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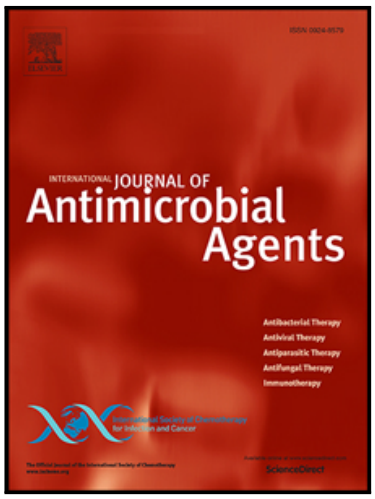
Published in:
International Journal of Antimicrobial Agents

Link to article, DOI:
10.1016/j.ijantimicag.2018.12.015

Publication date:
2019

Document Version
Peer reviewed version



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PII: S0924-8579(18)30385-6
DOI: https://doi.org/10.1016/j.ijantimicag.2018.12.015
Reference: ANTAGE 5623

To appear in: *International Journal of Antimicrobial Agents*

Received date: 16 April 2018
Accepted date: 23 December 2018

Please cite this article as: Niels Høiby , Kaj-Åge Henneberg , Hengshuang Wang , Camilla Stavnsbjerg , Thomas Bjarnsholt , Oana Ciofu , Ulla Rydal Johansen , Thomas Sams , Formation of Pseudomonas aeruginosa inhibition zone during Tobramycin disk diffusion is due to a transition from planktonic to biofilm mode of growth, *International Journal of Antimicrobial Agents* (2019), doi: https://doi.org/10.1016/j.ijantimicag.2018.12.015

Highlights

The formation of *P. aeruginosa* inhibition zone during Tobramycin agar diffusion susceptibility tests is due to a switch from planktonic growing bacteria to the biofilm mode of growth.
Small aggregates in the inhibition zone (young biofilms) containing ≤≈64 *P. aeruginosa* cells are killed by tobramycin. Larger aggregates survive and form the inhibition zone.
P. aeruginosa at the border of the stable inhibition zone and beyond continues to grow to a mature biofilm and produces large amount of polysaccharide containing matrix.
The inhibition zone gives the clinical important information, that biofilm growing bacteria are tolerant to the antibiotic and the clinician will *probably not* be successful to treat and eradicate biofilm infections with the conventional doses of tobramycin.

Formation of *Pseudomonas aeruginosa* inhibition zone during Tobramycin disk diffusion is due to a transition from planktonic to biofilm mode of growth

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Running title: Biofilm and inhibition zones

Key words: Pseudomonas aeruginosa, biofilm, agar diffusion, antibiotic susceptibility test, tobramycin

Abstract

Pseudomonas aeruginosa PAO1 (MIC 0.064µg/ml) was used to perform agar diffusion tests employing tobramycin containing tablets. The growth of the bacteria and the formation of inhibition zones were studied by stereomicroscopy and by blotting with microscope slides and staining with Methylene blue, Alcian blue and a fluorescent lectin for the *P. aeruginosa* PSL which was studied by confocal laser scanning microscopy. The diffusion of tobramycin from the deposit was modelled by using a 3D geometric version of Fick's 2nd law of diffusion. The time-dependent gradual increase of Minimal Biofilm Eradication Concentration (MBEC) was studied by the Calgary Biofilm Devise. The early inhibition zone was visible after 5 h incubation. The corresponding calculated tobramycin concentration at the border was 1.9µg/ml and increased to 3.2µg/ml and 6.3µg/ml after 7 and 24 h incubation. The inhibition zone increased to the stable, final zone after 7 h incubation. Bacterial growth and small aggregate formation (young biofilms) took place inside the inhibition zone until the small aggregates contained ≤≈64 cells and production of polysaccharide matrix including PSL had begun, thereafter the small bacterial aggregates were killed by tobramycin. The bacteria at the border of the stable

inhibition zone and beyond continued to grow to a mature biofilm and produced large amount of polysaccharide containing matrix. The formation of the inhibition zone during the agar diffusion antibiotic susceptibility test is due to a switch from the planktonic to the biofilm mode of growth and gives clinical important information about the increased antibiotic tolerance of biofilms.

Introduction

Soon after antibiotics began to be used to treat infections, the need for testing the susceptibility of the offending bacteria to various antibiotics became urgent, since occurrence of resistant mutants of otherwise susceptible species became a problem. The agar diffusion method became the most popular and clinical used method employing agar cups, disks or tablets as deposits (1-6). The method was repeatedly standardized internationally (7), and by CLSI in USA (www.clsi.org) and EUCAST in Europe (www.eucast.org) and is regarded as a good method for categorizing bacteria as susceptible (S), intermediate susceptible (I) and resistant (R) to systemic use of antibiotics in accordance with the harmonised breakpoints (8) (www.eucast.org, www.clsi.org). The diffusion of antibiotics from the deposit in a disk or tablet takes place in water within a 1% agar plate and follows Fick's 2nd law of diffusion. The size of the inhibition zone was shown to be dependent not only on the minimal inhibitory concentration (MIC) but also on the lag phase, the generation time of the bacterial strain and its inoculum, the amount of antibiotic in the tablet, and the diffusibility of the antibiotic, (www.eucast.org). The formation of the inhibition zone is readable after ≤5 hours incubation but increases marginally during the next hours

after which it becomes stable and the colonies at the border and beyond the inhibition zone continues to grow overnight.

These characteristics of the formation of the inhibition zone indicate, that it may be the transition from a planktonic mode of growth (the inoculum) to a biofilm mode of growth which is decisive for the formation of the inhibition zone. The present study, therefore, aims to investigate the influence of the transition from planktonic to biofilm mode on the formation of the inhibition zone of *P. aeruginosa* around a tablet containing tobramycin. The Pharmacokinetic/Pharmacodynamic characteristics of tobramycin is concentration dependent killing (9).

Materials and methods

Bacterial strain. *Pseudomonas aeruginosa* PAO1 (10) was used for the experiments. The planktonic minimal inhibitory concentration (MIC) of tobramycin measured by e-test is 0.064µg/ml for PAO1. Inocula of 10^7 Colony Forming Units (CFU)/ml (7) or 10^8 CFU/ml (EUCAST = MacFarlane 0.5 (www.eucast.org)) were used and the plates were inoculated by floating. The low inoculum gives a 2mm bigger zone diameter of inhibition and 1.5h longer time to study the growth of microcolonies before the inhibition zone is formed and *vice versa* concerning the high inoculum.

Antibiotic tablets and antibiotic susceptibility agar medium. NEOSENSITABS (9 mm diameter) with 40µg diffusible tobramycin/tablet (MW 467.54, formula C18H37N5O9) (ROSCO A/S, Tåstrup, Denmark). At temperature $T = 37^{o}\mathrm{C}$ the diffusion constant for tobramycin is around $D = 3.84\ 10^{-5}\ \mathrm{cm}^2/\mathrm{s} = 1.38\ \mathrm{mm}^2/\mathrm{h}$ (11).

Cation-adjusted horse blood agar susceptibility medium (SSI Diagnostica, State Seruminstitute, Hillerød, Denmark) was employed. For some experiments, where visualisation of the bacterial growth was required on transparent agar, LB agar plates were employed (SSI Diagnostica, State Seruminstitute, Hillerød, Denmark). Inoculation, incubation and reading of the plates and stereomicroscopy of the plates was done by two of the authors (NH, URJ) in the incubation cabinet at 37^{o}C employing an Olympus SZ61 stereomicroscope (Olympus, Tokyo, Japan). Microscopy examination of stained slides was done with an Olympus BX50 light microscope (Olympus, Tokyo, Japan). Confocal laser scanning microscopy was done with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

The gradual increase of Minimal Biofilm Eradication Concentration (MBEC) of tobramycin by increasing age (h) of the biofilm was determined in microtiter plates with the Calgary Biofilm Device method (Fig. 1 & supplemental to Fig 1), using an inoculum of 10^7 CFU/ml (12) (NUNC, Roskilde, Denmark), as reported previously (13).

Stains: Methylene blue (Sigma-Aldrich Denmark, Copenhagen, Denmark), Alcian blue (Sigma-Aldrich Denmark, Copenhagen, Denmark), PSL-specific FITC-HHA lectin (EY Laboratories, San Mateo, CA, USA) (14).

Calculation of the concentration of tobramycin in the agar plate.

The diffusion of antibiotics in the agar plate follows Fick's 2nd law of diffusion equation:

$$\frac{\partial c}{\partial t} = D\,\Delta c$$

where D is the diffusion constant for the antibiotic. This is valid when there is no significant consumption of antibiotics and when the agar concentration is just a few percent (15). We assume that there is no significant adsorption of tobramycin to agar.

Cylindrical geometry:

In the idealised situation where the antibiotics is added in a small cylindrical hole in the middle of the agar plate, this produces the Gaussian shaped solution

$$c(r,t) = \frac{1}{4\pi Dt}\exp(-r^2/4Dt)$$

Here we have normalized such that it integrates to one when integrated in the plane. If, instead, we wish to express the concentration as mass/volume we get

$$c(r,t) = \frac{m}{h_0}\frac{1}{4\pi Dt}\exp(-r^2/4Dt)$$

Here m is the mass of antibiotics added at the centre and h_0 is the height of the agar. In practice, we do not start with a concentration in a narrow cylinder at the centre. Rather, the antibiotics is added in a cylinder with finite radius r_0. The antibiotics is therefore already spread with the variance in a cylinder, $\sigma^2 = \frac{1}{2}r_0^2$. The radial variance of the Gaussian distribution is $\sigma^2 = 4Dt$. The time required to build up this variance is therefore

$$t_0 = \frac{r_0^2}{8D}$$

Including this advance spread we get the approximate solution

$$c(r,t) = \frac{m}{h_0}\frac{1}{4\pi D(t+t_0)}\exp(-r^2/(4D(t+t_0)))$$

In supplemental to Fig. 2, we show a comparison between the full calculation for selected radii and the shifted Gaussian version. The specific calculation concerning the tobramycin containing NEOSENSITABS® (ROSCO a/s, Taastrup, Denmark) is performed with cylinder radius $r_0 = 4.5\text{mm}$, agar height $h_0 = 6\text{mm}$, and tobramycin

mass $m = 40\mu g$. The radius of the small agar plate is $R = 45mm$ and the reflective boundary does not influence the results in Fig. 2 and supplemental to Fig. 2. For some experiments larger agar plates were used with a radius of 70mm.

Full 3D geometry:

If the deposition of antibiotics is as a tablet on top of the agar, we need to carry out a full 3-dimensional calculation. The NEO-SENSITABS® tablets are of radius $r_0 = 4.5mm$ and height $h_0 = 1.55mm$. Therefore, the thickness of the tablet cannot be assumed to be much smaller than the height of the agar. The v/v concentration of the water in the tobramycin tablets may be estimated by weighing the tablets before and after they are placed on the agar surface and filled with water by capillary forces. The weight of the tablets increases by 43µg leading to a porosity (volume fraction occupied by water) of $\varepsilon = 0.436$. The tortuosity of the porous channels will be taken as $\tau = \varepsilon^{-1/2}$ from the Bruggeman model (16). If we assume that the porosity does not vary in time the modified diffusion equation reads

$$\varepsilon \frac{\partial c}{\partial t} = \nabla \cdot \left(\frac{\varepsilon}{\tau} D \nabla c \right)$$

where $c(r, z, t)$ is the concentration of antibiotics in the water segment.

The diffusion equation is required to be continuous and respect mass conservation across the boundary between the tablet and the agar. At all other boundaries reflective boundary conditions are assumed. The initial concentration of tobramycin in the water fraction of the tablet is $c_0 = 930\mu g/ml$ when we assume that the tobramycin is quickly going into solution. The numerical solution of the diffusion equation is performed in COMSOL Multiphysics®. ver. 5.3.4 Comsol AB, Sweden.

Results

Minimum Biofilm Eradication Concentration (MBEC)

Fig. 1 shows that the MBEC of *P. aeruginosa* PAO1 to tobramycin increased with time from 0.5µg/ml (1 h) to 2-4µg/ml after 4-6h and 8-16µg/ml after 10-24h .

Calculated time-dependent concentration of tobramycin in the agar plate

Comparison between the full 3D calculation, the 3D calculation ignoring the porosity of the tablet, the full 2D calculation, and the approximate Gaussian solution described above is shown in supplemental to Fig. 2 [diffusion_models.eps]. In the 3D calculation, we are displaying the concentration at the agar surface since this is where the bacteria reside. It is seen that, particularly at early times compared to the vertical equilibration time and small radii, it is of importance to perform the full calculation. The concentration of tobramycin at radii ranging from 6.5mm to 15mm (lowest curve) as a function of time in the full 3D model including the effect of porosity of the NEOSENSITABS® is shown in Fig. 2 [diffusion_model_porous.eps]. The concentration at the top of the agar plate is plotted. For reference, the underlying, calculated, concentrations are listed in supplemental Table 3 as a function of time and radius. The concentration of tobramycin profile in agar 7h after placing the NEOSENSITAB® tablet is shown in Fig. 3.

Growth of P. aeruginosa on blood agar plates during susceptibility testing with tobramycin disks.

By counting the bacterial cells on the agar surface using stereomicroscopy and by blotting the growth on the surface of the blood agar with a microscopy slide, fix it with methanol and stain it with Methylene blue and study the results with a light microscope, the lag phase of *P. aeruginosa* after seeding the blood agar susceptibility medium was determined to be ≈ 2h. Thereafter the doubling time became ≈ 30min. during the 7h of observation. Formation of an inhibition zone around the tobramycin

disk was visible both with the naked eye and the stereomicroscope after 5h with an inoculum of 10^7/ml (Table 1) which means that the number of cells had increased by a factor $\approx 2^8$ (= 256). The inhibition zone was already visible after 3.5h when the inoculum was 10^8/ml.

Fig. 4 and Table 1 show the observed inhibition zone diameter and Table 2, Fig 2 and supplemental to Fig. 2 show the calculated results of the agar diffusion tests with tobramycin NEOSENSITABS® added at time 0 to +7h after the inoculation and onset of the incubation of the plates with *P. aeruginosa* PAO1 (Fig 4). As expected, it is seen that the later the tobramycin tablets were placed on the inoculated agar surface, the smaller became the inhibition zone indicating that the increasing amount of the visible growth could not be lysed by delayed placement of the tobramycin tablet on top of the growth.

The morphology of the bacterial growth inside and outside the inhibition zone as visualized by stereomicroscopy is shown in Fig. 5A, B, C. The bacterial matrix is clearly seen at the border and outside the border of the inhibition zone.

Subculture guided by stereomicroscopy after 24h incubation from the area inside the inhibition zone even up to the border of the zone and from the area where the early zone had been visible after 5h incubation failed to show any growth and the bacteria had therefore been killed by tobramycin (Fig. 5, 6, 7). On the other hand, subculture from the border of the final zone always showed growth so the bacteria had survived tobramycin. These results were confirmed by propidium iodide staining in a few experiments (Live/Dead cell viability assay (ThermoFisher, USA, data not shown).

The bacterial growth in the border of the inhibition zone had therefore obtained properties characteristics for biofilm growth which is observed from Fig. 7, C & E and thereby the growing biofilm aggregates had become more tolerant to tobramycin

than the seeded planktonic bacteria. The size of the dead bacterial aggregates inside the inhibition zones and the calculated and observed growth of the bacteria indicate, that when the aggregates are ≤ ≈64 cells (after 5h of incubation with a 2h lag phase, Fig. 8B) they are later killed inside the early inhibition zone by 3.2-6.3µg/ml tobramycin (Table 2, 7-24h). After 7h (the final inhibition zone), when the bacterial aggregates reach ≈256 cells, then they remain tolerant against the tobramycin 1.8-5.3 µg/ml (Table 2, 7-24 h) and continue to grow in agreement with the biofilm results of the MBEC shown in Fig. 1. The border of the inhibition zone was sharply marked and the bacterial cells – many with filamentous shape – were separated by a self-produced matrix which is characteristic for biofilm growth (Fig. 5B, Fig. 7 C & E).

Polysaccharides are present in the bacterial matrix of the P. aeruginosa aggregates and biofilm growth

Fig. 8 A shows, that the matrix produced by the bacterial growth at the border and especially outside the border of the inhibition zone contains polysaccharide which become visible by the Alcian blue stain. In Fig. 8C & D it is seen, that both the outline or surface of the bacteria – visualised as a blue-stained ring outlining the bacterial cell – and the extracellular matrix contain polysaccharide. At least part of the of polysaccharide is identified as PSL (Fig. 8E & F). Fig. 8B shows 2 polysaccharide containing bacterial aggregates which were located in the inhibition zone close to its border and had stopped growing. Each of these aggregates contains ≈64 cells but stereomicroscopy guided subcultures from such regions inside the inhibition zone never gave growth, indicating that these small aggregates had been killed by tobramycin.

Discussion

Diffusion of tobramycin takes place in the water segment of the medium used for susceptibility testing. Generally, therefore, the results of this study are independent of the medium used. Bacteria and fungi occur as individual (planktonic) cells or clustered together in aggregates (biofilms) which may or may not adhere to surfaces. Microbial biofilms are defined as structured consortia (aggregates) of microbial cells surrounded by a self-produced polymer matrix (17). Biofilm growing bacteria are much more tolerant (= reversible resistance induced by the biofilm mode of growth in contrast to genetic resistance caused by mutations or horizontal gene transference) to antibiotics than planktonic bacteria (17-19). Colonies of bacteria growing on agar plates are surface-adhering biofilms (20) and are therefore much more tolerant to antibiotics than the inoculated planktonic bacterial inoculum used for determining antibiotic susceptibility by the agar diffusion method. Additionally, the thicker the colony is, the longer is the diffusion time which scales as the square of the diffusion distance (21). In spite of the much higher tolerance to antibiotics of biofilms measured by MBEC (analogous to the planktonic Minimal Bactericidal Concentration = MBC), the pharmacokinetic/pharmacodynamic properties of antibiotics follow the same rules for planktonic and biofilm growing bacteria (13, 22, 23).

Generally, the *in vitro* formation of bacterial biofilms on a surface follows five steps: *1)* reversible adhesion, *2)* irreversible adhesion, *3)* the bacteria divide to form aggregates and extracellular matrix is produced forming a young biofilm, *4)* the biofilm maturates and becomes structured, *5)* dispersal of single cells and sludging off of biofilm aggregates (24). Our results from the present tobramycin agar diffusion assay agree with the first 4 steps. Planktonic bacteria are seeded and starts to grow

and form aggregates and when the aggregates become > ≈64 cells after 5h of incubation, they are more tolerant to tobramycin and continue to grow and form a mature biofilm 256 cells) after 7h of incubation which is completely tolerant to tobramycin. These observations correlate well to previously published results (25) which showed in a surface-attached bacterial colony model that the bacterial cells are entering in the tolerant, stationary growth after 6-7h. The correlation between the decreasing zone diameter and the delayed placing of the tobramycin tablet after seeding is in accordance with the results reported by Nichols et al. 1988 (11). The 4 step of biofilm formation correlates also with the gradual increasing MBEC.

The calculations of the tobramycin concentrations in the agar explains the formation of the early and the stable inhibition zones, although the growth conditions are different on agar plates and on pegs in microtiter plates as also reported by Abbanat et al. 2014 (20).

The formation of the biofilm matrix (step 3-4) was also shown to occur in our experiments. Generally, the biofilm matrix consists of polysaccharides, which is the most important component, proteins (e.g. pili, other adhesins, flagella) and eDNA produced by the bacteria, and *in vivo* also of components from the host (26, 27). We have now shown, that abundance of matrix polysaccharide is produced by the non-mucoid *P. aeruginosa* PAO1 during the formation of aggregates and biofilm and that the PSL polysaccharide is produced, which has been shown to be important for biofilm formation in non-mucoid strains (14, 28). Other polysaccharides which are stained by Alcian blue are e.g LPS and also alginate (29) which is produced in small amounts by the non-mucoid PAO1 and is further induced when a *P. aeruginosa* PAO1 biofilm is exposed to imipenem (30). Alginate is the dominating matrix polysaccharide in vitro and in vivo in biofilms of mucoid phenotypes (30).

There is an important and clinically relevant interpretation of the role of transition from planktonic mode of growth to biofilm mode of growth for the formation of the inhibition zone. The conventional interpretation of the diameter of the inhibition zone is that the category Susceptible (S) based on clinical breakpoints (8) indicates that it is *probably* successful to treat and eradicate infections caused by planktonic growing bacteria. However, the biofilm mode of growth in the border of the inhibition zone and the corresponding tobramycin concentration in the agar offers the new interpretation, that the agar diffusion assay shows that it is *probably not* successful to treat and eradicate biofilm infections caused by the bacteria in question at least if a conventional dosing regime is used. This interpretation underlines, that the conventional interpretation of agar diffusion – like microtiter dilution assays - does not provide key information to clinicians concerning treatment of biofilm infections (17). The agar diffusion assay in contrast to the microtiter dilution assay gives visual information about the increased antibiotic tolerance of the biofilm mode of growth. The Calgary device, which we used for determination the time-dependent increase of tolerance to tobramycin, is designed for measuring the MBEC of adhering biofilms and may be used for that purpose in the clinical microbiology laboratory (12, 13, 17).

In conclusion, the inhibition zone formation during agar diffusion susceptibility testing is due to a switch from planktonically growing bacteria to biofilm growing bacteria with increased tolerance to antibiotics. The inhibition zone gives the clinical important information, that biofilm growing bacteria are tolerant to the dose of antibiotic recommended for infections caused by planktonic growing bacteria. Therefore, if treatment failure or recurrence occur, clinicians should suspect that the reason may be that biofilm growing bacteria are causing the infection.

Declarations

Funding: No

Competing Interests: No

Ethical Approval: Not required

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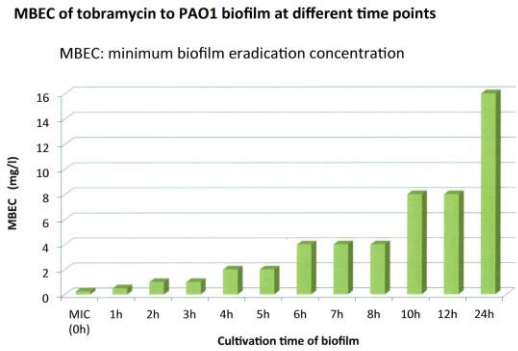
Figures and legends to the figures



MBEC and MIC (µg/ml)	MIC E-test	MIC microtiter	1h biofilm	2h biofilm	3h biofilm	4h biofilm	5h biofilm	6h biofilm	7h biofilm	8h biofilm	10h biofilm	12h biofilm	24h biofilm
PAO1	0.064	0.25	0.5	1	1	2	2	4	4	4	8	8	16

Fig. 1. Minimum Biofilm Eradication Concentration (MBEC) was performed using the Calgary Biofilm Device (12). MBEC was determined at the specified time (h) after start of the biofilm growth of *P. aeruginosa* PAO1 (13).

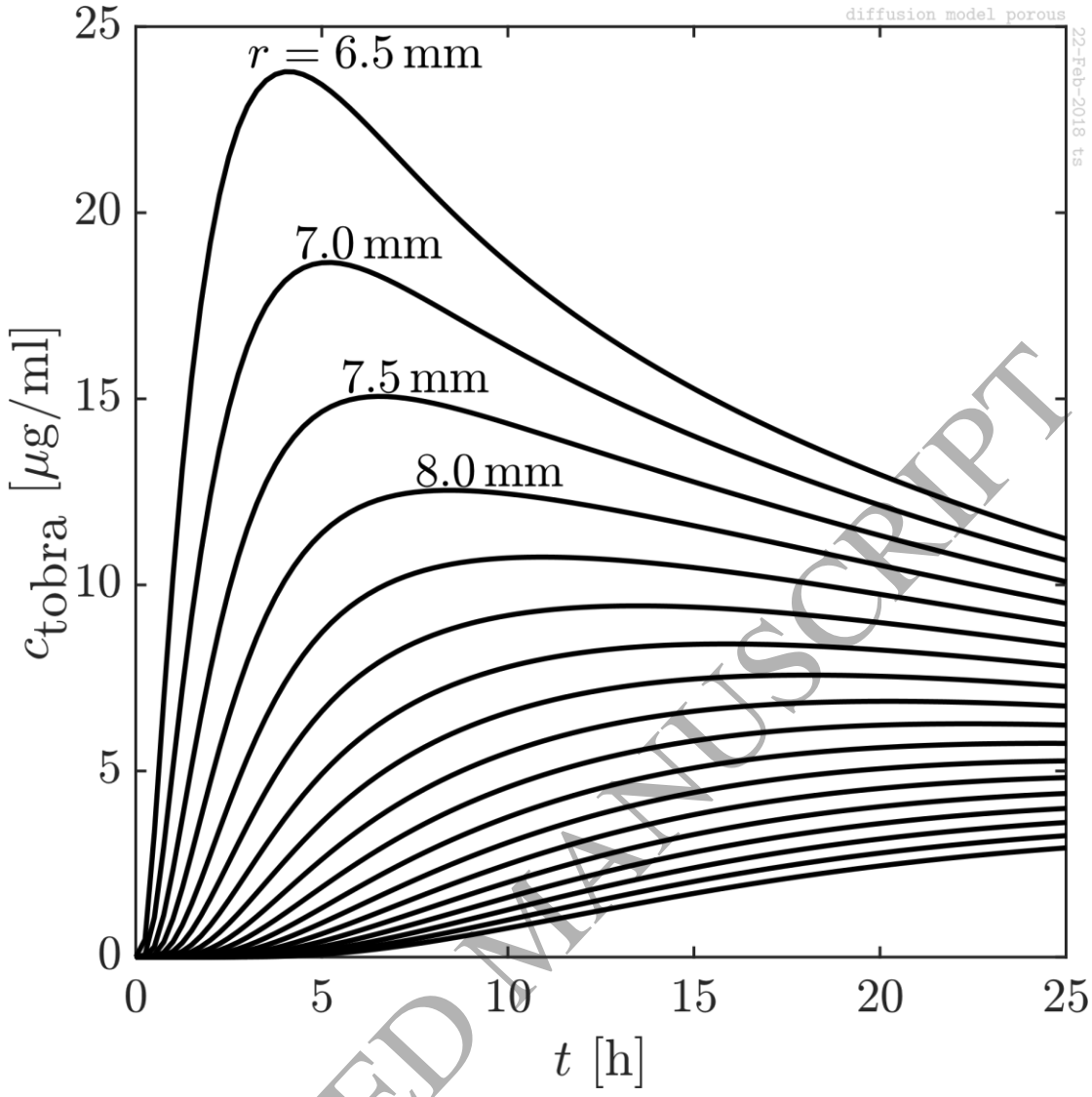


Fig. 2. Concentration of tobramycin at radii ranging from 6.5mm to 15mm (lowest curve) as a function of time in the full 3D model including the effect of porosity of the NEOSENSITABS® tablet [diffusion_model_porous.eps]. The radius is taken from the centre of the tablet. The concentration at the top of the agar plate is plotted.

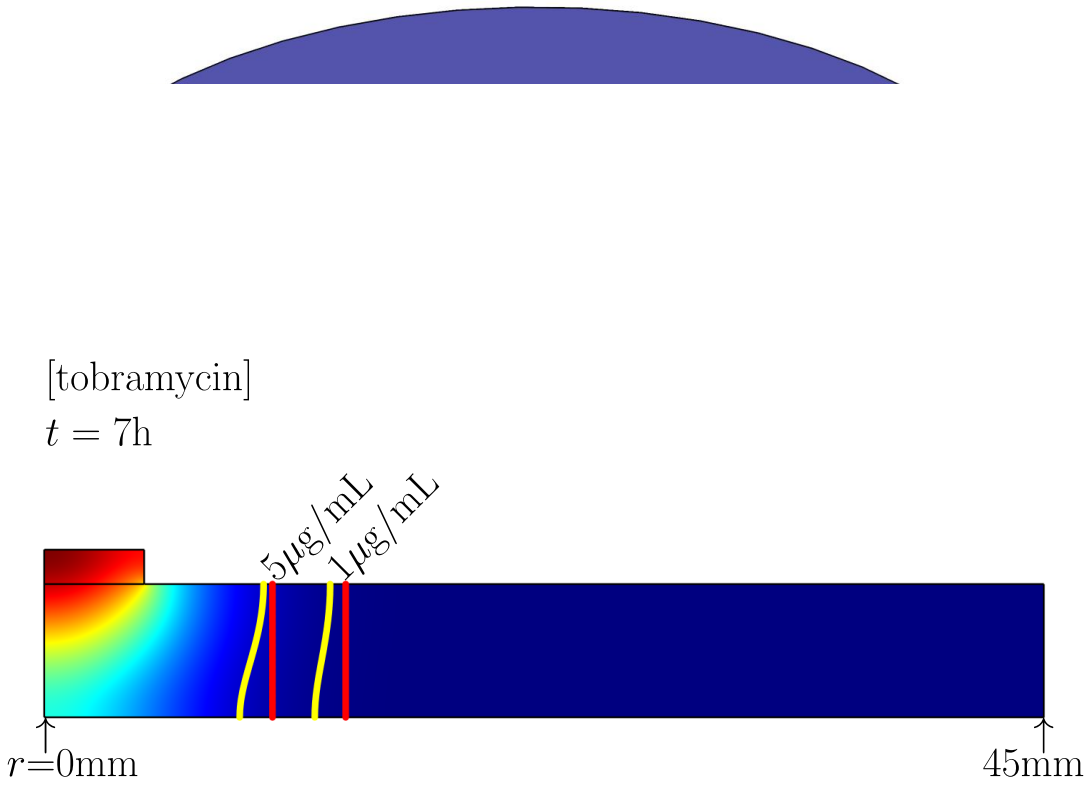


Fig. 3. Top: Illustration of the concentration of tobramycin profile in agar 7h after placing the NEOSENSITAB® tablet. Bottom: Cross section with indications of the 1µg/ml and 5µg/ml contours for the full calculation (yellow) and the shifted gaussian (red). [DiscDiffusionPorous.eps].

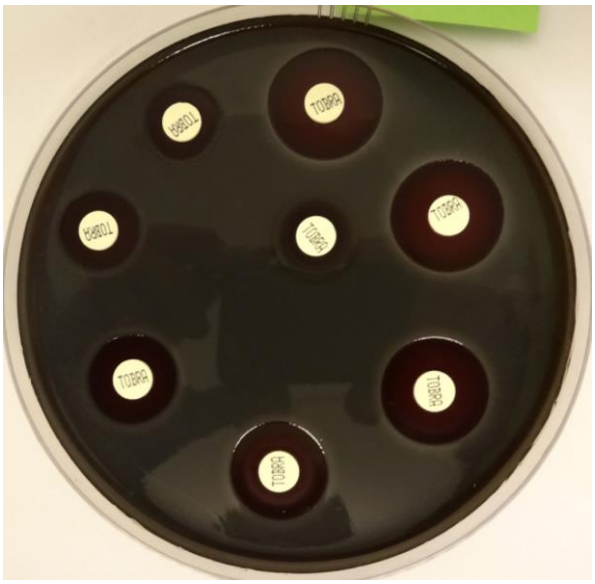
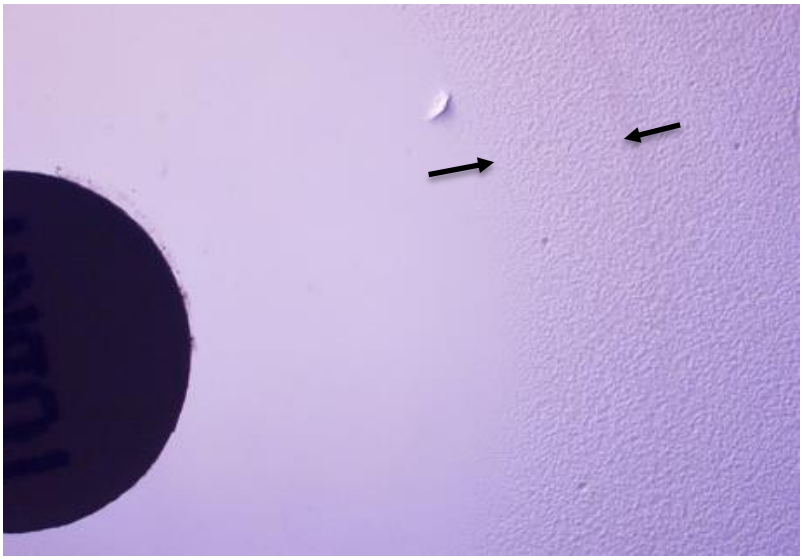
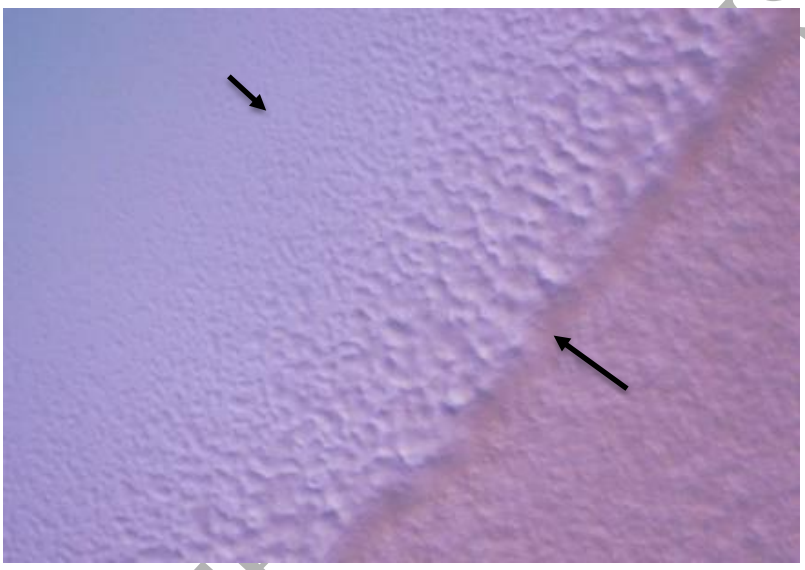


Fig. 4 Correlation between the diameter of the inhibition zone of *P. aeruginosa* PAO1 and the time after seeding of the agar plate (inoculum 10^7/ml) where the NEOSENSITABS® tobramycin tablet was placed on surface of the agar and the diffusion began. Top: time 0h, clockwise time +1h, +2h, +3h, +4h, +5h, +6h, +7h (center). The plates were incubated for 24h at 37°C.

A



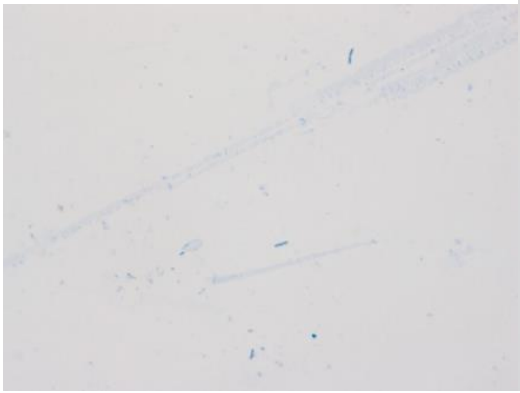
B



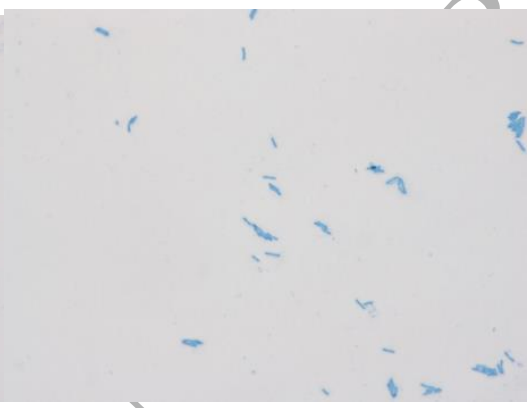
C

Fig. 5A, B, C. Stereomicroscopy photos (A: magnification x 40, B & C: x 100) of the *P. aeruginosa* PAO1 cells in the inhibition zone around a tobramycin NEOSENSITABS® tablet, 24h incubation. *A:* The tobramycin tablet is seen at the left side and the border of the inhibition zone is indicated by 2 arrows. *B:* close up, the arrows are placed as in A. The surface of the growth outside the inhibition zone shows presence of a self-produced matrix, C: the morphology of the bacteria inside the inhibition zone. The scratches in the agar surface in A and C are artificial landmarks to facilitate the microscopy.

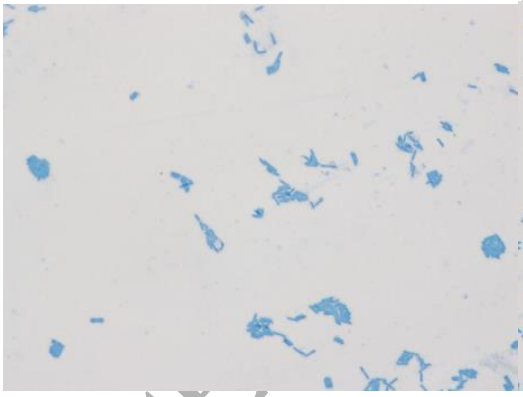
A - 1h



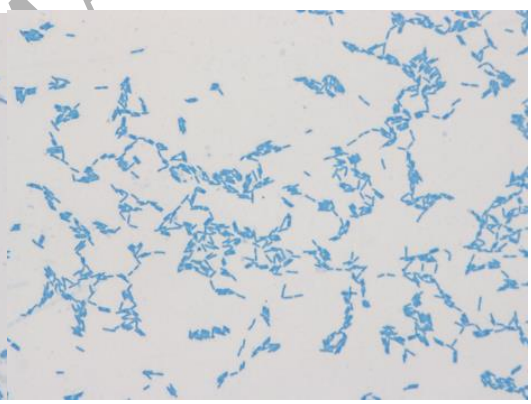
B - 3h



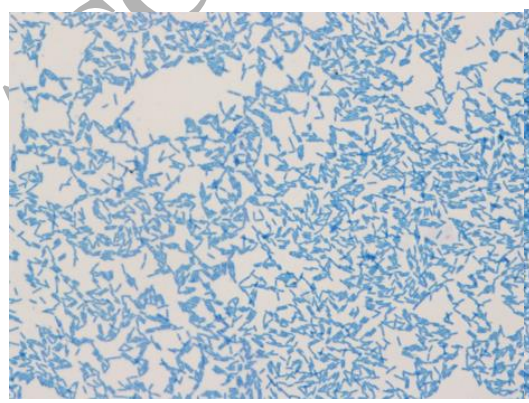
C - 4h



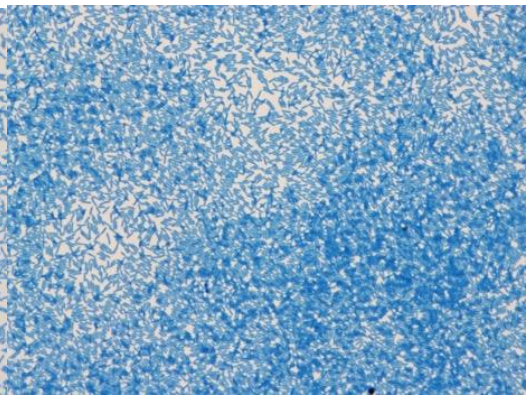
D - 5h



E - 6h



F - 7h



G - 24h

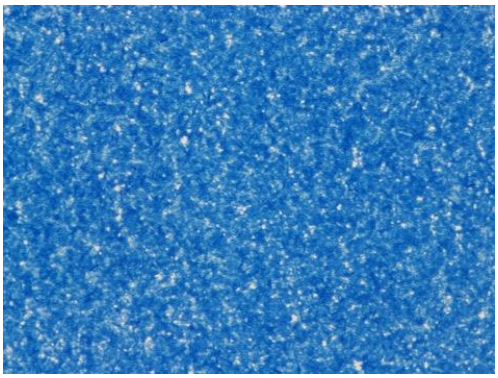
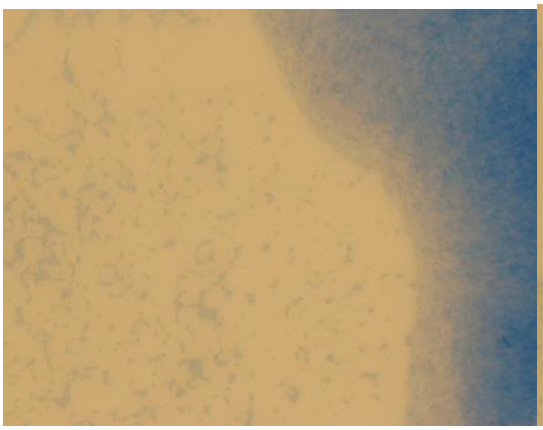
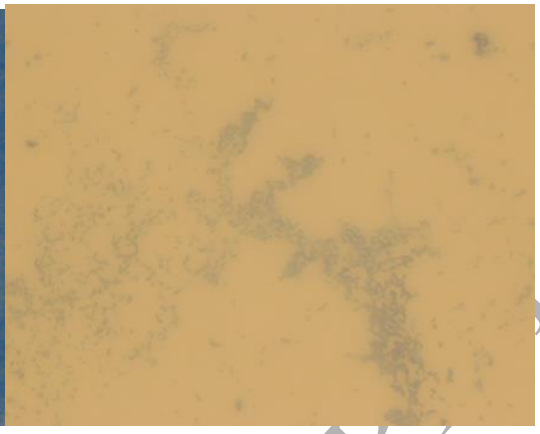


Fig. 6A, B, C, D, E, F, G. Growth of *P. aeruginosa* PAO1 (inoculum 10^7/ml) seeded on the agar surface with a tobramycin NEOSENSITABS® tablet and incubated at 37^{o}C for 24h. The surface - outside the area where the inhibition zone was expected - was blotted with microscope slides, fixed with methanol and stained with Methylene blue. 1000 x magnification. From top left A, after 1h incubation; B, after 3h; C, after 4h; D, after 5h; E, after 6h; F, after 7h; G, after 24h.

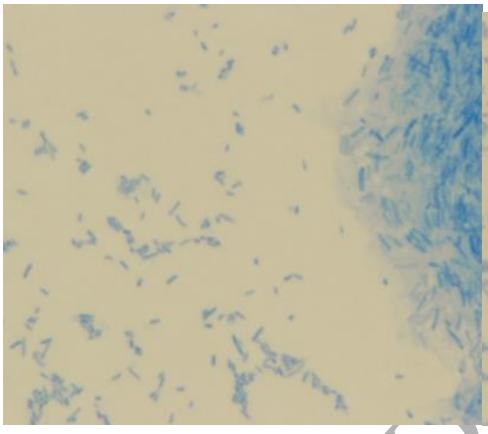
A



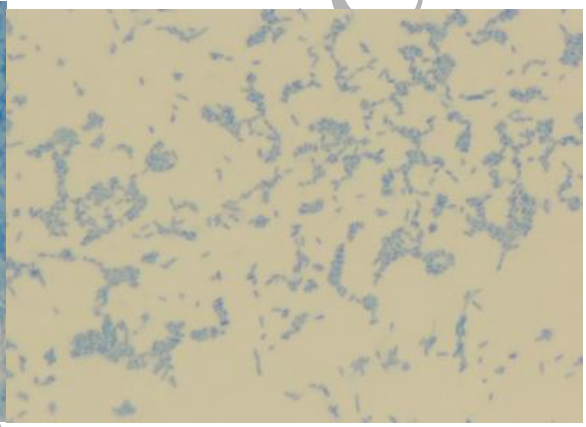
B



C



D



E

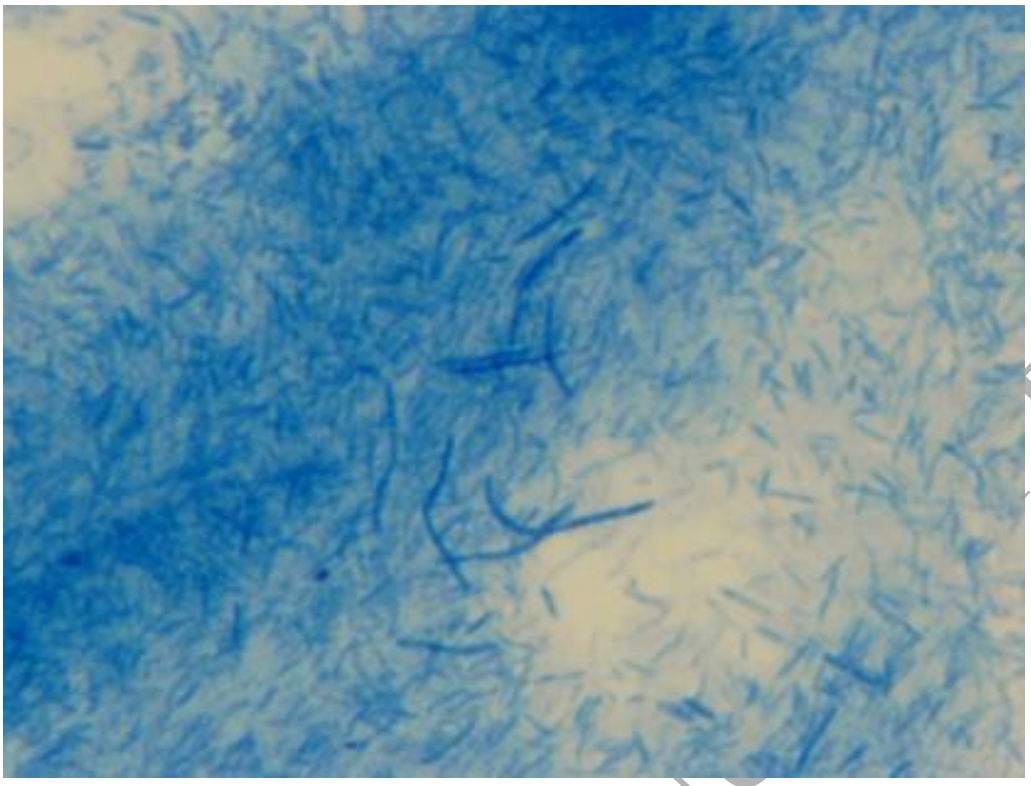
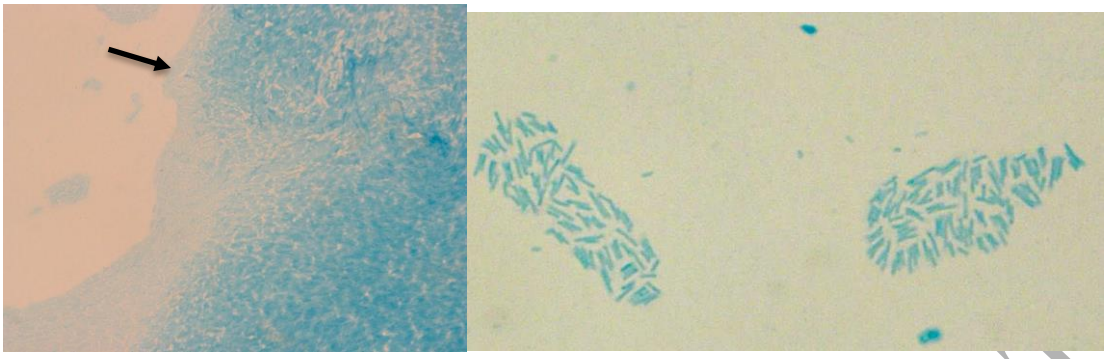
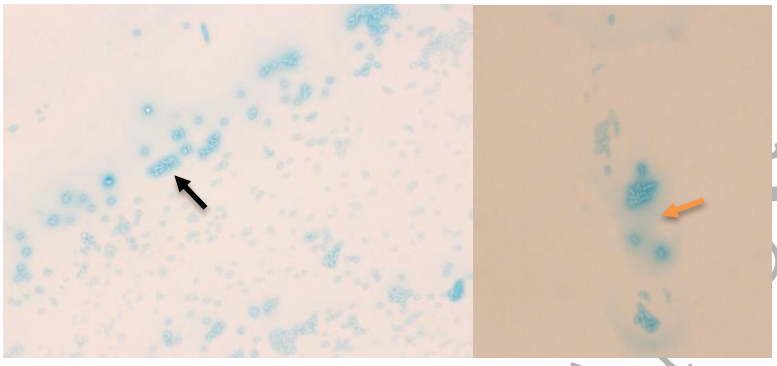


Fig. 7 A, B, C, D, E. Growth of *P. aeruginosa* PAO1 (inoculum 10^7/ml) seeded on the agar surface and incubated at 37^{o}C for 24h with a tobramycin NEOSENSITABS® tablet. The surface across the inhibition zone was blotted with microscope slides, fixed with methanol and stained with Methylene blue. 1000 x magnification. *A & C*, the border of the inhibition zone showing dense growth and a matrix between the bacterial cells. The bacterial aggregates inside the inhibition zone (also shown in *B & D*) are dead as shown by stereomicroscopic guided subculture. *E* shows filamentous bacterial cells in the border of the inhibition zone probably due to the influence of tobramycin and the biofilm mode of growth. The bacteria inside the border of the inhibition zone are viable and showed dense growth after stereomicroscopic guided subculture.

A B



C D



E F

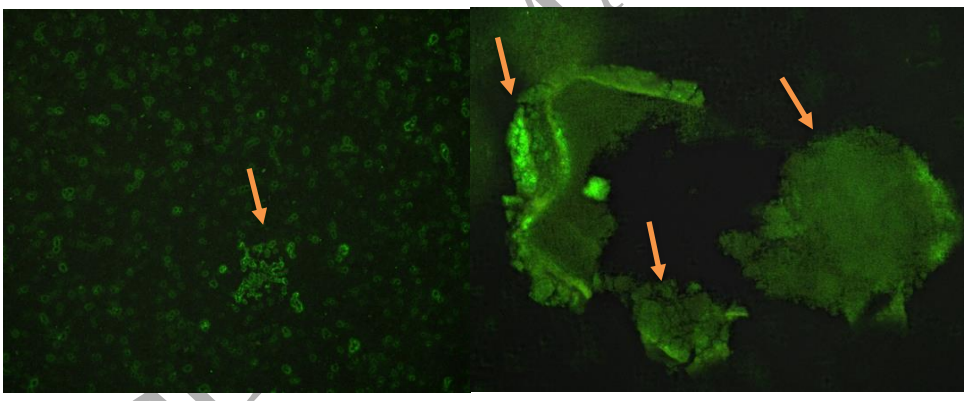


Fig. 8 A, B, C, D, E, F. Growth of *P. aeruginosa* PAO1 (inoculum 10^7/ml) seeded on the agar surface with a tobramycin NEOSENSITABS® tablet and incubated at 37°C for 24h. The surface across the inhibition zone was blotted with microscope slides, fixed with methanol and stained with Methylene blue. 1000 x magnification. *A-D:* polysaccharide production visualized by Alcian blue staining, black arrow= surface rings of polysaccharide on the bacterial cells, orange arrow = extracellular polysaccharide. *A*: The border of the inhibition zone (arrow). *B-D*: The area just inside the border of the inhibition zone. *E-F:* The bacterial growth outside the inhibition zone, *P. aeruginosa* PSL polysaccharide visualized by FITC-HHA lectin staining; orange arrows = green fluorescence stained PSL. E-F: Confocal laser scanning microscopy photo.

Tables

Table 1. Correlation between the diameter of the inhibition zone of *P. aeruginosa* PAO1 and the time (0h to +7h) after inoculation of the agar plate (inoculum 10^7/ml) where the tobramycin tablet (NEOSENSITABS®) was placed on surface of the agar and the diffusion began. The zone was read every 30min.

Tobramycin	*Inhibition zone (mm) read after incubation for:*				
placed at:	1-4h	5h	6h	7h	24h
0h	invisible	22	23	24	24 <*
+1h	”	19	22	23	23 <
+2h	”	18	19	19	19 <
+3h	”	14	16	16	16 <
+4h	”	n.e.z.	14	15	15 <
+5h	”	n.e.z.	n.e.z.	14	14 =**
+6h	”	n.e.z.	n.e.z.	n.e.z.	15
+7h	”	n.z.e.	n.z.e.	n.z.e.	15

n.e.z.: no evaluable zone, *< the inhibition zone increased from barely visible to 24h, =** the inhibition zone was stable from barely visible to 24h.

Table 2. Calculated tobramycin concentration in agar at the border of the early (small) and the final (large) *P. aeruginosa* PAO1 inhibition zone after diffusion for *n* h (MIC= 0.064 µg/ml). NEOSENSITABS® containing 40µg diffusible tobramycin was placed on the inoculated agar (inoculum 10^7/ml) immediately (0h) or 1h, 2h, 3h, 4h, 5h later. Same experiment as in Table 1.

		Tobramycin concentration in the border of the inhibition zone	
		Early inhibition zone after 5h incubation	*Final inhibition zone after 7h incubation*
Tobramycin placed at	*Diffusion time*	**22mm diameter**	**24mm diameter**
0h	5h	1.9 µg/ml	0.9 µg/ml
	7h	3.2 µg/ml	1.8 µg/ml
	24h	6.3 µg/ml	5.3 µg/ml
		19mm diameter	**23mm diameter**
+1h	4h	3.7µg/ml	0.8µg/ml
	6h	5.9µg/ml	1.9µg/ml
		18mm diameter	**19mm diameter**
+2h	3h	3.5µg/ml	2.3µg/ml
	5h	6.6µg/ml	4.9µg/ml
		14mm diameter	**16mm diameter**
+3h	2h	12.4µg/ml	4.7µg/ml
	4h	18.2µg/ml	10.1µg/ml
		-*	**15mm diameter**
+4h	1h	-	-
	3h	-	11.5µg/ml
		-	**14mm diameter**
+5h	0h	-	-
	2h	-	12.4µg/ml

*No zone