Synergistic Effect of Antibiotics against Bacterial Pathogens Causing Diarrhea Isolated from Children <5 Years of Age

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Abstract

Background: Infant mortality rate due to diarrhea is high in Pakistan. The alarming increase in antimicrobial resistance of bacterial pathogens causing diarrhea has made the treatment more complicated. This study was designed to determine the in vitro synergistic (combined) effect of different antibiotics against these pathogens in order to determine whether the combined antibiotic therapy may be used to treat diarrhea.

Objectives: To study the antibiotic resistance pattern of bacterial pathogens causing diarrhea isolated from children <5 years. To study the synergistic effect of antibiotics against bacterial pathogens causing diarrhea.

Study design, settings and duration: A cross sectional study conducted at Pakistan Health Research Council (PHRC), Jinnah Postgraduate Medical Center and National Institute of Child Health, Karachi over a period of twenty months.

Materials and Methods: Bacterial pathogens including *Salmonella, Shigella, Vibrio*, *Escherichia coli* and *Aeromonas* causing diarrhea were collected. Their sensitivity and resistance pattern was performed by disk diffusion method and combined the effect of existing antibiotics including ampicillin (AMP), cefotaxime (CTX), Cefixime (CFM), Co-trimoxazole (SXT) and Ofloxacin (OFX) was performed by checkerboard method. The study was approved by Ethical Review Board of National Institute of Child Health, Karachi.

Results: Most of the combinations showed indifferent and antagonistic activity against bacterial pathogens causing diarrhea. But only AMP-SXT and CFM-OFX yielded synergistic activity against Vibrio and Aeromonas respectively. AMP-CFM and AMP-OFX exhibit synergistic effect against *Escherichia coli* while no combination of antibiotics proved as synergistic for Salmonella and Shigella.

Conclusion: Little synergistic activity of different antibiotic combinations was observed against diarrheal isolates and most of the combinations exhibited indifferent and antagonistic activity. Hence this can be concluded that single antibiotics are effective than combination.

Key words: Synergistic Effect, antagonistic, indifferent, diarrhea, antibiotics, combinations, mortality, sensitivity, resistance pattern.

Introduction

Diarrhea is a worldwide health problem in the developing world. An estimated 100 million cases of acute diarrhea occurs annually in United States.[1] Pakistan is one of the top four states of the world with highest number of death ratio of children[2] younger than

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Authors Contribution

RI has done the conceptualization of project and literature search. RI and SB did the data collection, drafting, revision and writing of the manuscript. Statistical analysis was done by SEA.

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five years.[3] In Pakistan, around forty five lac cases of diarrhea were reported, among them 14% were children less than five years.[4] Diarrhea also caused 1.5 million deaths in 2009.[5] while in Karachi the incidence of treated diarrhea was just 48% per year.[6]

Although there are various causes of infectious diarrhea like virus, protozoan and helminthes, but the rate of bacterial diarrhea in Pakistan is 40.7%.[7]

Antibiotics play an important role in preventing deaths due to bacterial diarrhea but it is reported that its mortality rate is continuously increasing. In routine clinical practices, trimethoprim-sulfamethoxazole, cefixime, ceftriaxone, cefotaxime and fluoroquinolones are recommended to treat diarrhea. But literature suggests that these existing antibiotics are becoming less effective or pathogens have acquired resistance.

Internationally, over 33% of salmonella isolates were resistant to ampicillin and tetracycline and almost 100% Shigella resistant to trimethoprim sulfamethoxazole

(TMP-SMX) and 30% of *Campylobacter jejuni* isolates were resistant to ampicillin and ciprofloxacin.[8] *E.coli* and salmonella showed 27.02% and 72.22% resistance to ciprofloxacin,[9] while resistance to nalidixic acid in *S. para typhi. A* is increased with each subsequent year, it was reported as 69%, 72%, 77% and > 93% in 2006, 2007, 2008 and 2009 respectively.[10] In the latest research, 58%, 85%, 12.6%, 3.0% and 2.4% *Shigella* isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid, ofloxacin and ceftriaxone respectively.[11] Another research of Karachi is also the evident of high rate of resistance in Shigella against Ampicillin and nalidixic Acid 55.5% and 39% respectively.[12] Resistance to ampicillin and cotrimoxazole is also observed in *E.coli* as 72% and 78% respectively.[13]

This increasing resistance among diarrheal pathogens points towards the need of testing alternative options for treating diarrhea, synergism/ combined antibiotic effect is an important phenomenon through which diarrheal rate would be reduced. Synergistic effect is the activity of two drugs when they are used in combination, their effect is greater to the sum of their independent activity when studied separately.[14] Hence this research aims to study the synergistic (combined) effect of antibiotics against bacterial pathogens causing diarrhea which shows resistance against existing antibiotics.

Materials and Methods

It was cross sectional study and samples (bacterial pathogens) from children less than 5 years of age were obtained from National Institute of Child Health (NICH), Karachi and other private laboratories / clinic of Karachi. The collected samples were brought and processed in PHRC, JPMC Karachi. A standardized proforma was designed to record details of isolate, sensitivity pattern, antibiotics tested and combination results.

A total of 80 bacterial isolates were collected including *salmonella, shigella, vibrio, aeromonas* and *E.coli*, 46 (57.5%) were collected during March to December-2013 and 34 (42.5%) were collected during January to November-2014. Smooth lawn was made on Muller Hinton Broth through sterile cotton swab and antibiotic discs were placed on it, at appropriate distance according to Kirby Bauer disk diffusion method as recommended by Clinical and Lab Standard Institute (CLSI) guidelines and incubated at 37°C for 24 hours. After incubation, the zone of inhibition was measured. The antibiotics which showed intermediate zone or no zone were considered as less effective for that pathogen, so was selected for combined effect.

Standard powder form of antibiotics (stored at 2-8°C) was used for combined activity by checker board method.[15] The stock solutions and serial twofold dilutions of each antibiotic to at least double the MIC was prepared according to the recommendations of NCCLS guidelines,[14] Mueller-Hinton broth (50μl) was distributed to each well of the micro dilution plate. Inoculum (0.5 McFarland) was prepared from each isolate in Mueller-Hinton broth. MIC of each pathogen was determined by micro dilution method. Each micro titer well was inoculated with 100μl of a bacterial inoculum of 5×10^5 CFU/ml and incubated at 37°C for 24 hours under aerobic conditions. Inhibitory concentration (results) at which antibiotics exhibits synergistic effect was recorded.

According to NCCLS guidelines: ΣFIC was calculated as follows:

$$\Sigma FIC = FIC\ A + FIC\ B$$

Where: FIC A is the MIC of drug A in the combination/MIC of drug A alone
FIC B is the MIC of drug B in the combination/MIC of drug B alone
The combination was considered synergistic when the ΣFIC is ≤0.5
The combination was considered indifferent when the ΣFIC is >0.5 to <2
The combination was considered antagonistic when the ΣFIC is ≥2

The data feeding and analysis was done on computer package SPSS (Statistical Packages of Social Sciences) version 11.0. Clinical characteristics was summarized in terms of frequencies and percentages for qualitative variables (physical examination, microscopic examination, etc.), mean ± S.D, for quantitative variables (zone of inhibition of existing antibiotics). To find the resistant pattern of existing antibiotics and to check the dilution at which antibiotics exhibits synergistic effect. Study was approved by ethical review board of National Institute of Child Health, Karachi.

Results

Out of 80 pathogens, 51% were salmonella, 20% were shigella, 13% were vibrio, 14% were *E.coli* and only 3% were Aeromonas. Existing antibiotics including Ampicillin (AMP), Cefotaxime (CTX), Cefixime (CFM), Co-trimoxazole (SXT) and Ofloxacin (OFX) were checked for sensitivity by disk diffusion method and findings are given in Table.

The isolates which gave intermediate zone or no zone were selected for MIC and combined effect. By this method, most of the combinations showed indifferent and antagonistic activity. The combination of ampicillin and

Table: Sensitivity and resistance pattern.

Antibiotics		Salmonella n=41 (%)	Shigella n=16 (%)	Vibrio n=10 (%)	E.coli n=11 (%)	Aeromonas n=02 (%)
	S	25 (60.97)	08 (50.00)	01 (10.00)	03 (27.27)	01 (50.00)
	I	06 (14.63)	05 (31.25)	03 (30.00)	00 (00.00)	00
Ampicillin	R	10 (24.39)	03 (18.75)	06 (60.00)	08 (72.72)	01 (50.00)
	S	34 (82.92)	14 (87.5)	07 (70.00)	10 (90.90)	02 (100.0)
	I	01 (02.43)	00	00	01 (09.09)	00
Cefotaxime	R	06 (14.63)	02 (12.5)	03 (30.00)	00	00
	S	33 (80.48)	13 (81.25)	04 (40.00)	11 (100.0)	01 (50.00)
	I	00	01 (06.25)	00	00	00
Cefixime	R	08 (19.51)	02 (12.5)	06 (60.00)	00	01 (50.00)
	S	33 (80.48)	07 (43.75)	05 (50.00)	10 (90.90)	02 (100.0)
Co-trimoxazole	I	00	00	01 (10.00)	00	00
	R	08 (19.51)	09 (56.25)	04 (40.00)	01 (09.09)	00
	S	23 (56.09)	11 (68.75)	04 (40.00)	10 (90.90)	02 (100.0)
	I	02 (04.87)	01 (06.25)	00	01 (09.09)	00
Ofloxacin	R	16 (39.02)	04 (25.00)	06 (60.00)	00	00

Key (Zone of Inhibition): S = Sensitive, I = Intermediate, R = Resistant

cefixime showed as synergistic for a single isolate of *E.coli*. and ampicillin and co-trimoxazole exhibit synergistic effect against *Vibrio.* Similarly cefixime and ofloxacin showed synergy against *Aeromonas.*

Discussion

In fact antibiotics play an important role in preventing deaths due to bacterial diarrhea but it is reported that mortality rate due to diarrhea is continuously increasing. As research proved that in Pakistan "2.5 lac children die because of diarrhea annually."[16] In routine clinical practices, Trimethoprim-Sulfamethoxazole, Cefixime, Ampicillin, Cefotaxime and Fluoroquinolones are recommended to treat diarrhea. But literature suggests that these existing antibiotics are becoming less effective or pathogens have acquired resistance.

So the goal of this *in vitro* study was to access synergies (combinations) among antibiotic that are commonly used clinically for treatment of diarrhea. In this study, bacterial pathogens were obtained from clinical specimen of diarrheal patients and their sensitivity pattern was performed. Existing antibiotics including Ampicillin, Cefotaxime, Cefixime, Co-trimoxazole and Ofloxacin to cure diarrhea were checked for sensitivity by disk diffusion method. And the selection of antibiotics for synergy studies was based on antibiotic resistance.

As study results reflects that more than 50% isolates were *Salmonella* while *Aeromonas* were very less prevalent which is only 2.5%, Actually Aeromonas has a number of virulence factors and isolated as a single enteropathogen from 80% diarrheal and 20% asymptomatic cases [17] so may be this was the reason of less prevalence of *Aeromonas*. Study results showed that most of the isolates exhibit resistance against existing antibiotics so constant antibiotic surveillance is necessary

because the pathogens became extremely unaffected (resistant) to several antimicrobials. Therefore this study contributes to strengthening of the prevailing surveillance system and makes available aid for effective prevention and control strategies for antimicrobial resistance.[18]

In view of the emergence of multidrug-resistant strains, there is a need for therapeutic alternatives so the isolates which give intermediate zone or no zone were selected for MIC and combined effect by Checkerboard method. This assay was used to test the activities of drugs in combinations by determining the ΣFICs of all combinations tested.

By this method, most of the combinations show indifferent and antagonistic activity. The combination AMP-SXT and CFM-OFX yielded synergistic activity against *Vibrio* and *Aeromonas* respectively. The AMP-CFM and AMP-OFX yielded synergistic activity against *E. coli.* In comparison to other international studies, research also suggested that combined medication positively cures the infection of *H.pylori* in up to 90% of individuals.[19] Similarly, another study reported that combined therapy significantly reduced the mortality rate by 56% of patients [20] and can increase the single drug strength and slower the emergence of resistance.[21] The combination of cryptdin 2 and ampicillin was also found as extremely effective, which indicated *in vitro* synergy between the two agents.[22] Furthermore, as the results indicates that there is no combination of antibiotics proved as synergistic for *Salmonella* and *Shigella.* So the reason behind this may be the increasing resistance of pathogens. This widespread resistance could be attributed to excessive or indiscriminate use of antibiotics.[23] Finally emergence of pathogen's resistance to antibiotics is tending to be a serious problem. Furthermore, recurrent and lengthy use of antibiotics usually results in alteration of the intestinal flora.

One limitation of our study is that some combinations, such as CTX and CFM were not tested

because these two drugs belong to same generation so have same activity.

Little synergistic activity of different antibiotic combinations was observed against diarrheal isolates, and most of the combinations exhibited indifferent and antagonistic activity. Hence we may conclude that single antibiotics are effective than combination.

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