[7]. With increasing use of third generation cephalosporins, resistance is likely to spread against them as well. It has been estimated to be around 1% in cases with reduced ciprofloxacin susceptibility[8]. Lack of interpretive criteria like antibiotic zones and MICs of azithromycin for Salmonella Paratyphi in CLSI makes it difficult to report in microbiology lab reports. E-test is approved by Food and Drug Administration USA[9]. Azithromycin resistance in Salmonella Paratyphi A has already been reported but remains rare[10]. Despite in vitro sensitivity, first and second generation cephalosporins, tetracyclines and aminoglycosides are not effective in vivo.

Multi-drug resistant (MDR) strains (resistant to chloramphenicol, ampicillin and cotrimoxazole) are very common. Outbreaks of MDR Salmonella Paratyphi may be difficult to manage and the results can be devastating especially in developing countries where resources are already limited. Hence, there is dire need to explore new avenues for treatment of resistant Salmonellae. In this research we would analyze in vitro effect of new drugs, not usually used for treatment of Salmonella infections. This study was planned to explore long-term efficacy of antibiotics.

MATERIAL AND METHODS

It is a cross sectional observational research carried out at Department of Microbiology Armed Forces Institute of Pathology Rawalpindi. Research was conducted from Jun 2012 to May 2014, and we included all the isolates of Salmonella Paratyphi A which came to us in our study, the total number of which was 111. Sampling technique was non-probability convenient sampling.

Proper collection of blood was ensured especially with regard to adequate amount of blood added in blood culture bottle (10 ml for adults and 1-6 ml for kids). Due attention was given to disinfection of skin also. Blood cultures from different wards of the hospital received in the laboratory were incubated in BACTEC 9050 system. Other specimens like stool, pus & urine were also collected according to the standard protocols[11].

Table: Resistance percentage of Salmonellae Paratyphi to various antibiotics by Disk diffusion method.

Antibiotic	S. Paratyphi A (n=111)		
	Intermediate	Resistant	Sensitive
Ampicillin 30µg	12%	60%	28%
Chloramphenicol 30µg	1%	40%	59%
Cotrimoxazole 30µg	1%	38%	61%
Ceftriaxone 30µg	0%	7.9%	92.1%
Ciprofloxacin 5µg	63%	8%	24%
Cefpodoxime 10µg	10%	7.9%	81.1%
Doripenem 10µg	1.3%	0%	98.7%
Imipenem 10µg	5.3%	2.6%	92.1%
Ertapenem 10µg	7.9%	2.6%	89.5%
Aztreonam 30µg	6.6%	1.3%	92.1%
Moxifloxacin 10µg	14.6%	6.6%	79%
Cefepime 30µg	0%	0%	100%
Gatifloxacin 5µg	0%	1.3%	98.7%

Positive blood culture bottles were sub cultured on blood and MacConkey agar after gram stain findings. Only one isolate per patient was included in the study. For stool specimens' enrichment was done in Selenite broth. Non-lactose fermenting colonies growing on MacConkey agar or red/transparent colonies on XLD agar were identified by standard biochemical and serological tests[12].

Gram stain was performed on non-lactose fermenting colonies. Gram negative rods were dealt with further, and their motility was observed. The isolates were identified using phenotypic colony characteristics and confirmed by biochemical reactions with API 20 E (bioMerieux SA, Marcy I'Etoile, France).

Serotyping was done with specific antisera using polyclonal and monoclonal O, H and Vi antisera (Bio-Rad, Marnes-Ia-Coquette, France) according to the Kauffmann-White classification scheme[13]. Salmonella Paratyphi A was gram negative rod, was positive for glucose, arabinose and ODC but negative for LDC, citrate, urease, H2S and indole tests. Salmonella enterica serotype paratyphi B was confirmed when it showed agglutination with somatic antigen[1,4,5,12] and flagellar antigen H-a.

The isolates were tested for resistance to conventional antibiotics ampicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, doripenem, imipenem, ertapenem, aztreonam, moxifloxacin, cefpirome, cefepime, gatifloxacin, and chloramphenicol by Kirby-Bauer disc diffusion method[14]. Inoculum equivalent to 0.5 MacFarland turbidity was used.

The disks of antimicrobial drugs used were chloramphenicol (30 µg), co-trimoxazole (1.25/23.75 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and ceftriaxone (30 µg) doripenem (10 µg), imipenem (10 µg), ertapenem (10 µg), aztreonam (30 µg), moxifloxacin (5 µg), cefpirome (30 µg), cefepime (30 µg), gatifloxacin (5 µg), chloramphenicol (30 µg) and nalidixic acid (30 µg). All disks were of Oxoid company UK. The inoculated agar plates containing the suitable antibiotic discs were incubated for 16-18 hrs at 36^0C and inhibitory zone diameters obtained around the antibiotic discs were measured (table).

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 Etest was first verified with broth dilution MIC using cation adjusted Muller–Hinton broth for imipenem, aztreonam and cefpodoxime. 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 Clinical Laboratory Standards Institutes guidelines[15]. 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.

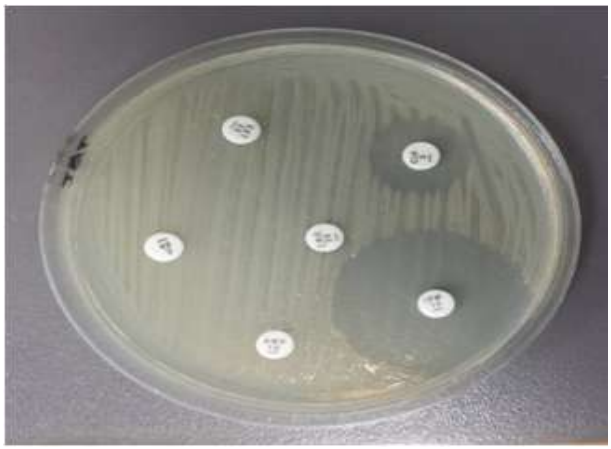

Figure-1: MDR Salmonella Paratyphi, Resistant to Ciprofloxacin and Ceftriaxone.

Based on recommendations of EUCAST[16] and one previous study[17] the cutoff for Azithromycin was taken to be ≥32 µg/ml. Isolates were preserved at -45 / -600C in nutrient agar with glycerol.

RESULTS

A total of 111 isolates were recovered from 2230 specimens. Out of 111 isolates 65 (59%) isolates were from blood culture, 23 (21%) isolates from stool, 8 (7%) from urine, 14 (13%) isolates from pus and 1 (0.9%) were from fluids. Median age of the patients was 22 years with range 1 to 77 years. They belonged to 12 different districts, all from north/north-west of the country but majority were from Rawalpindi. Highest number of culture positive cases 56 (51%) were between 8 and 17 years. Total number of cases less than 8 years of age were 9 (8.1%) while total cases more than 17 years age were 45 (41%). Gender was known for 87 (78.3%) isolates. Out of these 59 (53.2%) were males and the rest were females.

All isolates that were either MDR (resistant to ampicillin, chloramphenicol and co-trimoxazole) or were ciprofloxacin intermediate or resistant on disk diffusion technique were subjected to MIC test for the antibiotics for which E-strips were available (fig-1).

As far as the MIC is concerned (fig-2), for imipenem (mean MIC = 0.25 ± 0.005 mg/L) all isolates were sensitive. For aztreonam (mean MIC = 0.125 ± 0.93 mg/L), out of 96 isolates 6 (6.2%) were resistant and 18 (18.7%) were intermediate. For cefpodoxime (mean MIC = 2 ± 0.112 mg/L), out of 96 isolates 9 (9.3%) were resistant.

For azithromycin (fig-3) (mean MIC = 8 ± 1.31 mg/L)[16] out of 96 isolates 6 (6.2%) were resistant. For cefpirome (mean MIC = 0.094 ± 0.098 mg/L) and Tigecycline (mean 0.25 ± 0.017 mg/L) 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 (U. S. FDA) given in the package insert for treating Enterobacteriaceae (susceptible, 2 g/ml; 4 = Intermediate, resistant = 8 g/ml)[18] and those recommended by the European Committee on Antimicrobial Suscepti-bility Testing (EUCAST) (susceptible = 1 g/ml; resistant, 2 g/ml). According to both criteria all the isolates were sensitive.

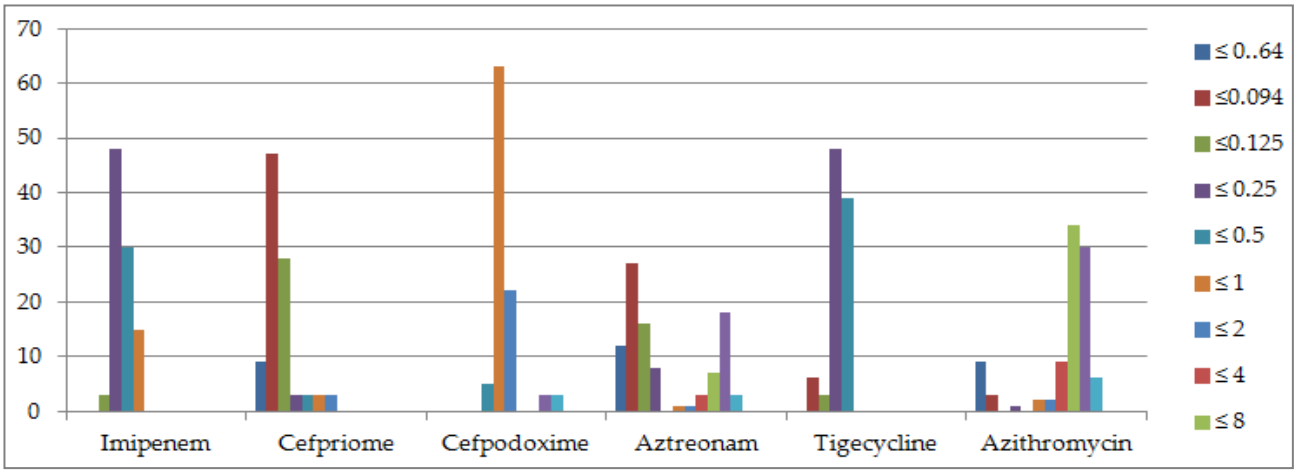

Figure-2: MIC of antibiotics for Salmonella Paratyphi A.

For cefpirome no previous breakpoints could be found. The range of MIC was from 0.047 to 0.75[15]. Six isolates (5.4%) of S. Paratyphi A were MDR.

DISCUSSION

In this study we tried to find solutions to the emerging problem of resistance in Salmonella Paratyphi. Since study was based in laboratories of tertiary care hospitals, hence the isolates were a mixture of extraintestinal and intestinal specimens. Azithromycin has the advantage of being available in oral preparation and can be given safely to children, MIC90 for azithromycin in Salmonella enterica serotype Paratyphi

(Salmonella Paratyphi) in our isolates was 16 µg/ml while earlier studies have reported MIC to be in the range of 4-16 microgram/ml[19-21]. Resistance of Salmonella Paratyphi A to azithromycin and treatment failure has already been reported[10]. It requires large clinical trials to prove its efficacy and to establish breakpoints. Most antibiotic sensitivity standards including CLSI and EUCAST do not mention breakpoint for azithromycin against Salmonella Paratyphi. This study would pave the way for breakpoint determination for azithromycin, after clinical correlation.

Azithromycin is concentrated manifold intracellularly and hence may show better clinical cure rate. Thus, there is speculation that intracellular MICs may not be represented fully by the currently available in vitro MIC testing methods. As this was a laboratory based study and the patients were not easily accessible, the therapeutic efficacy of these drugs was not possible. Furthermore, over-the-counter availability of effective drugs like quinolones and cephalosporins hampered such a move. Large scale randomized clinical trials of the new in vitro effective drugs is warranted. Since Salmonella Typhi and Paratyphi A are pathogenic only in humans, the trial in animals would remain dubious.

In 1999, thirty seven isolates of Salmonella were examined and all were sensitive to ciprofloxacin[22]. Because of rising resistance to ciprofloxacin, it is no more an ideal alternative as shown by increased MIC to 8 µg/ml[19]. Ciprofloxacin resistant strains have risen sharply in last 3 years. It may be due to excessive use of ciprofloxacin to treat typhoid fevers that has selected out resistant strains.

For imipenem and tigecycline, the difference in MIC90 and MIC50 was minimal. Amongst the antibiotics tested, tigecycline had the lowest MIC90 and MIC50 levels. However, resistance to ceftriaxone has been reported due to plasmid mediated cephalosporinases and extended spectrum beta-lactamases[23]. Hence, testing Salmonella isolates to ceftriaxone remains mandatory. In one study by CDC, ceftriaxone resistance was absent[24].

In this study trials of treatment of typhoid fever with azithromycin, imipenem, tigecycline, cefpirome, cefepime, cefpodoxime, gatifloxacin and aztreonam has been suggested, based on their in vitro activity against Salmonella enterica serovar Paratyphi A. In a previous study MIC range of Cefpirome for Salmonella species was 0.094 to 0.91 but in our study it was 0.06 to 1.6 for Salmonella Paratyphi A[25,26].

Due to frequent power outages in our country, we frequently faced problems like

Figure-3: MIC of MDR Salmonella Paratyphi against azithromycin.

incubator failure resulting in no growth on culture plates or requirement of repetition of biochemical tests. Many isolates could not be saved properly due to the same reason. There was poor yield of Salmonellae in many culture results. One of the likely reasons is the easy availability and widespread use of antibiotics by the patients early in disease.

From our study it is inferred that conventional anti-typhoid drugs are not effective in clinical isolates of Salmonella Paratyphi A. The study would help in formulating empiric therapy in developing as well as developed countries. Strong collaboration is desired between clinicians and microbiologists for treatment of bacterial diseases and judicious use of antibiotics to avoid the development of drug resistance.

Tigecycline, aztreonam and cefpirome have shown low MICs against Salmonella enterica serotype paratyphi A. Their efficacy however must be proven by clinical trials. Except azithromycin, the other drugs tested are not available in oral form. Tigecycline is notorious for development of resistance during therapy also. 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. The possible variables of MIC are antimicrobial potency, pH, agar depth and incubation temperature.

In a previous study from Karachi none of the isolates were resistant to ceftriaxone[19], however in our study 8% isolates of Salmonella Paratyphi A were resistant. This shows emerging resistance to cephalosporins and need to explore for alternatives. MIC90 of 178 isolates was compared by Cooper et al and found it to be 0.06µg/ml in 2001. It increased to 0.25 in 2005[7]. Rising and alarming trend has been noted in our study. High cost, requirement of parenteral administration and poor intracellular penetration makes ceftriaxone a difficult therapeutic option. It highlights the need to monitor emergence of resistance in typhoid salmonellae for third generation cephalosporins and to search for more alternatives. The acquisition of ESBL in MDR cases would be a disaster, compromising the utility of third generation cephaloporins in these cases. Since ESBL genes are mostly located on plasmids, further spread of this resistance genes is expected.

In this study we have given MICs of various antibiotics against Salmonella Paratyphi A. We expect that these MICs would be utilized by renowned antimicrobial susceptibility testing agencies like CLSI and EUCAST in establishing breakpoints for these antibiotics against Salmonella Paratyphi.

Studies comparing association of MICs of new drugs (Tigecycline, cefpodoxime, azithromycin, imipenem, cefpirome, aztreonam, cefepime, gatifloxacin and doripenem) with treatment failures are required before these drugs are marketed for clinical use in typhoid. It would be required to establish MIC breakpoints for these drugs as well. Tissue concentration achieved, side effects and intracellular penetration would be the main deciding factors in therapeutic response.

CONCLUSION

Imipenem, azithromycin, tigecycline, aztreonam, cefpodoxime and cefpirome are potential therapeutic agents for resistant Salmonella Paratyphi infections. Gatifloxacin is a possible alternatives. 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.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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