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Research paper



Investigation on the Effect of Antibiotic and Multivitamin in the Formation of Biofilm in Urinary Tract Infection (UTI) Causing Pathogens

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Abstract

Urinary catheters make humans vulnerable to Urinary Tract Infection (UTI) by damaging the natural barrier of the body. Bacteria which are commonly related to this infection are *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*. In this study *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were obtained from the hospital. Levofloxacin is a newly developed fluoroquinolone antibiotic, which is commonly used in clinical practice. The antibacterial effect of levofloxacin was studied using disk diffusion method. In this method the diameter of zone of inhibition in the presence of multivitamin was smaller than without multivitamin. The statistical analysis showed a significant difference in antibiotic sensitivity with and without multivitamin ($p<0.05$). In the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), It was observed that in the absence of multivitamin *Klebsiella pneumoniae* growth stopped at the concentration of 200 µg/ml and in the presence of multivitamin the growth stopped at the concentration of 400 µg/ml. The growth of *Pseudomonas aeruginosa* stopped at the concentration of 400 µg/ml in the absence of multivitamin and in the presence the growth was stopped at the concentration of 800 µg/ml. Similar effect was studied in biofilm form as well by measuring the absorbance at 600nm. Using the absorbance values the biofilm growth curve was carried out and in the presence of multivitamin, both bacteria in single and consortia form stayed in stationary phase longer than without multivitamin. These findings demonstrate that a higher concentration of antibiotic is required to inhibit the growth of bacteria when supplemented with multivitamins and in turn increases the development of antibiotic resistance under biofilm condition.

Keywords: *Biofilm; Levofloxacin; MBC; MIC; UTI*

1. Introduction

Urinary tract infections (UTIs) are common and affects all age groups [1]. Nosocomial urinary tract infections are acquired in hospitals. The important reason for this infection is the biofilms associated with catheters [2]. The most common bacteria that cause biofilm formation are *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Escherichia coli* [3]. Here biofilm causes persistent infections by overcoming the host defense mechanisms. The virulence factors which support them in invasion include fimbriae, adhesions, etc. Biofilms consist of the bunch of microorganisms, which are associated with living or non-living surfaces. Certain group of microorganisms in the biofilm secrete intracellular polymeric substances and they can be homogeneous or heterogeneous [1]. The microorganisms itself form a matrix of extracellular polymeric substance (EPS) to attach to each other which then will be bond tighter. The matrix formation by microorganism make them resistant to most of the antibiotics [4].

Formation of biofilm occurs immediately after the insertion of the catheter. Bacteria attached to the conditioned protein surfaces, which is near to the catheter surface. The planktonic bacteria with the help of the flagella move towards the conditioned surfaces and attach there. In addition to the catheters, the biofilm is present in uroepithelium, prostrate stone, etc. The biofilm formed in the urinary catheter can cause pyelonephritis by infecting the renal tissues [5].

The major anxiety on infection is the challenge in their elimination as inner cells in a biofilm are protected from the immune mechanism of the host and from the impact of antibacterial agents [6]. A biofilm will minimize the growth rate, which will automatically reduce the movements of antimicrobial agents [7]. The resistance formed by bacteriae in biofilm is due to certain intrinsic mechanism and this is also one of the factors causing it to withstand antibiotic sensitivity [8]. In the matter of mixed bacterial growth, bacteria had already become antibiotic sensitive can become resistant in succeeding antibiotic susceptibility test from a mechanism called horizontal gene transfer and the resistant bacteria can change to sensitive bacteria when they grow within a biofilm [6]. The microorganisms like *Proteus sp.* produce urease enzyme that hydrolyze urea to ammonia ions this increases the pH of the urine, and results in the formation of magnesium and phosphorous crystal. These crystals can protect bacteria from antibiotics. [2].

Quinolones and fluoroquinolones are synthetic antibiotics which have bactericidal property and broad spectrum activity towards infection causing pathogens. They are used for the treatment of uncomplicated UTI, nosocomial UTI and pyelonephritis. Among fluoroquinolones, levofloxacin is a recently developed antibiotic and widely used in treatment [9]. It is the L isomer of ofloxacin and gives the same antibacterial effect of ofloxacin but with half

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the dose. It is a broad spectrum antibiotic which shows strong antibacterial effects on *E. coli, K. pneumonia, Salmonella sp*. etc. It is found that levofloxacin is a suitable antibiotic for UTI [10].
A multivitamin is a supplement which has a range of low dose vitamins and minerals that is suggested to be consumed daily as dietary intake [11]. It might contain more than one vitamin such as vitamin A, β-carotene, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B6 (pyridoxine), vitamin B12 (cyanocobalamin), vitamin C, vitamin D, vitamin E, folic acid, niacin and calcium [12]. The consumption of vitamins along with antibiotics may show beneficial [13]. Supplementation of multivitamins helps in preventing DNA damage and they contain antioxidants [14].
The aim of this study is to check the effectiveness of antimicrobial agent with the presence of multivitamin and to investigate the role of multivitamin in the formation of biofilm and how induces resistance towards antimicrobial agents.

2. Methods

P. aeruginosa and *K. pneumoniae* were selected for this study. The pure culture of bacteria was obtained from Hospital Kuala Lumpur. Before storage bacterial strains were checked for their purity and viability by streaking them in selective media. Then observed their colony morphology and Gram staining on the overnight culture. The identity of the organisms was confirmed by performing biochemical tests. The preparation of working stocks was done by plating the strains in Nutrient Agar slant and stored at 4^0c. The glycerol stocks were kept under -20^0C and -80^0C. the working stocks were prepared freshly once in two weeks' time to ensure the viability of the strain. Levofloxacin stock was prepared by mixing pure levofloxacin with 0.1% Dimethyl sulfoxide (DMSO) with the final concentration of 100µg/ml. The multivitamin solution was prepared by mixing the with water to the final concentration of 0.05ml/ml.

2.1. Antibiotic Susceptibility test (Kirby-Bauer Method)

Antibiotic susceptibility test was carried out by using disk diffusion and the tests were repeated three times. Levofloxacin antibiotic disks were prepared aseptically in the concentration of 40µg/disk, 200µg/disk, 400µg/disk and 2000µg/disk. The disk was placed about 24mm apart from each to avoid overlapping. The same method was repeated on another set of Mueller Hinton (MH agar) and multivitamin solution of 0.05ml/ml of MH [15].

2.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC of the levofloxacin against the organisms was detected by the tube dilution method [16]. In this logarithmic dilution method was employed and the concentration of antibiotic used was ranging from 0µg/ml to 51200µg/ml with and without multivitamin.
MBC was performed to check at which concentration does the antibiotic eradicate the bacteria. From the MIC broth, one loop of broth was taken and streaked onto the nutrient agar plate. The plates were incubated at 37°C for 24 hours. The highest concentration of antibiotic kills the bacteria is defined as MBC [17].

2.3. Seed preparation and Biofilm formation:

The seed for the biofilm formation was prepared by adding 5ml of overnight cultured broth to 5ml of PBS (1:1) and centrifuged at 3000 rpm for 10 minutes repeated for three times. Discarded the supernatant after centrifugation. 50 ml of Tryptic Soy Broth (TSB) containing 0.24% of glucose added into the sediment and mixed well. Biofilm formation was carried out in flat bottom 96 well microtiter plate with a single organism and mixed organisms known as consortia. The consortia of bacteria were prepared in the ratio of 20:80, 40:60, 60:40 and 80:20. Two microtiter plates were used for the formation of biofilm, one set for single organism biofilm and another set for consortia biofilm and both sets were incubated with and without the multivitamin. Cultured TSB (200µl) added to all the wells except 12th well, which serves as the control. Accordingly, levofloxacin was added to the concentration ranging from 0 µg to 51200µg/ml of total. Upon addition of all the components, the first reading was taken at 0 hours and followed by 2, 4, 8, 12, 24, and 48 hours by using an ELISA Microtiter plate reader (Labtech LT-5000 Plate Reader) at 600nm absorbance. In between the plates were kept in the incubator at 37°C and graph was plotted with absorbance against hours of incubation. After 48 hours of incubation the contents were aspirated, and plates were washed with PBS. The microtiter plates were stained with 0.25% crystal violet and for the observation of biofilm [18].

2.4. Statistical analysis:

The zone of inhibitions from antibiotic sensitivity tests were recorded and statistically analysed using SPSS software version 23. The data were analysed with independent t-test and $p<0.05$ was accepted as statistically significant. [19].

3. Results and Discussion:

3.1. Antibiotic Susceptibility test using disk diffusion method with and without multivitamin:

In this study, we have investigated the antibacterial activity of levofloxacin against uropathogens in planktonic and biofilm form in the presence and absence of multivitamins *Invitro*. The results obtained from the Kirby- Bauer disk diffusion antibiotic susceptibility testing showed that an increase in the concentration of antibiotic to inhibit the growth of bacteria. The size of the zone of inhibition increases as the concentration of antibiotic increases. In addition, the zone of inhibition on MH agar containing multivitamin is smaller when compared to the zone of inhibition on MH agar without multivitamin. The table.1 shows that the zone of inhibition on MH agar containing multivitamin is smaller when compared to the zone of inhibition on MH agar without multivitamin. The antibacterial effect of levofloxacin against *Klebsiella pneumonia* at the concentration of 200 µg/disk and 400 µg/disk was significantly higher without multivitamin ($p<0.05$) as indicated by higher zone of inhibition in MH agar without multivitamin.

Table 1: The influence of multivitamins on the diameters of the zone of inhibition produced by levofloxacin.

Bacterial strains		Diameter of zone of inhibition	
	Antibiotic concentration	Mean ±SD	
	µg/disk	Control	+ multivitamin
K.pneumoniae	2000	2.4±0.010	2.5±0.12
	400	1.50±0.10	1.9±0.10***
	200	1.1±0	1.4±0.10***
	40	0	0
P. aeruginosa	2000	2.2±0.10	2.4±0.058
	400	0	0
	200	0	0
	40	0	0

*** Numerical value that are significantly different from the values for the corresponding controls ($P<0.05$).

As per the observation, Fig.1 &2 shows that at 40µg/disk, 200µg/disk and 400µg/disk bacteria exhibit smaller zone of inhibition compared to 2000µg/disk. The highest zone of inhibition can see in 2000µg/disc. It is due to the high levofloxacin concentration.

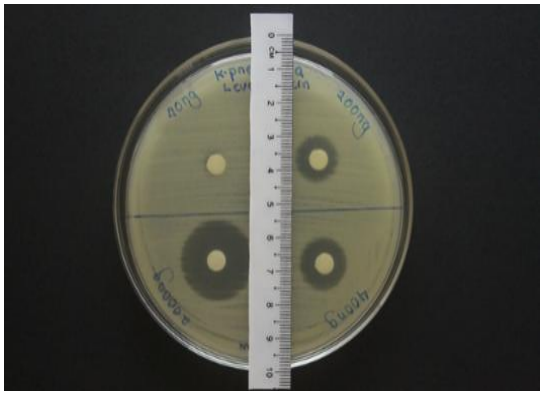

Fig.1: Antibiotic sensitivity pattern of levofloxacin against *K. pneumoniae* with multivitamin at the concentration of 40µg/disk, 200µg/disk, 400µg/disk and 2000µg/disc.

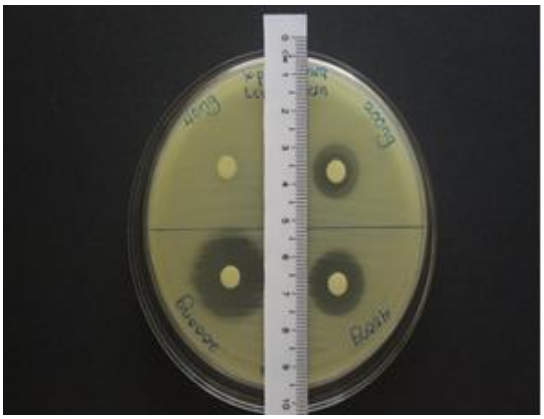

Fig.2: Antibiotic sensitivity pattern of levofloxacin against *K. pneumoniae* without multivitamin at the concentration of 40µg/disk, 200µg/disk, 400µg/disk and 2000µg/disc.

For levofloxacin diameter of inhibition in the presence of multivitamin was lower than corresponding control (Fig.3 &4), suggesting that the multivitamin interfered with antibacterial activity.

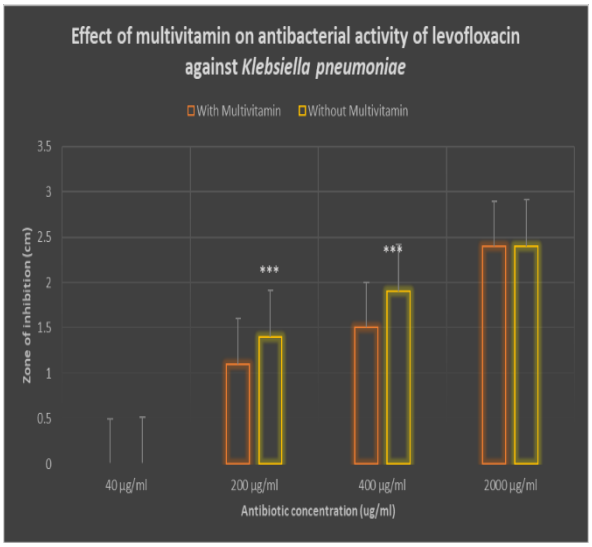

Fig.3: Effect of multivitamin on antibacterial action of levofloxacin for *K. pneumoniae* in the presence and absence of multivitamin expressed as mean ± standard error. Groups represented by *** indicates significant difference with $p<0.05$.

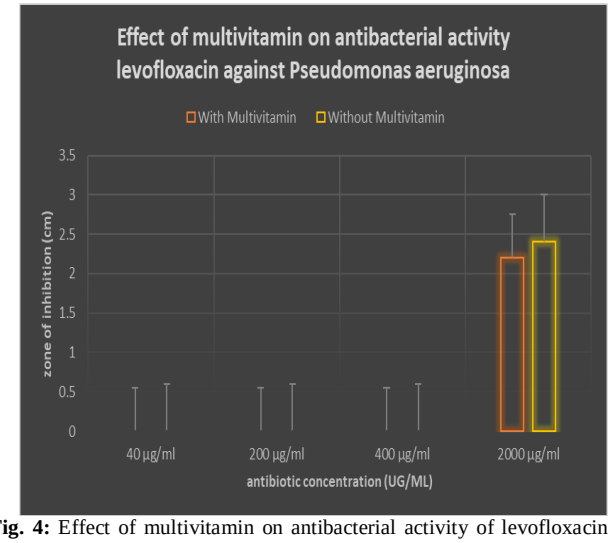

Fig. 4: Effect of multivitamin on antibacterial activity of levofloxacin at various concentration against *Pseudomonas aeruginosa* expressed as mean ± standard error.

3.2. Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC) of levofloxacin against UTI pathogens:

The results of the MIC which is done in the tube dilution method had shown almost similar pattern of results as the disk diffusion antibiotic sensitivity test. Based on results shown inTable.2 below, the reading of *P. aeruginosa* and *K. pneumoniae* showed less turbidity in the control tubes whereas the tubes containing multivitamin had more turbidity in the higher concentration of antibiotic for both organisms.

Table 2: MIC results *of K. pneumonia* and *P. aeruginosa* in tube dilution method. (+) refer to turbidity whereas (-) refer to the absence of turbidity.

Antibiotic concentration (ng/ml)	0	25	100	200	400	800	1600	3200	6400	12800	25600	51200
*Lev	0	1	2	3	4	5	6	7	8	9	10	11
K. pneumonia												
*C	+	+	+	-	-	-	-	-	-	-	-	-
*MV	+	+	+	+	-	-	-	-	-	-	-	-
P. aeruginosa												
C	+	+	+	+	-	-	-	-	-	-	-	-
MV	+	+	+	+	+	-	-	-	-	-	-	-

*Lev= levofloxacin: *C= control: *MV=Multivitamin

Data obtained from the MBC reading was almost like MIC reading.

During this observation, the minimum concentration of antibiotic required to inhibit the growth of *K. pneumoniae* was 100µg/ml. In the presence of multivitamin, the concentration required was increased to 200µg/ml. The minimum inhibitory concentration needed for *P. aeruginosa* was 200µg/ml but in the presence of multivitamin it raised up to 400µg/ml. The MIC result was confirmed by the minimum bactericidal concentration test. The MBC confirmed on the growth variation of bacteria in the presence and absence of multivitamin. The figure.5 shows that *P. aeruginosa* can grow in the nutrient agar containing levofloxacin until the concentration of 400µ/ml, after that there is no growth in the media. The plate with multivitamin showed growth of the same bacteria at 200µg/ ml. The same growth pattern was observed in the case of *K. pneumoniae*.



Fig.5: MBC of levofloxacin against *P. aeruginosa* with (Right)and out multivitamin (Left).

Our study is consistent with the findings of Goswami *et al (*2007) [19]. The study stated that the presence of glutathione and ascorbic acid help *E. coli* to survive from the antibacterial actions of aminoglycosides by a mechanism, that is still not fully understood.

3.3. Biofilm formation

The development of biofilms was confirmed by visual observation using Crystal Violet staining. The reading of the biofilm formation of both single organism and consortia pointed out the difference in absorbance. Both gave an increased absorbance in multivitamin treated condition compared to non-treated with multivitamins.

The biofilm formation rate was slow in the control plate, whereas, biofilm treated with multivitamin rate was very obvious. The obtained result clearly showed that the multivitamin increased the rate of biofilm formation by increasing its resistance towards the antimicrobial agent.

3.3.1. Single organism Biofilm

The biofilm growth curve was constructed to determine the various phases that occur during biofilm development and the duration of each stage. The growth curve of *P. aeruginosa* at the concentration of 51200µ/ml of levofloxacin with and without multivitamin was shown in the Fig.6. In the presence of multivitamin, the growth of bacteria was higher compared with the growth of bacteria in absence of multivitamin. In the multivitamin treated condition bacteria stays longer in stationary phase.

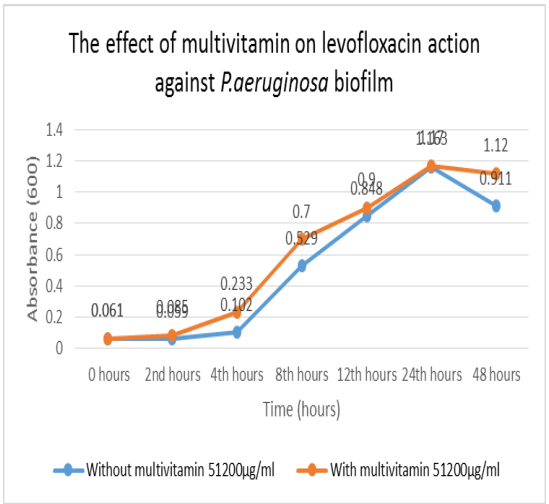

Fig.6: Growth curve of *P. aeruginosa* at 51200µg/ml of levofloxacin with and without multivitamin in biofilm

The growth curve of *K. pneumoniae* in a biofilm condition was shown in Fig.7. In which the bacterial growth curve shows a rise after 2 hours for both with and without multivitamin. It is consistent up to the 24th hour. After the 24th hour, the growth curve without multivitamin was in a decline phase. As the incubation time increases the growth curve with multivitamin stays in a stationary phase more than that of without multivitamin.

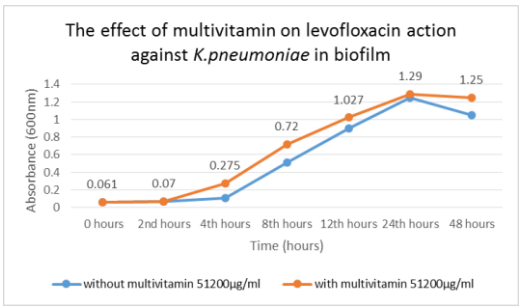

Fig.7: Biofilm growth of *K.pneumoniae* at 51200µg/ml of levofloxacin with and without multivitamin.

3.3.2. Mixed Biofilm

Biofilm formed with mixed species of *K. pneumoniae* and *P. aeruginosa* showed sensitivity towards levofloxacin in the absence of multivitamin. In a condition with higher concentration of levofloxacin, the absorbance showed lower values as incubation time increased and slowly attaining the stationary phase towards 48th hours of incubation. This can be visualized clearly in Fig.8 with a concentration of 1000 µg/ml of levofloxacin in a ratio of 80:20 (*P. aeruginosa: K. pneumoniae*). In the presence of multivitamin, the absorbance is higher, and it takes time to decline compared to the condition without multivitamin.

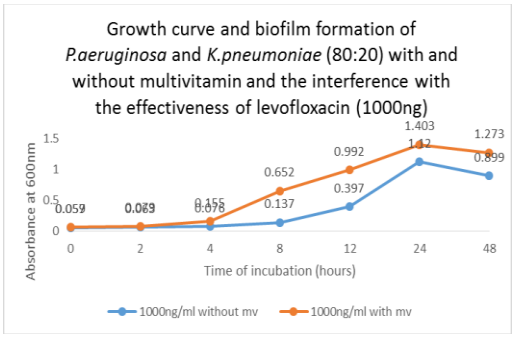

Fig.8: Growth curve of *P. aeruginosa* and *K. pneumoniae* in the ratio of 80:20 with and without multivitamin.

In biofilm condition when there is decrease in the availability of nutrition, bacteria enter a stationary phase and grow very slowly or sometimes stop growing and in this zone bacteria less susceptible to antibiotics [20]. In our findings, the reduction of antibiotic sensitivity in the presence of multivitamin is different from the studies by Eman El-Gebaly (2012) [21]. The study showed that the biofilm formation upon combination with the antibiotic levofloxacin and vitamin C will be inhibited rather than enhancing the biofilm formation in catheters.

Antibiotic resistance is the most emerging problem in medical history which may be due to many factors during the treatment procedure. According to Masadeh *et al* (2016), the involvement of ciprofloxacin can generate reactive oxygen species. But the antioxidants present in the multi-vitamin can protect the bacteria from these reactive oxygen species. In our present studies this condition was proved. The antibiotic sensitive test showed less effectiveness towards the bacteria treated with multivitamins [22]. The antibiotic sensitivity tests on biofilm condition also showed the same results. The bacteria in the biofilm treated with multivitamin stayed longer in a stationary phase than the condition without multivitamin. The current study showed that the presence of multivitamins makes the bacteria more resistant towards levofloxacin.

4. Conclusions

In conclusion, these results showed the rescue effect of multivitamins on bacteria from levofloxacin in planktonic as well as biofilm form. The antioxidants present in the multivitamin lower the

antibiotic activity of levofloxacin. The observation might have significant associations toward the therapeutic characters since multivitamins with the antioxidant property are sometimes prescribed along with the antibiotics during the course of treatment. The consumption of antibiotics may lead to the loss of normal flora and the multivitamin offsets the damage of it. Under such conditions, the effectiveness of the antibiotic considerably decreased due to the increased level of antioxidants. This research can be further investigated on the cellular damage and DNA damage studies using comet assay (single-cell gel electrophoresis).

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