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Evaluation of Phenotypic Variations in the Antibiotics Sensitivity of Escherichia Coli by Repeated Exposure

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Abstract

Enterobacteriaceae, in particular Escherichia coli, are habitual residents of the gastrointestinal tract, capable of causing a large number of infections. The MIC varies according to the bacterial strains and the antibiotics used, hence the need to carry out antibiotic sensitivity tests. The objective of this study is to evaluate the behavior of Escherichia coli after repeated exposure to the same antibiotic to demonstrate a possible correlation between excessive intake of antibiotics and bacterial resistance. A prospective and descriptive study was carried out in the Microbiology Laboratory of Fundamental and Applied Biochemistry (Faculty of Sciences Antananarivo) during the month of November 2019. The strains studied were the reference strain Escherichia coli ATCC 25922 provided by the Laboratory and two clinical strains from the Microbiology Laboratory of the Joseph Ravoahangy Andrianavalona University Hospital Center Antananarivo. Repeated exposure to Tobramycin and Ofloxacin of these strains were performed. The results of our study showed that most E. coli is exposed to the antibiotic, the more it develops resistance. The evolution of E. coli's sensitivity is different in the presence of Tobramycin with MICs up to 4 times the starting value while in the presence of Ofloxacin, the MIC increases to 125 times the initial value. This difference may be due to the different target of the antibiotic which causes the bacteria to develop variable mechanisms to escape it.

Keywords: E. *coli;* MIC; Antibiotics; Repeated exposure

Introduction

Enterobacteriaceae, in particular Escherichia coli, are habitual residents of the gastrointestinal tract, capable of causing a large number of infections [1]. The discovery of antibiotics at the beginning of the twentieth century was a real revolution in the medical field [2]. These molecules are powerful weapons capable of eradicating infectious diseases of bacterial origin. However, excessive, and uncontrolled use of antibiotics is one of the first pathways to bacterial resistance [3, 4]. Antibiotic resistance has gradually developed. It results from the repeated administration of antibiotics in humans or animals which creates conditions, called "selection pressure" favoring the acquisition and dissemination of strains resistant to antibiotics [5]. Reducing the excessive use of antibiotics and respect adequate therapeutic doses are part of the basic recommendations to fight against the spread of resistant bacteria. Indeed, it is recommended that an antibiotic should be used at least in doses 4 times higher than the minimum inhibitory concentration (MIC) to ensure good therapeutic success [6].

The MIC varies according to the bacterial strains and the antibiotics used, hence the need to carry out antibiotic sensitivity tests, especially on newly isolated strains [7]. However, it is also important to verify the existence of a variation in the effectiveness of these antibiotics on bacterial strains previously exposed to these same

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antibiotics at doses normally inhibiting their normal growth.

The objective of this study is to evaluate the behavior of Escherichia coli after repeated exposure to the same antibiotic to demonstrate a possible correlation between excessive intake of antibiotics and bacterial resistance.

Material And Methodology

A prospective and descriptive study was carried out in the Microbiology Laboratory of Fundamental and Applied Biochemistry (Faculty of Sciences Antananarivo) during the month of November 2019. The strains studied were the reference strain Escherichia coli ATCC 25922 provided by the Laboratory and two clinical strains from the Microbiology Laboratory of the Joseph Ravoahangy Andrianavalona University Hospital Center Antananarivo. Repeated exposure to Tobramycin and Ofloxacin of these strains were performed.

Escherichia coli strains were cultured in LB-MOPS medium (Luria Bertani, supplemented with a buffer system of 3- (N-morpholino) propanesulfonic acid which will stabilize the pH). After weighing the LB and MOPS, the powders are dissolved in distilled water in order to have a homogeneous mixture. The pH of the medium is regulated before autoclaving with 0.2N HCl or 1N KOH up to 7.2. The solid culture medium used in this work was Columbia agar of concentration 42.5 $g.l^{-1}$.

The antibiotics tested were Ofloxacin and Tobramycin, supplied by the Laboratory of Microbiology of Fundamental and Applied Biochemistry (Faculty of Sciences Antananarivo). A stock solution of antibiotics is prepared to have a concentration of 6mg.ml-1 for Tobramycin and 1.5$mg.ml^{-1}$ for Ofloxacin. Then the antibiotics are diluted in DMSO (Dimethylsulfoxide). To have these concentrations, the following formula is adopted:

DMSO volume = net weight weighed / Final concentration

The research for the MIC was then carried out which is the lowest concentration of a range of dilutions of antibiotic of half in half, which involves an inhibition of any visible bacterial growth after 24 hours of culture at 37°C [8, 9]. This value characterizes the bacteriostatic effect of an antibiotic.

As principle, the dilution method is used both in liquid medium than in solid medium. It consists in mixing the bacterial strain and the culture medium with a decreasing concentration of reason 2 antibiotics. The manipulation is done in a series of test tubes (for the macrodilution method) and the microplate or wells (for the method in microdilution).

A strain preculture of E. coliof 18h was prepared by introducing 500µl of stock strain of E. coli in 5ml of LB-MOPS liquid medium (pH = 7.2). The whole is incubated with shaking at 37°C for 24 hours. After centrifugation (3200 g for 5 min), the bacterial suspension was washed in 5 ml of LB-MOPS. The inoculum was obtained by adjusting the optical density of the bacterial population to a value of 0.04 at the wavelength of 600nm using a spectrophotometer.

Microdilutions were performed on a 96-well plate. For the first four lines (A, B, C and D), we deposited 3µl of Ofloxacin and 297µl of bacterial suspension in the first wells. The final concentration of Ofloxacin is then reduced to one hundredth of its initial concentration, i.e. 15 $µg.ml^{-1}$. Then, we put 150µl of bacterial suspension in the last eleven wells. This manipulation has been done for more certainty in the results.

For the last four lines (E, F, G and H), we deposited 3 µl of Tobramycin (TM) and 297 µl of bacterial suspension in the first wells. The concentration of Tobramycin in the first well is then reduced to one hundredth of its initial concentration, i.e. 60 $µg.ml^{-1}$. The rest of the operation is the same as that previously done for Ofloxacin.

A successive dilution is then carried out. Using a micropipette, we took 150µl of solution from the first well and pour it into the second well on the same line. Similarly, we took the same volume from the second well and transferred it to the third well. The same procedure was repeated until well number 11. The operation is stopped at the eleventh well so that the twelfth well can be used as a positive control of the manipulation. This successive dilution technique makes it possible to have a decreasing antibiotic concentration of reason 2. Finally, the plate is then incubated with shaking at 37° C for 24 hours. After 24 hours of incubation, the MIC is determined by the minimum molar concentration where there is no visible growth, i.e. it is determined by the last well where there is no clouding medium observable with the naked eye. The presence of visible growth is determined by adding 40 µl of p-iodonitrotetrazolium chloride (INT) at 0.2 $mg.ml^{-1}$. The pink or red coloration on the culture medium indicates the presence of bacterial growth after 30 minutes of incubation at 37° C.

For repeated exposure, the bacterial strain must be exposed to this same antibiotic throughout the treatment. From the colonies of the spot corresponding to the well of the IJC, 3 bights of the strain of E. coli were seeded in LB-MOPS for a pre-culture of 18 hours at 37° C with stirring. After washing and centrifugation, the optical density is measured at 600nm and should have

a value of 0.04. A culture on a 96-well plate for the determination of a new MIC is then carried out according to the same procedure. After incubation, adding INT to 0.2mg.ml^{-1} will confirm the presence of bacterial growth in the 96-well plate. These stages constitute a second cycle of exposure to an antibiotic. The operation is repeated until the fifth generation of the Escherichia coli strain exposed to the same antibiotic.

Results

Three strains of E. coli were studied by looking for their respective MICs with Tobramycin and Ofloxacin.

Sensitivity to Tobramycin: Table 1 shows the results for the MIC(pg.ml-1) of Tobramycin as a function of the generations of E. coli.

Table 1: distribution of the MIC of E. coli strains to Tobramycin according to generations

Generation	MIC ATCC 25922	MIC E. coli 1	MIC E. coli 2
G1	7,5	9,375	4,6875
G2	7,5	75	9,375
G3	30	75	9,375
G4	30	75	9,375
G5	30	75	9,375

Table 1 shows the respective MIC values for tobramycin as a function of the generations of E. coli. The lowest MIC value was 7.5 pg.ml-1 for the strain ATCC 25922, 9.375 for the strain E. coli 1 and 4.6875 for the strain E. coli 2 for the first generations of E. coli tested respectively with Tobramycin. The maximum bacteriostatic activity was 30 pg.ml-1 for the ATCC 25922 strain (4 times the initial strain), 75 pg.ml-1 for the E. coli 1 strain (8 times the initial strain) and 9.375 for the E. coli 2 strain (twice the initial strain). These results are observed since the third generation for the ATCC 25922 strain and for the 2nd generation for the 2 other strains which no longer varies until the fifth generation (Table 1).

Table 2: Distribution of the MIC of E. coli strains to Ofloxacin according to generations

Generation	MIC ATCC 25922	MIC E. coli 1	MIC E coli 2
G1	0,12	0,9375	0,9375
G2	0,46	37,5	1,875
G3	15	37,5	1,875
G4	15	37,5	1,875
G5	15	37,5	1,875

Sensitivity to Ofloxacin: Table 2 shows the respective MIC values for Ofloxacin as a function of the generations of E. coli.

Table 2 shows the respective MIC values for Ofloxacin as a function of the generations of E. coli. The lowest MIC value was 0.12 pg.ml-1 for the strain ATCC 25922, 0.9375 pg.ml-1 for the strain E. coli 1 and 2 for the first generations of E. coli tested respectively with Ofloxacin. The maximum bacteriostatic activity was 15 pg.ml-1 for the ATCC 25922 strain (125 times the initial strain), 37.5 pg.ml-1 for the E. coli 1 strain (40 times the initial strain) and 1.875 for the E. coli 2 strain (twice the initial strain). These results have been observed since the third generation for the ATCC 25922 strain and for the 2nd generation for the 2 other strains which no longer varies until the fifth generation (Table 2).

Discussion

To assess phenotypic variations in Escherichia coli susceptibility to antibiotics after repeated exposure under in vitro conditions, we investigated the minimum inhibitory concentration (MIC) of Tobramycin and Ofloxacin. The MIC identifies the sensitivities of bacterial strains when faced with an antibiotic. It also makes it possible to adapt the dosages by comparison of MICs and serum dosages [10].

The results of our study showed that most E. coli is exposed to the antibiotic, the more it develops resistance. The evolution of E. coli's sensitivity is different in the presence of Tobramycin with MICs up to 4 times the starting value (Table 1) while in the presence of Ofloxacin, the MIC increases to 125 times the initial value (Table 2). This difference may be due to the different target of the antibiotic which causes the bacteria to develop variable mechanisms to escape it. E. coli's strategies to escape the action of antibiotics are numerous such as target modifications, enzyme inactivation or the efflux pump system [11, 12].

Compared to data from CA-SFM (2019), E. coli is resistant to Tobramycin from the first generation for the three strains. However, it should be emphasized that the culture medium used for the determination of the MIC is not identical (LB-MOPS medium versus Mueller-Hinton medium), which makes the comparison not very timely.

Regarding sensitivity to Tobramycin, studies by Brilt MR, Dhondikubeer andal. [13, 14] have shown that Tobramycin has antibacterial activity against Gram negative bacteria, in this case E. coli, and has an MIC value between 4 and 8 pg.ml-1.

Based on the results of our study, we found that E. coli has a basic sensitivity to Tobramycin at

a concentration of 7.5 pg.ml-1 for ATCC 25922, 9.375 for E. coli 1 and and 4.6875 for E. coli 2 for the first exposure to antibiotic which evolves towards resistance from the 3rd generation for the ATCC 25922 strain and from the 2nd generation for the E. coli 1 and 2 strain. This modification of the MIC is probably linked to a selection of E. coli resistant preexisting or the appearance of an inducible resistance. Development of resistance to Tobramycin de novo seems unlikely since the MIC stabilizes in the 5th generation.

This resistance to Tobramycin is due to several mechanisms, the most frequent of which is the result of the production of enzymes which covalently modify the antibiotics which then become inactive either by acetylation, phosphorylation or by nucleotidylation [15]. Studies by Menard andal. [16] showed that the strains of E. coli studied in their work are resistant to Tobramycin at MIC values of 8 pg.ml-1 and that the overproduction of the enzyme [APH (3')] - gives E. coli resistance to Tobramycin. Inactivation enzymes AAC (3) -IIa and AAC (6') - Ib are also potential resistance agents for E. coli against Tobramycin [17].

Other resistance mechanisms may also be involved, such as target alteration (16S RNA), impermeability or active efflux of the antibiotic [18, 19]. The methylation of the nucleotide G1405 in position N7 in the 16S rRNA generates a mechanism of resistance to Tobramycin in E. coli described by Rosenberg andal. [20].

E. coli active efflux pumps against Tobramycin are numerous but AcrD-TolC is undoubtedly the most used to expel antibiotics out of the cell [20, 21, 22].

Concerning the sensitivity to Ofloxacin, the value of the initial MIC of the first generation ATCC 25922 strain of E. coli tested with Ofloxacin in our study is 0.12 pg.ml-1 (Table 2). These values conform to the reference set up by the CA-SFM [7]. In addition, these results corroborate the results of studies by Neu andal. [23] who shows that Ofloxacin has an antibacterial effect and who found an MIC value of 0.12 pg.ml-1. For the other 2 strains, they are very resistant to Ofloxacin because we found a minimum MIC of 0.9375 pg.ml-1 for the 2 strains of E. coli for the first generations which will become even more resistant to the 5th generation (Table 2).

Thus, the results obtained in this study show that in the first generation of the strain ATCC 25922 from E. coli, the strain is sensitive. Then, an intermediate phenotype characterizes the second generation. The first sign of resistance occurs in the third generation, i.e. the third exposure of E. coli to Ofloxacin with an MIC of 15 pg.ml-1. From the third generation, the strain became resistant to Ofloxacin. The difference between the value of the first generation MIC (0.12 pg.ml-1) and the third generation MIC (15 pg.ml-1) would mean that resistance mechanisms have been activated by E. coli to escape the action of Ofloxacin or that preexisting resistant populations were selected as is the case for the 2 other strains of E. coli that have already been resistant to Oflocaxine.

However, it should be noted that by their mechanism of action, fluoroquinolones are very mutagenic. Thus, these results could also be due to the fact that E. coli has developed resistance acquired through repeated exposure. The main cause of this resistance is due to mutations in the determining regions (GyrA, GyrB) and to the prevalence of plasmid mediated resistance genes in E. coli [24, 25, 26]. Other mechanisms such as overexpression of efflux pumps and alteration of permeability are also possible.

The protection of topoisomerase by Qnr proteins would give E. coli resistance to Ofloxacin which can increase the MIC value by 10 to 100 times [27]. Another resistance mechanism involves a mutation in the modifying enzyme AAC (6') - Ib-cr which is capable of modifying the structure of Ofloxacin [28]. It describes that the enzymatic modification by AAC (6') - Ib-cr is the most widespread resistance mechanism in E. coli. This mechanism can increase the MIC from 2 to 4 times its initial value [29].

A mutation in the gene encoding DNA Gyrase-A (ATP-dependent) causes a high level of bacterial resistance by enzymatic inactivation and reduces the accumulation of Ofloxacin in E. coli [23, 30]. The expression of efflux pumps is one of the resistance mechanisms of E. coli. AcrAB-TolC is an E. coli efflux system which may decrease the susceptibility of Ofloxacin [31]. QepA, a proton-dependent transporter, is a new efflux system that can increase the MIC value from 2 to 64 times its original value [22, 28, 29].

Determining the biochemical and molecular mechanisms of antibiotic resistance in E. coli should be undertaken to confirm likely resistance phenotypes.

Conclusion

This work has demonstrated that E. coli reacts differently to antibiotics by developing complex resistance mechanisms after they are exposed to the same antibiotic. In a bacterial population, the development of resistance to an antibiotic is the result of stressors linked to the presence of the antibiotic. The increase in the value of the MIC is a direct interpretation of this resistance. The value of the MIC of E. coli to Tobramycin and ofloxacin is highly variable for the initial MIC up to the 5th generation after repeated exposure. This significant increase in MIC reflects the

resistance of the strain which is generally due to the production of an enzyme which inactivates antibiotics or by overexpression of efflux pumps or by protection of the target of the antibiotic. We have thus found that the dose prescribed for taking an antibiotic (value 4 times the MIC) has been greatly exceeded if the bacteria is repeatedly exposed to the same antibiotic. A control of the consumption of antibiotics is strongly recommended as well as compliance with the necessary dose required for taking the antibiotic.

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