



Phenotypic Variation of Tobramycin and Ofloxacin Resistance of *Pseudomonas aeruginosa* by Repeated Exhibition

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Authors' contributions

This work was carried out in collaboration among all authors. Author AMA designed the study. Author CEM wrote the protocol and managed the literature searches. Author BA and TR managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Pseudomonas aeruginosa has the ability to resist almost all available antibiotics by rapidly accumulating multiple resistance mechanisms and thus lead to a therapeutic impasse and higher mortality in infected patients.

The objective of this study was to assess the phenotypic variation in resistance to tobramycin and ofloxacin from *Pseudomonas aeruginosa* by repeated exhibition after determination of the minimum inhibitory concentration.

This is a prospective and descriptive study carried out in the Laboratory of Microbiology of Fundamental and Applied Biochemistry (Faculty of Sciences, Antananarivo) during the month of

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January 2020. The strains studied were the virulent wild strain of *Pseudomonas aeruginosa* PAO1 supplied by the Laboratory and two clinical strains of *Pseudomonas aeruginosa* from the Microbiology Laboratory of the Joseph Ravoahangy Andrianavalona University Hospital Center, Antananarivo.

The strains of *P. aeruginosa* were cultured in the liquid culture medium (which is Luria Bertani, added with a buffer system of 3- (N-morpholino) propanesulfonic acid (LB-MOPS) which will stabilize the pH and a solid culture medium which is Columbia agar. Repeated exhibition to Tobramycin and Ofloxacin from these strains have been made. The MIC is determined by a visual evaluation of the turbidity of the various wells of the microplate.

The MIC value of *Pseudomonas aeruginosa* with tobramycin and ofloxacin is very variable for the initial MIC until the 5th generation after repeated exhibition. More *Pseudomonas aeruginosa* is exposed to an antibiotic many times, the more it develops resistance to this antibiotic, even being sensitive at the start. That is to say, clinically, the dose prescribed for the antibiotic has been greatly exceeded if *Pseudomonas aeruginosa* is repeatedly exposed to the same antibiotic.

Keywords: Resistance; *P. aeruginosa*; tobramycin; ofloxacin; repeated exhibition.

1. INTRODUCTION

Antibiotics are the best ally in the immune defense system of an organism invaded by pathogenic bacteria [1]. The misuse of these antibiotics has led to the emergence of resistance in bacteria.

Today, the level of antibiotic resistance has reached a worrying threshold for many bacteria. Infections with bacteria resistant to one or more antibiotics have become common and some pathogens are now resistant to all classes of antibiotics and their combinations. This is how multiresistant bacteria appear [1,2].

Known as a bacteria responsible for infections, diseases in plants and animals, several human diseases and nosocomial diseases such as pneumonia, urinary tract infection, bacteremia, infection of the operating site especially in immunocompromised patients, *Pseudomonas aeruginosa* is indeed a model of a pathogenic organism [3,4,5]. In the treatment of infections caused by *P. aeruginosa*, almost all classes of antibiotics are used repeatedly and excessively. However, the effectiveness of these antibiotics can vary depending on the bacterial strain and how to use these antibiotics. In addition, *Pseudomonas aeruginosa* has the ability to resist almost all available antibiotics by rapidly accumulating multiple resistance mechanisms and thus lead to a therapeutic impasse and higher mortality in infected patients [6].

The objective of this study was to assess the phenotypic variation in resistance to tobramycin and ofloxacin from *Pseudomonas aeruginosa* by

repeated exhibition after determination of the minimum inhibitory concentration (MIC).

2. METHODS

This is a prospective and descriptive study carried out in the Laboratory of Microbiology of Fundamental and Applied Biochemistry (Faculty of Sciences, Antananarivo) during the month of January 2020. The strains studied were the virulent wild strain of *Pseudomonas aeruginosa* PAO1 supplied by the Laboratory and two clinical strains of *Pseudomonas aeruginosa* from the Microbiology Laboratory of the Joseph Ravoahangy Andrianavalona University Hospital Center (CHU JRA), Antananarivo.

The strains of *P. aeruginosa* were cultured in the liquid culture medium (which is Luria Bertani, added with a buffer system of 3- (N-morpholino) propanesulfonic acid (LB-MOPS) which will stabilize the pH and a solid culture medium which is Columbia agar.

Repeated exhibition to Tobramycin and Ofloxacin from these strains have been made. Repeated exhibition results in a cycle repeated several times. A cycle includes a preculture of *Pseudomonas aeruginosa* followed by its culture with an antibiotic after the determination of the MIC in antibiotics in *Pseudomonas aeruginosa* and this cycle is repeated until the 5th generation of the bacteria. The antibiotics are prepared so as to obtain a concentration equal to 150 µg.ml⁻¹ for ofloxacin and 6 mg.ml⁻¹ for tobramycin from a stock solution of antibiotic of 12 mg.ml⁻¹ for each [7].

A stock strain of *Pseudomonas aeruginosa* PAO1 of 500 µl is cultured in 5 ml of LB-MOPS

medium and incubated at 37°C with shaking at 175 rpm for 18 hours, as well as for the 2 clinical strains. The culture of the strains is done by the microdilution method on a 96-well microplate. After 18 hours of growth, the strains were recovered by centrifugation with stirring at 3200 rpm for 5 min. The pellet is then recovered and washed twice with 5 ml of sterile LB-MOPS medium. The washing is done as follows: addition of the sterile medium, homogenization using the vortex, followed by centrifugation for 5 min, and recovery of the pellet. Then, the bacterial density at 600 nm is adjusted so that the starting bacterial density in each condition tested is between 0.02 and 0.03 corresponding to an inoculum of 10^7 CFU.ml⁻¹. To fill the wells of the plate, the total volume of the bacterial suspension is equal to 12 ml, hence the volume of strain to be suspended in LB-MOPS medium is equal to 42 µl at a bacterial density of 0.021 [7].

For culture, the final concentration of the antibiotic is thus reduced to one tenth of its initial concentration, i.e. 15 µg.ml⁻¹ for the OFL and to one hundredth of its initial concentration, i.e. 60 µg.ml⁻¹ for the TOB. The volume of antibiotic to be taken is 30 µl for the OFL and 3 µl for the TOB [7].

For each line, 297 µl of bacterial suspension supplemented with 3 µl of Tobramycin and 270 µl of bacterial suspension supplemented with 30 µl of Ofloxacin are deposited in the first well of the plate. Then, 150 µl of bacterial suspension are

deposited in each well of the ten other wells. And for the last well, 150 µl of sterile LB-MOPS medium serving as a control in order to check if there was no contamination in the manipulations [7].

Successive dilutions are made from column 1 to column 11 inclusive. 150 µl of the solution from the first well is taken using a micropipette and returned to the second well on the same line. In the same way, the same volume is taken from the second well and is transferred to the third well. The same procedure is repeated until the eleventh well and the 150 µl recently withdrawn from the eleventh well are discarded. Thus, the concentration of Antibiotic obtained is in decreasing order of reason 2 (Fig. 1) [7].

The MIC is determined by a visual evaluation of the turbidity of the various wells of the microplate. The MIC is indicated by the well which contains the lowest concentration of antibiotic with no visible growth, i.e. no disorder is visible after 24 hours of incubation of the microplate.

But to confirm the accuracy of the results, the use of a growth indicator is essential which is the INT. 40 µl of 0.2% p-iodonitrotetrazolium chloride (INT) was added to each well of the microplate and after incubation for 30 min at 37°C at 175 rpm, a colorless INT becomes red, which indicates visible bacterial growth. Therefore, the last well which does not represent the red color of the INT indicates the MIC (Fig. 2) [7].

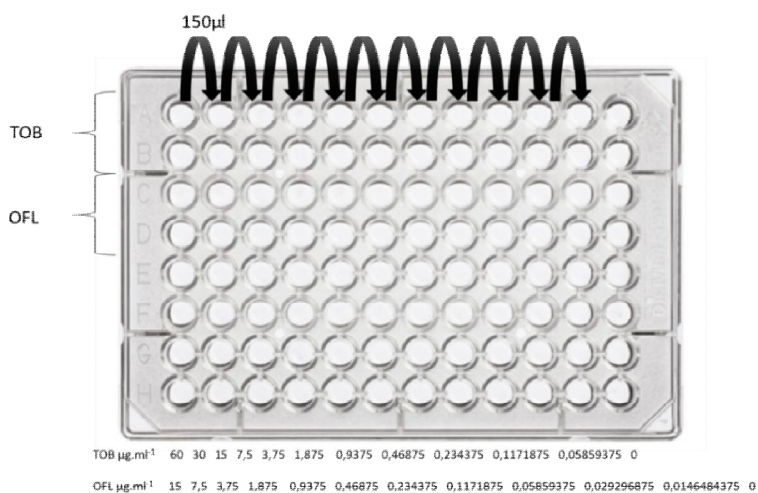


Fig. 1. Successive dilution method on the microplate
 Finally, the plate is maintained at 37°C for 24 hours in a shaking incubator at 175 rpm

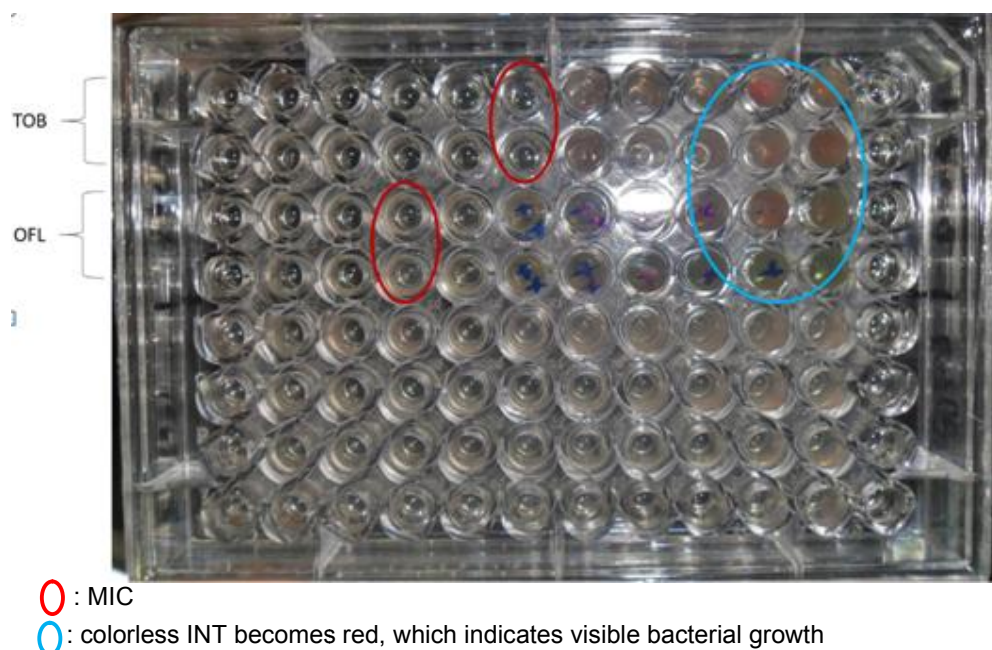


Fig. 2. The MIC after using INT

Table 1. The values taken for the preparation of bacterial suspensions of other generations

Generation	Total volume of SB (ml)		Volume of strain to be suspended (µl)		DO at 600 nm	
	TOB	OFL	TOB	OFL	TOB	OFL
2	25	25	54	59.5	0.036	0.028
3	10	10	22.7	136	0.025	0.036
4	12	12	26.4	25,8	0.023	0.027
5	4	4	7.6	8	0.028	0.033

Regarding repeated exposure: the preculture of the next generation is made from *Pseudomonas aeruginosa* strains indicating the MIC of the previous generation. This means that the strain representing the generation 1 MIC is subcultured for the generation 2 preculture and the strain representing the generation 2 MIC is subcultured for the generation 3 preculture and so on until generation 5. Transplanting of the strain is done from the culture in agar medium to 5 ml of LB-MOPS medium by the three-year method and is incubated at 37°C with shaking at 175 rpm for 18 hours [7].

The same process is adopted for cultures of other generations.

For the preparation of the bacterial suspensions, the values are summarized in Table 1.

For the microdilution method, the tests were carried out four times and the incubation for 18 hours at 37°C with shaking at 175 rpm [7].

3. RESULTS

3.1 *Pseudomonas aeruginosa* and Ofloxacin

Table 2 summarizes the MIC results of the five generations of the 3 *Pseudomonas aeruginosa* strains against ofloxacin by repeated exposure.

Concerning the PAO1 strain, for the first generation, the lowest MIC value was 1.875 µg.ml⁻¹ of ofloxacin. We found a MIC at 3.75 µg.ml⁻¹ for generation 2, a MIC at 7.5 µg.ml⁻¹ for generation 3. For the 4th and 5th generation, the MICs were 15 µg.ml⁻¹ (Table 2).

For the *P. aeruginosa* 1 strain, in the presence of ofloxacin, the lowest value of the MIC was 1.171 µg.ml⁻¹ for the first generation, then, the second generation had a MIC at 18.75 µg.ml⁻¹ and from the 3rd to the 5th generation, and the MIC was 37.5 µg.ml⁻¹ (Table 2).

And concerning the *P. aeruginosa* 2 strain, only the first generation had a MIC at 300 $\mu\text{g.ml}^{-1}$ and from the 2nd to the 5th generation, the MIC was 600 $\mu\text{g.ml}^{-1}$ (Table 2).

3.2 *Pseudomonas aeruginosa* and Tobramycin

The results of the MICs of the generations of *Pseudomonas aeruginosa* in the presence of tobramycin are summarized in Table 3.

In the presence of tobramycin, the first generation of the PAO1 strain had a MIC at 1.875 $\mu\text{g.ml}^{-1}$, the second generation had a MIC at 7.5 $\mu\text{g.ml}^{-1}$. For the 3rd generation, the MIC was at 15 $\mu\text{g.ml}^{-1}$ and for the 4th and 5th generation, the MICs were 30 $\mu\text{g.ml}^{-1}$ (Table 3).

For the *P. aeruginosa* 1 and 2 strains, only the first generations have an MIC of 4.687 $\mu\text{g.ml}^{-1}$ and 300 $\mu\text{g.ml}^{-1}$ respectively. From the 2nd to the 5th generation, the MICs were 75 $\mu\text{g.ml}^{-1}$ for the *P. aeruginosa* 1 strain and 600 $\mu\text{g.ml}^{-1}$ for the *P. aeruginosa* 2 strain (Table 3).

4. DISCUSSION

The evaluation of the phenotypic variation of resistance to tobramycin and ofloxacin from *Pseudomonas aeruginosa* by repeated exposure was carried out by observation of the evolution of the MIC of the antibiotics tested according to the successive generations of *P. aeruginosa*.

4.1 Sensitivity to Ofloxacin

Faced with ofloxacin, the variation in sensitivity of the PAO1 strain increases to more than 2 times the value of the initial MIC for the 2nd generation, then to more than 4 times for the 3rd generation and it is constant from the 4th generation with a value of 8 times the initial value. The MIC values range from 1.875 $\mu\text{g.ml}^{-1}$ to 30 $\mu\text{g.ml}^{-1}$.

For the *P. aeruginosa* 1 strain, in the presence of ofloxacin, the MIC value already goes from 16 times to the 2nd generation and from 32 times from the 3rd to the 5th generation. The MIC values range from 1.171 $\mu\text{g.ml}^{-1}$ to 37.5 $\mu\text{g.ml}^{-1}$.

And concerning the *P. aeruginosa* 2 strain, the first generation already had an MIC at 300 $\mu\text{g.ml}^{-1}$ and goes to 600 $\mu\text{g.ml}^{-1}$ for the rest of the generations.

Compared with data from the Antibiotic Committee of the French Society of Microbiology 2019 [8], *Pseudomonas aeruginosa* was resistant to ofloxacin from its first generation for all 3 strains. And this resistance of *P. aeruginosa* evolves a little more until the MIC stabilizes at the 3rd generation for the PAO1 strain, and at the 4th and 5th generation for the *P. aeruginosa* 1 and 2 strains. Considerable increase in the MIC value according to the generations of *P. aeruginosa* tested with ofloxacin. Ofloxacin has bactericidal activity on *P. aeruginosa* but this activity remains inconsistent as the generations of *P. aeruginosa* change.

Table 2. Distribution of MICs ($\mu\text{g.ml}^{-1}$) of the 3 strains of *P. aeruginosa* in the presence of ofloxacin according to the generations

Generation	MIC PAO1	MIC <i>P. aeruginosa</i> 1	MIC <i>P. aeruginosa</i> 2
G1	1.875	1.171	300
G2	3.75	18.75	600
G3	7.5	37.5	600
G4	15	37.5	600
G5	15	37.5	600

Table 3. Distribution of MICs ($\mu\text{g.ml}^{-1}$) of the 3 strains of *P. aeruginosa* in the presence of tobramycin according to generations

Generation	MIC PAO1	MIC <i>P. aeruginosa</i> 1	MIC <i>P. aeruginosa</i> 2
G1	1.875	4.687	300
G2	7.5	75	600
G3	15	75	600
G4	30	75	600
G5	30	75	600

According to our results, against ofloxacin, *P. aeruginosa* was already resistant from its first exposure and the more the exposure of *Pseudomonas aeruginosa* is repeated, the more the MIC in each generation increases by a factor of 2 until its stability at 32 times the value of the initial MIC. The resistance of *P. aeruginosa* in its first exposure to ofloxacin would signify the response to stress due to the presence of ofloxacin in its environment, known as inducible resistance.

The evolution of this MIC value in each successive generation of *P. aeruginosa* explains a very high resistance to ofloxacin. This resistance was caused by the activation of the various resistance mechanisms that *P. aeruginosa* possess to escape the action of the antibiotic. However, it should be noted that by their mode of action, fluoroquinolones are very mutagenic. Thus, these results could also be due to the fact that *P. aeruginosa* may have developed acquired resistance over repeated exposures. The acquired resistance of *P. aeruginosa* to fluoroquinolones is mainly linked to chromosomal support mechanisms involving target modification, membrane impermeability and efflux over expression [9,10]. In addition, according to the ANSM [11], *P. aeruginosa* is inconstantly sensitive to ofloxacin and resistance is most frequently acquired by the deployment of various membrane mechanisms.

In target modification, mutations in the *gyrA* protein (DNA gyrase subunit) or in the *parC* subunit of topoisomerase lead to a reduced affinity for fluoroquinolones. Indeed, these mutations introduce amino acid substitutions in the QRDR regions where the antibiotics bind. Alterations in the *gyrA* subunit are frequent and significantly increase the MIC of all fluoroquinolones; amplifying resistance to very high levels [12,13,14].

For membrane impermeability, this is linked to a modification of LPS or an alteration of the porin OprF and which gives *Pseudomonas aeruginosa* a low level resistance to fluoroquinolones (MIC increased by a factor of 2 to 8) [15].

Efflux overexpression via MexAB-OprM, MexXY-OprM and MexEF-OprN is caused by the mutation of the regulatory regions *nalB*, *nfxB* and *nfxC* associated with mutations in target enzymes. And this over expression seems to cause a variable elevation of MICs (at least this

doubles the MICs) to all quinolones leading to moderate resistance [14,16,17].

Apart from these chromosome-supported resistance mechanisms, a mechanism of plasmid resistance by target protection has been described for a short time. The plasmid providing one or more genes responsible for the production of proteins with repeated pentapeptide motifs which protect the DNA gyrase and topoisomerase complexes from the action of fluoroquinolones. This new mechanism is responsible for a low level of resistance against quinolones [18].

4.2 Sensitivity to Tobramycin

Concerning the sensitivity to tobramycin, for the strain PAO1, the variation in the sensitivity of the strain increases to more than 4 times the value of the initial MIC for the 2nd generation, by 8 times for the 3rd generation and by 16 times the value initial for the 4th and 5th generation.

For *P. aeruginosa* 1 and 2 strains, the MIC values vary from 2 times to 16 times the initial MIC values.

Compared with data from the Antibiotic Committee of the French Society of Microbiology 2019 [8], the PAO1 strain is sensitive to tobramycin during its first exposure but it becomes resistant since the 2nd generation. This resistance persists and increases until a stabilization of the MIC in the 5th generation indicates the least possibility of acquiring new resistance to tobramycin in *Pseudomonas aeruginosa*. For strains *P. aeruginosa* 1 and 2, they have already been resistant since the first generation. Tobramycin has a bactericidal activity on *P. aeruginosa* on the PAO1 strain. But this sensitivity has evolved towards resistance since the 2nd generation and the more the exposure is repeated, the more the CMI values increase by a factor of 2 to a factor of 16.

For strains of *P. aeruginosa* 1 and 2, they were already resistant from the first generation, and this increases after repeated exposure of the strains.

The modification of the sensitivity of the PAO1 strain of the first generation in resistance since the 2nd generation or the resistance from the first generation for the *P. aeruginosa* 1 and 2 strains was probably linked to the preexistence of a resistant bacterial subpopulation or the appearance of adaptive resistance.

This resistance to tobramycin is due to several mechanisms to combat the action of the antibiotic.

Although enzymatic modification constitutes the main mechanism of resistance to amino glycosides, low membrane permeability, overproduction of active efflux systems or even modifications at target level can explain the increased resistance of the pyocyanic bacillus to this class of antibiotics [19].

Pseudomonas aeruginosa has modifying enzymes of the aminoglycosides, classified into three groups: Aminoglycosides APH phosphatransferases, Aminoglycosides adenyl transferases AAD = Aminoglycosides nucleotidyltransferase ANT and Aminoglycosides acetyltransferases AAC [20]. These enzymes are stereospecifics which attach phosphate, adenylyl or acetyl groups to the –NH₂ or –OH functions of these antibiotics, thus preventing their attachment to the ribosome [21]. Their genes are frequently carried by transposons and / or integrons and induce a constitutive expression of the enzymes responsible for high level resistance to aminoglycosides [19].

Resistance to aminoglycosides by impermeability can be explained on the one hand by the production of defective lipopolysaccharides thus altering the absorption of the aminoglycosides through the external membrane [22], on the other hand by anomalies on the electron transport chain causing a decrease in the absorption of aminoglycosides at the level of the cytoplasmic membrane, thus limiting the intracellular accumulation of aminoglycosides which does not allow reaching a sufficient ribosome inhibition threshold [23].

The overproduction of the efflux systems extruding the aminoglycosides, namely MexEF-OprN and MexXY-OprM, is another mechanism to which *Pseudomonas aeruginosa* has access in order to better resist aminoglycosides. However, this mechanism only causes a slight rise in MICs, generally leading to moderate resistance to aminoglycosides (factor 2 to 8) [23,24].

The most recent resistance is resistance linked to the modification of the ribosomal target on which the aminoglycoside binds [17]. A mutation in ribosomal proteins at the A-site of 16S rRNA prevents binding of the antibiotic to the ribosome and appears to cause high level resistance to all

of the aminoglycosides [25]. However, this resistance only appears on clinical isolates of *P. aeruginosa* [17,19] and that this is a rare phenomenon as per Toumi [20].

For adaptive resistance, in the work led by Hocquet et al. [26], it was reported that the ephemeral and reproducible nature of this phenomenon precludes the intervention of genetic mutations. Likewise, ribosomal alterations or the presence of modifying enzymes are unlikely due to the lack of specificity of adaptive resistance. But they indicated the implication of the overproduction of the MexXY – OprM efflux system in this phenomenon. And the overproduction of this efflux system occurs during "swarming" or rapid proliferation of hyperflagellated cells during their differentiation [2].

At the end of this study, we found that the more *Pseudomonas aeruginosa* is exposed to an antibiotic many times, the more it develops resistance to this antibiotic, even being sensitive at the start. That is to say, clinically, the dose prescribed for the antibiotic has been greatly exceeded if *P. aeruginosa* is repeatedly exposed to the same antibiotic.

It would be necessary to widen the study on many strains by applying other antibiogram methods such as MIC by E-test, and by testing other classes of antibiotics which have different modes of action than those used in this study and in genotypically characterizing antibiotic resistance.

5. CONCLUSION

The MIC value of *Pseudomonas aeruginosa* with tobramycin and ofloxacin is very variable for the initial MIC until the 5th generation after repeated exhibition. The PAO1 strain has sensitivity to tobramycin only at the first exposure. This significant increase in MIC reflects the resistance of the strain.

After being exposed to antibiotics, strains of *P. aeruginosa* had different ways of developing its resistance mechanisms. The variations in the value of the MIC are the direct interpretation of this resistance.

The correlation between the excessive intake of antibiotics and the variation in the resistance of bacteria will allow us to understand the behavior of a bacterium in the face of antibiotics, to know

the serious consequences on the excessive and excessive use of antibiotics and the importance of respecting the prescribed indications for the use of antibiotics.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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