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In vitro Development of Resistance to Azithromycin in *Pseudomonas aeruginosa*

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Abstract

Antimicrobial resistance has been considered as a growing public health problem all over the world. Due to improper use of antibiotics many bacterial species including the *Pseudomonas aeruginosa* become resistant. So, the objectives of this study were to determine the success or failure of antibiotic therapy. Tests were performed *in vitro* and measured the growth response of an isolated bacterium to a particular drug. This study determined the zone of inhibition, minimum inhibitory concentration (MIC) and time at which bacteria showed resistance. From sensitivity test, it showed that *P. aeruginosa* is sensitive to azithromycin antibiotic. MIC level of *P. aeruginosa* that was found from the analysis was 3 µg/ml and from further analysis it was found that *P. aeruginosa* grew resistance when it got up to 2.5 µg/ml concentration of antibiotic below MIC level for 24 hours. When the *P. aeruginosa* was treated with the concentration upto 2.5 µg/ml for 24 hours then it showed growth at the concentration of MIC level. It means that *P. aeruginosa* got the drug below MIC level for a certain period and became resistant to azithromycin.

Key words: *In vitro,* Azithromycin, Resistance, *Pseudomonas aeruginosa*.

Introduction

The antimicrobial resistance is increasing worldwide at an alarming rate. According to world health organization (WHO), antimicrobial resistance is one of the greatest threats to human health. In 2001, at least one of the currently available antibiotics became resistant due to pathogenic bacteria (Cragg and Newman 2001). In recent years, indiscriminate uses of antibiotics lead to antibiotic resistance (Haque *et al.* 2014). The prevalence of drug resistant lead to high mortality rates in immune compromised persons (Valan *et al,* 2009).

Most of the people in Bangladesh specially who are living in lowers socio-economic condition and lacking minimum health related facilities are often seen to take antibiotic at an irregular course. That's why microorganisms get the concentration or amount of drug which are enough to make them resistant to that drug. This bad habit are generating some unavoidable problems. This trend is directing our public health to a danger point .This is seen to impart a negative effect on the pharma antibiotic market and also increasing medical treatment cost.

P. aeruginosa is an aerobic bacterium with unipolar motility (Ryan and Ray 2004). *P. aeruginosa* can secrete a variety of pigments, including pyocyanin, pyoverdine, and pyorubin (King *et al.* 1954). Some *Pseudomonal* species were considered the causative agents of old diseases now are being reexamined for their potential use as biological warfare agents.

So, on the basis of the socio-economic condition of our country and health status of peripheral countryman it is urgently needed to carry out more and more fruitful studies about the antibiotic resistance and direct the unconscious people to a

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position where they will be more conscious about the importance of taking the full course antibiotic.

Materials and Methods

Collection and Pure Culture of Isolates: In this study total ten samples were collected from the drains of General Hospital, Prime Hospital, Royal Hospital and the wastewater channel of Globe pharmaceuticals in Noakhali. Bacterial strains were isolated from the samples and minimal inhibitory concentrations were determined. The collected samples were directly plated on nutrient agar plate through cotton bud and incubated at 37ºC for 24-48 hours. After the incubation period the plates were observed for any kind of growth on the media. The numbers of smooth colonies were counted. Then the colonies were randomly selected and streaked on the nutrient agar plate for purification. Pure culture of twenty different isolates was formed and the collected isolates were stored at -50^0C. Among twenty isolates, Isolate 2 was considered as *P. aeruginosa* using biochemical test.

Identification of bacterium using different agar media: For identification of *P. aeruginosa*, MacConkey agar, Eosine methylene Blue (EMB) agar, Cetrimide agar, Mannitol salt agar (MSA) and cystine lactose electrolyte deficient medium (CLED) were used.

Biochemical test: Some biochemical tests were also carried out for the characterization of *P. aeruginosa* such as Oxidase test, Urease test, Indole test, Citrate test and Triple Sugar Iron (TSI) test. Morphological characters of selected isolate were observed by cultural and microscopic methods. By the cultural method using selective media such as CLED agar media, Cetrimide agar, blood agar, macconkey agar media colony characteristics were observed. Microscopic methods were used for the study of size, shape, arrangement, color etc. Biochemical tests were used which were more important than morphological characteristics in the identification of bacteria up to species using "Bergey's Manual of bacteriology", 9^{th} edition.

Antibacterial assay against P. aeruginosa: Antibacterial activity of antibiotics was evaluated *in vitro* against *P. aeruginosa* by disc diffusion assay (Bauer *et al.* 1966). *P. aeruginosa* bacterium was streaked on two petri-plates. The plates were occupied by ten antibiotic disks and kept for incubation for 24 hours. Then the zone of inhibition was measured using a millimeter scale.

Minimum Inhibitory Concentration (MIC) tests: MIC test of azithromycin was done by serial tube dilution technique or turbidimetric assay against *P. aeruginosa* (Reiner, 1982). 15 test tubes were taken and each of the test tubes was filled with 9 ml McFarland solution. Then the antibiotic solutions were added. Different concentrations such as1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 µg/ml were prepared and incubated at 24 hrs. Finally, the growth of bacteria was observed to find out the minimum concentration at which bacteria could not grow.

Resistance development test: As the MIC level for *P. aeruginosa* against azithromycin antibiotic was 3 µg/ml so antibiotic solution of 1.0, 1.5, 2.0, 2.5, 3.0 µg/ml concentration were prepared .These solution of azithromycin was added to the test tube containing 9 ml nutrient broth solution. Sample containing bacteria and drug (1.0, 1.5, 2.0 and 2.5 µg/ml) were observed after 24 hours to see the growth of *P. aeruginosa*. If growth was observed, then the bacteria was treated with drug at MIC level and observed after 24 hrs. If growth was found at MIC level then it could due to resistance developed in microorganisms.

Results and Discussion

P. aeruginosa is a gram-negative rod-shaped bacterium that can cause disease in plants, animals and humans. It is an opportunistic pathogen, often causing nosocomial infections. In addition to causing serious and life threatening diseases, this organism exhibits innate resistance to many antibiotics and can develop new resistance after exposure to antimicrobial substances.

In biochemical analysis, the selected bacterial strain showed negative in gram staining, indole test and urease test whereas positive result found in citrate and oxidase test (Table 1).

Isolate 2 contains large genome (5.5-7.5 Mb) and encodes between 5,500 and 6,500 open reading frames (Klockgether *et al.* 2011). It was analyzed that 5,021 genes are present across the first five genomes with at least 70% sequence identity .This set of genes is considered as *P. aeruginosa* core genome (Mathee *et al.* 2008).

Isolate 2 showed saffranin or red color which indicates that the strain is gram-negative. Morphological, cultural and biochemical analysis were done and it was confirmed that the Isolate 2 is *P. aeruginosa.* Disc diffusion method was used to measure bacterial susceptibility (Bauer *et al*, 1966). Ampicillin (10 µg), Cloxacillin (1 µg), Amoxacillin (20 µg), Ceftriaxone (30 µg), Clindamycin(30 µg), Ciprofloxacin (30 µg), Azithromycin (5 µg), Gentamicin (15 µg), Cotrimoxazole (10 µg) and Chloramphenicol (25 µg) antimicrobial disks were used.

Table 1. Identification of *P. aeruginosa.*

Test Performed	Isolate 2
MacConkey agar media	White or colorless colony
Eosine methylene blue agar media	Pinkish mucoid colonis
Cystine lactose electrolyte deficient medium	Green Colonies
Mannitol salt agar media	No Growth
Cetrimide agar media	Yellow green to blue colonies
Tripple Super Iron test	Red slant and red butt
Indole Test	Negative
Citrate Test	Positive
Oxidase test	Positive
Urease Test	Negative
Gram staining	Negative

Table 2. Zone of inhibition of different antibiotics against *P. aeruginosa.*

Name of bacterium	Tested Antibiotic	Disk potency	Zone of inhibition(mm)
Pseudomonas aeruginosa	Ampicillin	10µg	12
	Cloxacillin	1 µg	13
	Amoxacillin	20 µg	15
	Ceftriaxone	30 µg	16
	Clindamycin	30 µg	16
	Ciprofloxacin	30 µg	17.5
	Azithromycin	5 µg	18
	Gentamicin	15 µg	13.5
	Cotrimoxazole	10 µg	13
	Chloramphenicol	25 µg	15

From Table 2 it is seen that azithromycin shows largest zone of inhibition that means it is more sensitive to *P. aeruginosa* bacteria.

Table 3. Minimum Inhibitory Concentration (MIC) test of azithromycin against *P. aeruginosa.*

Number of test tubes	Concentration (µg/ml)	Growth of *p. aeruginosa*
1	1	+
2	2	+
3	**3**	-
4	4	-
5	5	-
6	6	-
7	7	-
8	8	-
9	9	-
10	10	-
11	11	-
12	12	-
13	13	-
14	14	-
15	15	-

The sign "+" = growth "-" = no growth. Table 3 showed no growth of *P. aeruginosa* at or above 3 µg/ml of azithromycin. Therefore, 3 µg/ml was the MIC level.

Table 4. Resistance development of *P. aeruginosa* against azithromycin.

Sample containing bacteria	Addition of azithromycin solution		Bacterial growth
9 ml	1.0 µg/ml	Subjected in incubator for 24 hrs	+
9 ml	1.5 µg/ml		+
9 ml	2.0 µg/ml		+
9 ml	2.5 µg/ml		+
9 ml	Treated the bacteria with the antibiotic at MIC (3 µg/ml) level		Bacterial growth was observed

From Table 4 it is seen that as *P. aeruginosa* bacteria gets drug up to 2.5µg/ml for 24 hrs then it shows growth at MIC level that means the bacteria get resistant.

Conclusion

In 21st century, drug resistance among bacteria is a global health problem. There is no doubt that we are now facing greater antibiotic resistance challenges than ever before, limiting treatment options for patients with severe infections. Now-a-days new antimicrobial agents have failed to keep pace with the ingenuity of resistant bacteria. So, we have to adapt this threat by reducing unnecessary antibiotic prescribing, both qualitatively and quantitatively. Therefore, we need to minimize the risk of spreading resistant bacteria, and we have to find novel compounds active against resistant bacteria.

Declaration of Interests

The authors have no conflicts of interests.

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