British Microbiology Research Journal
14(4): 1-7, 2016, Article no.BMRJ.25463
ISSN: 2231-0886, NLM ID: 101608140

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In-vitro Resistance Development by Ciprofloxacin and Azithromycin in *Shigella dysenteriae*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MSI and MAH designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors AKS, MAR and MAUI managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/25463
Editor(s):
(1) Arun Chauhan, Department of Immunology and Microbiology, School of Health & Medicine, University of North Dakota, Grand Forks, ND, USA.
Reviewers:
(1) Akobi Oliver Adeyemi, Federal Medical Centre, Niger State, Nigeria.
(2) Charbell Miguel Haddad Kury, Medical School of the Municipality of Campos dos Goytcazes, Rio de Janeiro, Brazil.
(3) Ronald Bartzatt, University of Nebraska, USA.
Complete Peer review History: http://sciencedomain.org/review-history/14450

Original Research Article

Received 4th March 2016
Accepted 23rd April 2016
Published 4th May 2016

ABSTRACT

Introduction: Drug resistance is a growing public health issue among the health care professionals. Due to improper use many bacterial species including the *Shigella dysenteriae* become resistant to the standard therapy.
Objectives: In the present study, we have shown the bacterial resistance development in the *Shigella dysenteriae* species against Ciprofloxacin and Azithromycin and therefore decrease in their antibiotic susceptibility together with DNA and morphological alteration by the presence of sub inhibitory concentration of antibiotics.
Methods: Disc diffusion method was applied to examine resistance of *Shigella dysenteriae* against ciprofloxacin and azithromycin and genomic anomalies were observed in ciprofloxacin resistant and sensitive strains by agarose gel electrophoresis.
Results: Sub culturing in the ciprofloxacin reduced the ZOI from 22 to 15 mm at their MIC on 13th passages but still the sensitivity was preserved in azithromycin (20 µg/dics) ZOI of 21 mm. In case

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of Azithromycin, zone reduced from 24 to 18 mm at their MIC after 13 subcultures, but it raised high to 25 mm for both ciprofloxacin (20 µg/dics). As documented of genomic changes in ciprofloxacin resistant strain (Azithromycin not included) by agarose gel electrophoresis, six different DNA bands found whereas one DNA band found in ciprofloxacin sensitive strain.

Conclusion: So it is clear that the misuse of existing antimicrobials may enable the microorganism to develop their less susceptible strain, by changing the restriction fragment length point which was appeared in the agarose gel, to that antimicrobial agent.

Keywords: Shigella dysenteriae; ciprofloxacin; antibacterial susceptibility; genomic change; zone of inhibition.

1. INTRODUCTION

Shigellosis, an acute diarrhoeal disease caused by a rod shaped, Gram negative endotoxin (lipopoly-saccharide) releasing bacillus of any of this four species naming *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei* [1,2]. It has been reported that about 140 million people suffer from shigellosis with estimated 6,00000 deaths worldwide [3,4].

Shigellosis is a common problem in Bangladesh and it alone accounts for 20% of child death every year.

Antibacterial resistance development in many pathogenic bacterial species including *Shigella dysenteriae* is a common scenario in the clinical environment. Antibiotics that are being used in the last couple of years in Bangladesh, is progressively becoming resistant to this *Shigella* species [5]. Ampicillin and the combination of sulphamethoxazole-trimethoprim, which were extensively used in the last two decades, are now appeared as ineffective to different strains of *Shigella* species [6]. Development of resistance occurred even to newer and more potent antibiotics. In recent year *Shigella* strain emerged resistance to ciprofloxacin in India and Bangladesh [7,8-9]. Mahbubur R et al. [10] isolated 3,337 Shigella strains from Dhaka Hospital of ICDDR,B between 2001-2002 and tested about 266 (8%) of *Shigella* isolates against various antimicrobial agents. Among 12% *Shigella* isolates shows intermediate rate of resistance to ciprofloxacin and 16% shows moderate and 62% shows intermediate rate of resistance to azithromycin. Debdutta B et al. [11] collected 412 stool samples from G.B plant hospital and Primary Health Centers thought-out the Andaman and Nirobar Islands during 2006-2009.They recovered Shigella isolates from 50 of 412 stool samples and tested against 22 antimicrobial agents. It was observed that about 80% of *Shigella* isolates were resistance to ciprofloxacin.

The increasing resistant species may be the result of misuse or overuse of antimicrobial drugs. It was previously shown that pneumococcal MICs (minimum inhibitory concentrations) are increased by the sequential subcultures in sub inhibitory concentration of azithromycin and cefprozil [12]. This antibiotic exposure can lead to morphological change to Gram negative bacilli like *P. aeruginosa,* which is also a cause of endotoxin release. There are many reports concerning the morphological change associated to antibacterial agent's exposure [13].

For the differentiation and to find the phylogenetic relationship among different microorganisms, restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified 16S-rDNA is a suitable technique [14]. If there is any variation in the DNA due to repetition of base pairs, it will alter the length between two points of the DNA where it is cut by restriction enzyme. So there will be RFLP in the DNA of separate strains of same species. This kind of polymorphism is appearing in the electrophoresis gel from the PCR product.

The present study describe the antibiotic resistant development of *Shigella dysenteriae* by subcultures in the sub inhibitory concentration of Ciprofloxacin (fluoroquinolones group), Azithromycin (macrolide group) and their morphologic, genetic and antibiotic susceptibility analysis.

2. MATERIALS AND METHODS

2.1 Collection, Pure Culture Preparation and Identification of *Shigella dysenteriae*

Slant culture of sensitive *Shigella dysenteriae* Strains were collected from ICDDR, B (International Centre for Diarrheal Disease Research, Bangladesh). Pure culture was obtained by serial dilution technique [15] followed

by identification by some non microscopic studies including bacterial colony study in both nutrient broth and agar medium, agar slant study, microscopic observation of the strain, and Gram's staining.

2.2 Biochemical Test

Some biochemical test was also carried out for the characterization of the microorganism such as- Oxidase test, Urease test and Indole test, [16-18].

2.3 Determination of MIC

MIC of ciprofloxacin, and azithromycin for *S. dysenteriae* strain were calculated using the serial tube dilution method [19]. In brief description, microorganisms were grown in different tubes containing different concentrations of antibiotics, where the clear solution in the test tube indicate the ability of the antibiotic to inhibit the growth and turbid indicate the viability of microorganisms after incubation period. The lowest concentration that made the clear solution was termed as the MIC.

2.4 Development of Resistant Strains of *S. dysenteriae*

Kensuke Nagai (2000) previously reported that the presence of substandard level of antibiotic in a culture medium may develop resistance to *S. Penumoniae*. In order to prepare resistant strain in a sensitive *S. dysenteriae in vitro,* we applied two antibiotics at their MIC i.e. 12 µg/ml-ciprofloxacin and 2 µg/ml-azithromycin with frequent discontinuation of drugs. Freshly cultured inoculums (20 µL) were seeded in test tubes containing 2 ml autoclaved nutrient broth and were subjected to antibiotic. After 2 hrs of treatment, 20 µL aliquot of nutrient broth was transferred to a 2 mL of antibiotic free broth and incubated for 22 Hrs. Again 20 µL of culture was transferred to the antibiotic containing media for 2 Hrs of treatment. In this way, we performed 16 serial passages and among them bacterial culture from 8^{th} and 13^{th} passages were tested with standard disc containing antibiotics (Ciprofloxacin and Azithromycin) for the measurement of inhibitory zone. Same passages were conducted in another test tube containing sensitive *Shigella* strains in antibiotic free media which was taken as positive control.

2.5 Antimicrobial Susceptibility Test

Bacterial susceptibility was measured by standard disc diffusion method of Bauer et al. [20]. Ciprofloxacin (Jingxin Pharma, China) and Azithromycin (Shanghi Modern Pharma, China) active were purchased from local market as dried powder. Ciprofloxacin and Azithromycin treated *Shigella* strains (from 8^{th} and 13^{th} passages) were tested using Ciprofloxacin (20 µg/disc) and Azithromycin (20 µg/disc) respectively. Control strains were also tested against Ciprofloxacin (20 µg/disc) and Azithromycin (20 µg/disc).

2.6 Microscopic Observation of Both Sensitive and Resistant Strain of *S. dysenteriae*

Microscopic observation of *Shigella* strains (both sensitive and resistant) was done by Hanging drop technique. One drop of nutrient agar from both sensitive and resistant strains of each antibiotic treatment was placed on glass slide and stained by crystal violet and observed in the microscope (at 200 x magnification) by placing a cover slip [21].

2.7 Determination of Polymorphisms in the Ciprofloxacin Resistant (13^{th} Passage) *S. dysenteriae* by PCR (Polymerase Chain Reaction) and Gel Electrophoresis Techniques

DNA was extracted by collecting pellet from 3ml of bacterial culture in two separate eppendorf tube followed by suspending in 100 µL lysis buffer and keeping in the ice for 5-10 mins. The pellet was again subjected to 200 µL of freshly prepared alkali lysis buffer and kept in ice after mild vortexing. Ice-cold potassium acetate (150 µL) was added and kept in ice for another 15 minute after gently shaking. This mixture was then centrifuged at 15000 rpm for 10 min at 4ºC and pellet was washed with 70% ethanol and dried. Finally it was suspended in de-ionized water.

For the PCR, a cocktail components was prepared in a tube by adding Taq polymerase buffer 2.5 µl, dNTPs (Sigma, USA) 0.5 µl, 27f=5´ AGATTTGATCCTGGCTCAG 3´, 1100γ= 5´ GGGTTGCGCTCGTTG 3´ and 1114f= 5´ GCAACGAGCGCAACCC 3´ random primer (Sigma, USA) 1.5 µl, extracted DNA 1.0 µl, and Taq DNA polymerase (Genei, India) 0.2 µl. Then total volume was made up to 25 µl by adding distilled water. As a control, only water was taken in another tube without DNA. After a brief spinning the reaction was carried out in the following conditions: 7 min at 94ºC followed by

30 cycles in 94ºC in 1min, 61ºC for 1 min, 72ºC for 2 min, and 72ºC for 7 min for final extension and kept at 4ºC until tested.

And for the electrophoresis purpose, agarose gel was casted in a plastic case and placed in the electrophoretic chamber suspending in the Tris-borate EDTA (TBE) buffer. DNA ladder and the DNA sample (mixing with gel loading buffer containing 0.25% bromophenol blue, 0.25% xyline cyanol, 30% glycerol in water) were loaded in different lane of the gel and subjected to 10 volt/cm for electrophoretic separation. In a gel documenter (Alpha Innotech, U.S.A), DNA bands were appeared.

2.8 Statistical Analysis

Experimental data was analyzed by SPSS version 21. Mean and standard deviations were calculated to evaluate this study.

3. RESULTS

3.1 Biochemical Analysis

In biochemical analysis, the selected bacterial strain showed negative in Gram staining, Oxidase, and Urease test where as positive result found in Indole test. Results are shown in Fig. 1.

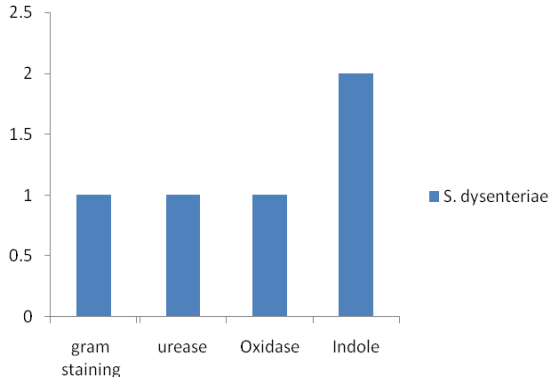

Fig. 1. Biochemical profiling of *Shigella dysenteriae*. Negative and positive results were recorded with 1 and 2 respectively

3.2 Anti-bacterial Susceptibility Test

Anti-bacterial susceptibility of ciprofloxacin, and azithromycin against drugs treated *Shigella dysenteriae* was evaluated by using disc diffusion methods. Results are shown in Fig. 2.

It was observed that the initial zone of inhibition of ciprofloxacin (20 µg/dics) against sensitive *Shigella dysenteriae* strain was 22 mm. After 8th serial passage at MIC level of ciprofloxacin the zone decreased to 15 mm. In the subsequent transfer at 13th passage there is no significant change ($p>0.05$) in zone of inhibition. The initial zone of inhibition of azithromycin (20 µg/dics) against sensitive *Shigella dysenteriae* strain was 24 mm. After 8th serial passage at MIC level of azithromycin the zone decreased to 18 mm. In the subsequent transfer at 13th, there is no significant change in zone of inhibition.

3.3 Changes in Microscopic View of *S. dysenteriae* Strain

From microscopic view it was observed that sensitive strain of *Shigella dysenteriae* was comparatively large in size and rod shaped. But after development of resistance it became small and oval shaped. Results are shown in Fig. 3.

3.4 Changes in Genome level of *S. dysenteriae* Strain

In case of sensitive strain, one DNA band found at 400 bp of ladder DNA. But in case of resistant strain six bands found at 200 bp, 300 bp, 400 bp 500 bp, 600 bp, and 700 bp of ladder DNA. It is clear that DNA of two strains was not same. In resistant strain, DNA, the primer randomly match at six position and amplified the DNA. On the other hand, in case of sensitive strain the primer match in one position and amplified. For this reason six bands were detected in resistant strain and one was in sensitive strain. So, there was a difference between the DNA of sensitive and resistant strain.

4. DISCUSSION

Drug resistant pathogens are critical threats for the global public health. Bacteria can develop different resistance mechanisms against antimicrobial agents and make treatment more challenging. In order to elucidate the morphological, genetic and ZOI pattern changes that may attain by subcultures in the sub inhibitory concentrations due to resistance development in microorganisms, we investigated *Shigella dysenteriae* species by ciprofloxacin and azithromycin.

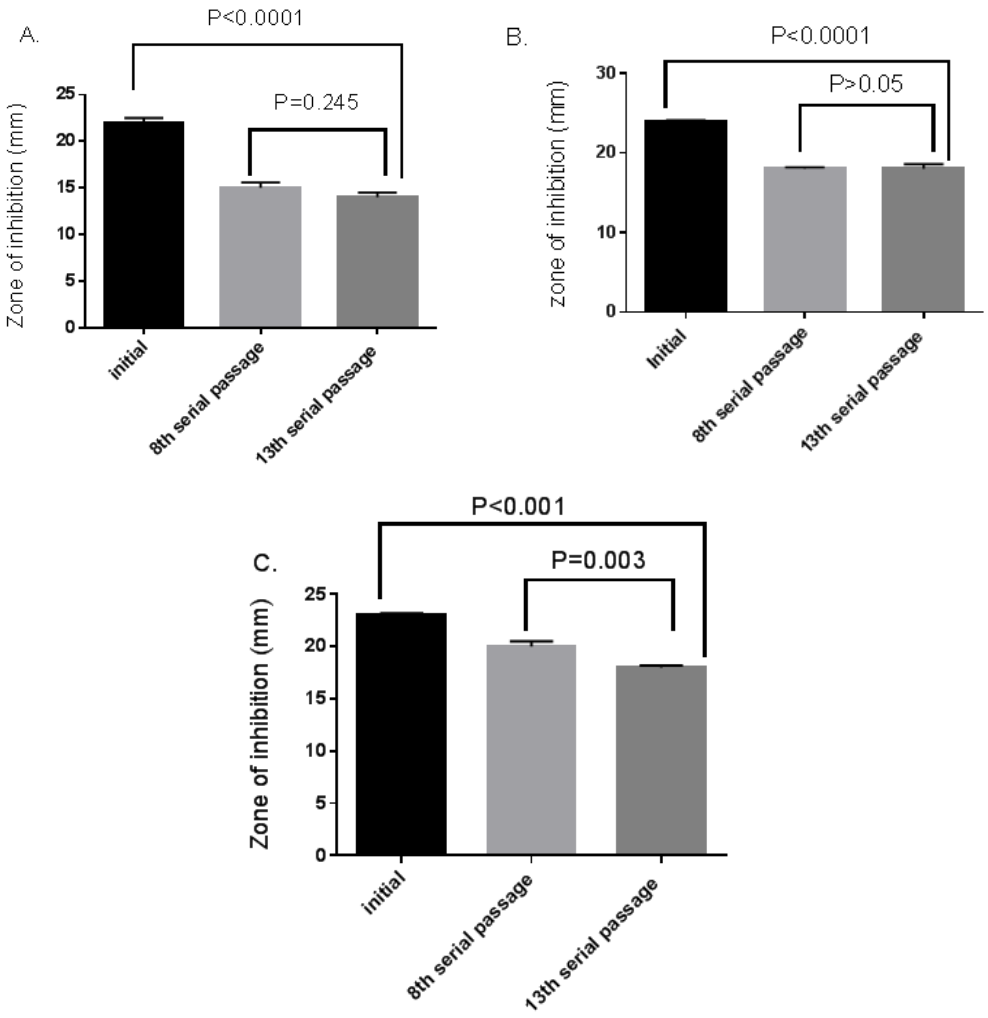

Fig. 2. ZOI of *S. dysenteriae* strains of different antibiotics at their MICs. A. Zone of inhibition of ciprofloxacin recorded and found statistically significant level (P<0.05) midst initial and 13th serial passages. B. Significant p value found (P<0.05) in azithromycin resistance development midst initial and 13th serial passages. C. Explored the zone of inhibition of standard antibiotics (Kanamycin)

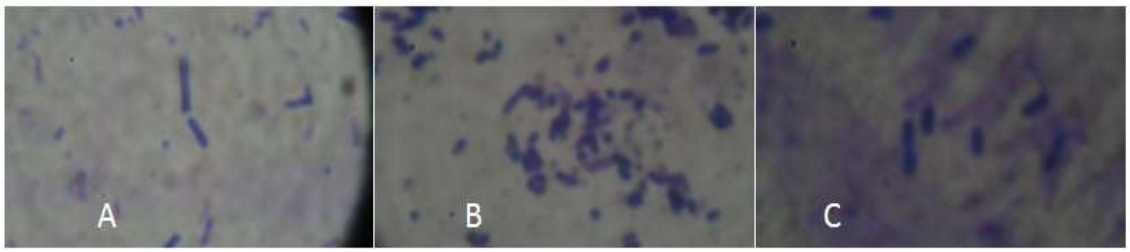

Fig. 3. Shows the 200X magnified view of sensitive and resistant *Shigella dysenteriae* species by advanced biological microscope, B1 series, Motic, China (Software: Motic image plus 1.0). Here A, B and C slide represents Sensitive strain, Ciprofloxacin-resistant, and Azithromycin resistant, respectively

We have exposed the sensitive isolates of *Shigella dysenteriae* to two different antibiotics at its MIC, above and below level to develop the resistance among them. And we found very little differences in these three different treatment groups in terms of their ZOI pattern. Finally we chose to treat the MIC level as a subinhibitory concentration for the resistant strain development. The antibiotic sensitivity pattern for different passages was shown different (Fig. 1). Subculturing in the Ciprofloxacin reduced the ZOI from 22 to 15 mm at their MIC. In case of Azithromycin, zone reduced from 24 to 18 mm at their MIC after 13 subcultures. Nilima Lankeshwar et al. [22] studies in 2013 and found that the *S. dysenteriae* resistant strain showed resistant to 10 µg/ml concentration of ciprofloxacin with no zone of inhibition, whereas in our study, ZOI reduced to 15 mm from 22 mm at their MIC (20 µg/ml) after development of resistance. From microscopic view it was observed that sensitive strain of *Shigella dysenteriae* was comparatively large in size and rod in shape, but the resistant strains look small and oval, instead. Study shows that *P. aeruginosa,* a Gram negative species, alters its morphology by carbapenem treatment at its sub-inhibitory level [23,24]. This changing in shape to oval depends on antibiotic concentration and exposure time [23].

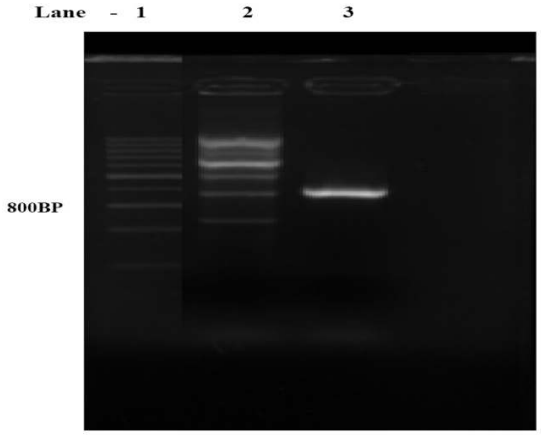

Fig. 4. PCR analysis antibiotic resistant determinants with reference strain. Here lane 1, is for DNA ladder, lane 2 is from transformed cells (Ciprofloxacin treated) and lane 3 is obtained from DNA of sensitive *Shigella* strain (reference strain). In the sensitive strain we found only one band but two or more bands were present in the transformed cells

Also PCR was carried out to observe the changes in the genomic DNA in those resistant strain in the presence of universal primers 27f (5´- AGATTTGATCCTGGCTCAG -3´), 1100γ (5´- GGGTTGCGCTCGTTG-3´) and 1114f (5´-GCAACGAGCGCAACCC-3´). We amplified the 16s rRNA gene from the plasmid DNA by these primers and separated in the agarose gel. Interestingly primer was matched in almost five positions, due to restriction fragment length polymorphisms, of that ciprofloxacin resistant strain and amplified, where as matching was found to have only one position, at 800 base pair, for the sensitive strain (Fig. 4).

5. CONCLUSION

Development of resistance to antibiotic is a serious healthcare problem for the treatment of infectious diseases. From our study it is clear that, strains of *Shigella dysenteriae* became resistant to Ciprofloxacin and Azithromycin due to irrational uses of these antibiotics. Morphological and genetic characteristics of resistant *Shigella* strains were quite different from that of sensitive *Shigella* strains. More studies should be carried out in respect to the development of resistance, morphological and genetic changes in microorganisms due to improper uses of antibiotics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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