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

Niels Høiby[a,b,*], Kaj-Åge Henneberg[c,d], Hengshuang Wang[a,b], Camilla Stavnsbjerg[b], Thomas Bjarnsholt[a,b], Oana Ciofu[b], Ulla Rydal Johansen[a], Thomas Sams[c,d]

[a] *Department of Clinical Microbiology, Rigshospitalet, 2100 Copenhagen, Denmark*
[b] *Department of Immunology and Microbiology, Costerton Biofilm Center UC-CARE, Faculty of Health Sciences, University of Copenhagen, 2200 Copenhagen, Denmark*
[c] *Biomedical Engineering, Department of Electrical Engineering, Technical University of Denmark, 2800 Lyngby, Denmark*
[d] *Biomedical Engineering, Department of Health Technology, Technical University of Denmark, 2800 Lyngby, Denmark*

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ABSTRACT

Pseudomonas aeruginosa PAO1 (tobramycin MIC = 0.064 μg/mL) was used to perform agar diffusion tests employing tobramycin-containing tablets. Bacterial growth and 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. Diffusion of tobramycin from the deposit was modelled using a 3D geometric version of Fick's second law of diffusion. The time-dependent gradual increase in the minimum biofilm eradication concentration (MBEC) was studied using a Calgary Biofilm Device. The early inhibition zone was visible after 5 h of incubation. The corresponding calculated tobramycin concentration at the border was 1.9 μg/mL, which increased to 3.2 μg/mL and 6.3 μg/mL after 7 h and 24 h, respectively. The inhibition zone increased to the stable final zone after 7 h of incubation. Bacterial growth and small aggregate formation (young biofilms) took place inside the inhibition zone until the small aggregates contained less than ca. 64 cells and production of polysaccharide matrix including PSL had begun; thereafter, the small bacterial aggregates were killed by tobramycin. 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. Formation of the inhibition zone during agar diffusion antimicrobial susceptibility testing is due to a switch from a planktonic to biofilm mode of growth and gives clinically important information about the increased antimicrobial tolerance of biofilms.

1. Introduction

Soon after antibiotics began to be used to treat infections, the need to test the susceptibility of the offending bacterium to various antibiotics became urgent since the occurrence of resistant mutants of otherwise susceptible species became a problem. The agar diffusion method became the most popular and clinically used method employing agar cups, disks or tablets as deposits [1–6]. The method has been repeatedly standardised internationally [7], by the Clinical and Laboratory Standards Institute (CLSI) in the USA (http://www.clsi.org) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe (http://www.eucast.org), and is regarded as a good method for categorising bacteria as susceptible, intermediate-susceptible or resistant to the systemic use of antibiotics in accordance with the harmonised breakpoints [8]. Diffusion of antibiotics from the deposit in a disk or tablet takes place in water within a 1% agar plate and follows Fick's second law of diffusion. The size of the inhibition zone was shown to be dependent not only on the minimum inhibitory concentration (MIC) of the bacterium 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 (http://www.eucast.org). Formation of the inhibition zone is read-

* Corresponding authors.
E-mail addresses: hoiby@hoibyniels.dk, hoiby@dadlnet.dk (N. Høiby), tsams@dtu.dk (T. Sams).

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able after ≤5 h of incubation but increases marginally during the next few hours, after which it becomes stable and colonies at the border and beyond the inhibition zone continue to grow overnight.

These characteristics of inhibition zone formation indicate that it may be the transition from a planktonic mode of growth (the inoculum) to a biofilm mode of growth that is decisive for inhibition zone formation. Therefore, the present study aimed to investigate the influence of the transition from a planktonic to a biofilm mode of growth on the formation of the inhibition zone of *P. aeruginosa* around a tablet containing tobramycin. The pharmacokinetic/pharmacodynamic (PK/PD) relationship of tobramycin is concentration-dependent killing [9].

2. Materials and methods

2.1. Bacterial strain

Pseudomonas aeruginosa PAO1 [10] was used for the experiments. The planktonic MIC of tobramycin measured by Etest is 0.064 μg/mL for PAO1. Inocula of 10^7 CFU/mL [7] or 10^8 CFU/mL (EUCAST) (=0.5 MacFarland standard) were used and the plates were inoculated by floating. The low inoculum gives a 2 mm larger zone of inhibition and a 1.5 h longer time to study the growth of microcolonies before the inhibition zone is formed.

2.2. Antimicrobial tablets and antimicrobial susceptibility agar medium

Neo-Sensitabs (Rosco Diagnostica A/S, Tåstrup, Denmark) (9 mm diameter) with 40 μg of diffusible tobramycin/tablet (molecular weight 467.54 g/mol, formula $C_{18}H_{37}N_5O_9$) were used. At a temperature of 37°C, the diffusion constant (D) for tobramycin is ca. $3.84 \cdot 10^{-5}$ cm^2/s = 1.38 mm^2/h [11].

Cation-adjusted horse blood agar susceptibility medium (SSI Diagnostica, Hillerød, Denmark) was used. For some experiments, where visualisation of bacterial growth was required on transparent agar, Luria–Bertani agar plates were used (SSI Diagnostica). Inoculation, incubation and reading of the plates as well as stereomicroscopy was performed by two authors (NH and URJ) in an incubation cabinet at 37°C using an Olympus SZ61 stereomicroscope (Olympus, Tokyo, Japan). Microscopic examination of stained slides was performed with an Olympus BX50 light microscope (Olympus). Confocal laser scanning microscopy was performed with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

The gradual increase in the minimum biofilm eradication concentration (MBEC) of tobramycin by increasing age of the biofilm was determined in microtitre plates (Nunc A/S, Roskilde, Denmark) using the Calgary Biofilm Device method (Fig. 1; Supplementary Fig. S1) with an inoculum of 10^7 CFU/mL [12] as described previously [13].

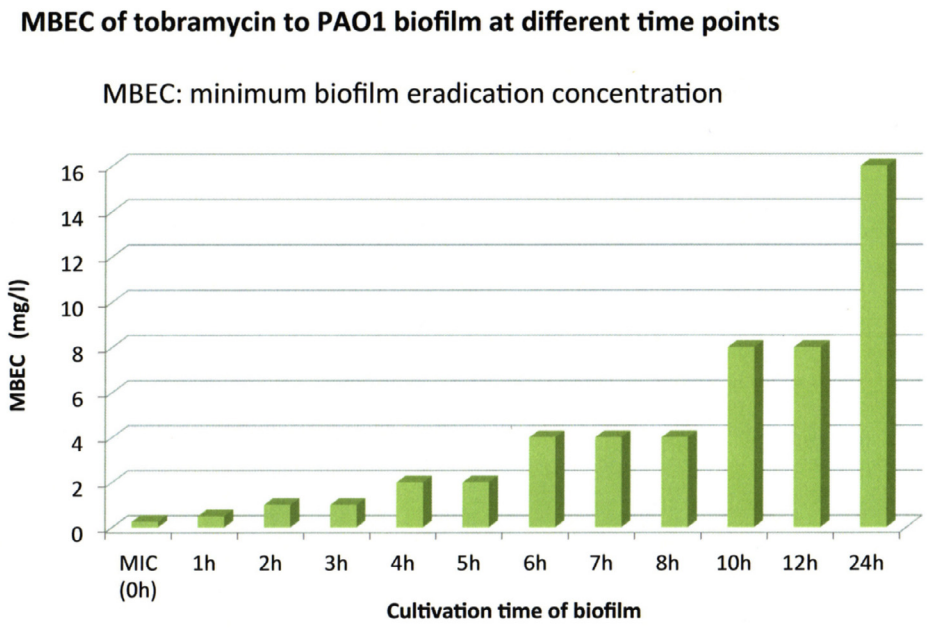
2.3. Stains

Methylene blue (Sigma-Aldrich Denmark A/S, Copenhagen, Denmark), Alcian blue (Sigma-Aldrich Denmark A/S) and PSL-specific fluorescein isothiocyanate-conjugated *Hippeastrum* hybrid lectin (Amaryllis) (FITC-HHA lectin) (EY Laboratories, San Mateo, CA) were used [14].

2.4. Calculation of the concentration of tobramycin in the agar plate

Diffusion of antibiotic in the agar plate follows Fick's second law of diffusion:

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



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) determined using the Calgary Biofilm Device [12]. The MBEC was determined at the specified time (h) after the start of biofilm growth of *Pseudomonas aeruginosa* PAO1 [13]. MIC, minimum inhibitory concentration.

where c is the concentration of antibiotic, t is the time and D is the diffusion constant for the antibiotic. This is valid when there is no significant consumption of antibiotic and when the agar concentration is just a few percent [15]. We assume that there is no significant adsorption of tobramycin to agar.

2.5. Cylindrical geometry

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

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

Here we have normalised such that it integrates to 1 when integrated in the plane. If, instead, we wish to express the concentration as mass/volume we get:

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

Here m is the mass of antibiotic 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 antibiotic is added in a cylinder with finite radius r_0. The antibiotic 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:

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

In Supplementary Fig. S2 we show a comparison between the full calculation for selected radii and the shifted Gaussian version. The specific calculation regarding the tobramycin-containing Neo-Sensitabs® tablet is performed with cylinder radius $r_0 = 4.5$ mm, agar height $h_0 = 6$ mm and tobramycin mass $m = 40$ µg. The radius of the small agar plate is $R = 45$ mm and the reflective boundary does not influence the results in Fig. 2 and Supplementary Fig. S2. For some experiments, larger agar plates were used with a radius of 70 mm.

2.6. Full three-dimensional (3D) geometry

If the deposition of antibiotic is as a tablet on top of the agar, we need to carry out a full 3D calculation. The Neo-Sensitabs® tablets are of radius $r_0 = 4.5$ mm and height $h_0 = 1.55$ mm. Therefore, the thickness of the tablet cannot be assumed to be much smaller than the height of the agar. The v/v concentration of 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 43 µg 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 antibiotic 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 $c_0 = 930$ µg/mL when we assume that the tobramycin is quickly going into solution. The numerical solution of the diffusion equation is performed in COMSOL Multiphysics® v.5.3.4 (Comsol AB, Stockholm, Sweden).

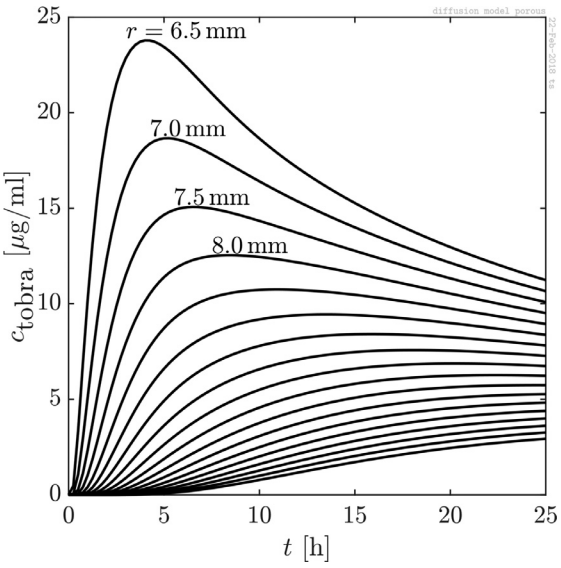


Fig. 2. Concentration of tobramycin (c_{tobra}) at radii ranging from 6.5 mm to 15 mm (lowest curve) as a function of time (t) in the full three-dimensional (3D) model including the effect of porosity of the Neo-Sensitabs® tablet. The radius is taken from the centre of the tablet. The concentration at the top of the agar plate is plotted.

3. Results

3.1. Minimum biofilm eradication concentration

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

3.2. 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 Supplementary Fig. S2. 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 with the vertical equilibration time and small radii, it is of importance to perform the full calculation.

The concentration of tobramycin at radii ranging from 6.5 mm to 15 mm (lowest curve) as a function of time in the full 3D model including the effect of porosity of the Neo-Sensitabs® is shown in Fig. 2. The concentration at the top of the agar plate is plotted. For reference, the underlying calculated concentrations are listed in Supplementary Table S1 as a function of time and radius. The concentration of the tobramycin profile in agar 7 h after placing the Neo-Sensitabs® tablet is shown in Fig. 3.

3.3. Growth of Pseudomonas 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

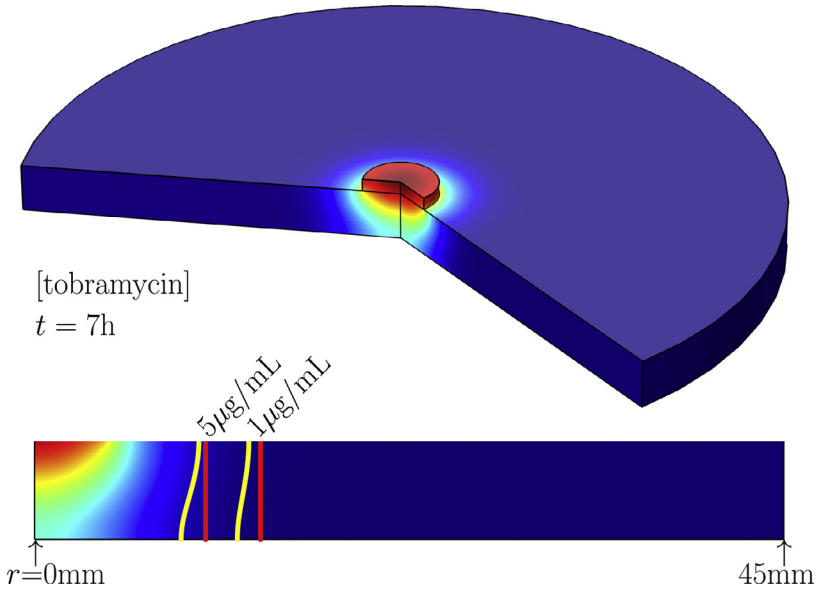


Fig. 3. Top: Illustration of the concentration of tobramycin profile in agar 7 h after placing the Neo-Sensitabs® tablet. Bottom: Cross-section with indications of the 1 µg/mL and 5 µg/mL contours for the full calculation (yellow) and the shifted Gaussian (red).

Table 1
Correlation between the diameter of the inhibition zone of *Pseudomonas aeruginosa* PAO1 and the time (0–7 h) following inoculation of the agar plate (inoculum 10^7 CFU/mL) when the tobramycin Neo-Sensitabs® tablet was placed on surface of the agar and the diffusion began. The inhibition zone was read every 30 min.

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

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

blood agar with a microscopy slide, fixing with methanol and staining with methylene blue and studying the results with a light microscope, the lag phase of *P. aeruginosa* after seeding the blood agar susceptibility medium was determined to be ca. 2 h. Thereafter, the doubling time became ca. 30 min during the 7 h of observation. Formation of an inhibition zone around the tobramycin disk was visible both with the naked eye and with the stereomicroscope after 5 h with an inoculum of 10^7 CFU/mL (Table 1), which means that the number of cells had increased by a factor ca. 2^8 (= 256). The inhibition zone was already visible after 3.5 h when the inoculum was 10^8 CFU/mL.

Fig. 4 and Table 1 show the observed inhibition zone diameter, and Table 2, Fig. 2 and Supplementary Fig. S2 show the calculated results of the agar diffusion tests with tobramycin Neo-Sensitabs® added at time 0–7 h 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 the inhibition zone became, indicating that the increasing amount of 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 visualised by stereomicroscopy is shown in

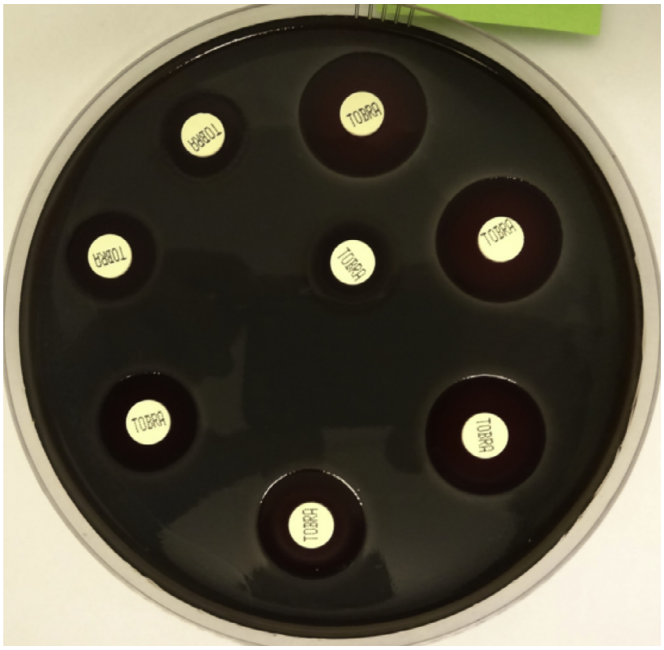


Fig. 4. Correlation between the diameter of the inhibition zone of *Pseudomonas aeruginosa* PAO1 and the time after seeding of the agar plate (inoculum 10^7 CFU/mL) when the Neo-Sensitabs® tobramycin tablet was placed on surface of the agar and the diffusion began. Top: time 0 h; clockwise, time 1, 2, 3, 4, 5 and 6 h; and centre, time 7 h. Plates were incubated for 24 h at 37°C.

Fig. 5. The bacterial matrix is clearly seen at the border and outside the border of the inhibition zone.

Subculture guided by stereomicroscopy after 24 h of 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 5 h of incubation failed to show any growth and the bacteria had therefore been killed by tobramycin (Figs. 5–7). On the other hand, subculture from the border of the final zone always showed growth, therefore the bacteria had survived tobramycin. These results were confirmed by propidium iodide staining in a few experiments (LIVE/DEAD® Cell Viability Assay; Thermo Fisher

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

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

MIC, minimum inhibitory concentration.
[a] –, indicates no zone.

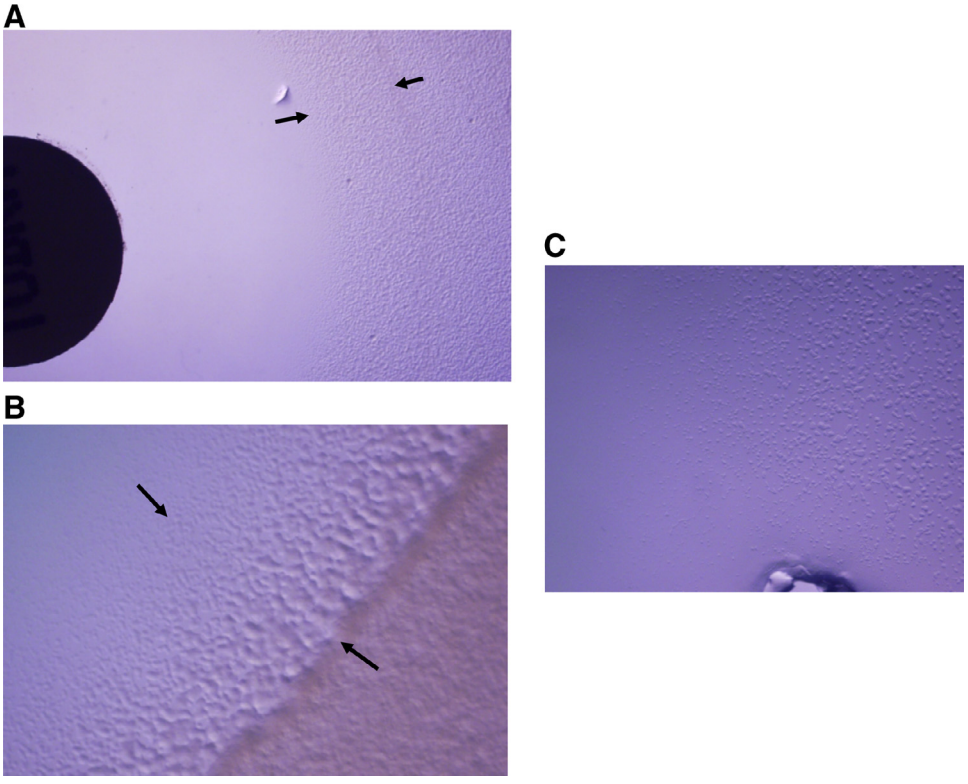


Fig. 5. Stereomicroscopy photos of *Pseudomonas aeruginosa* PAO1 cells in the inhibition zone around a tobramycin Neo-Sensitabs® tablet after 24 h of incubation. (A) The tobramycin tablet is seen at the left side and the border of the inhibition zone is indicated by two arrows. (B) Close up, the arrows are placed as in (A). The surface of the growth outside of the inhibition zone shows the presence of a self-produced matrix. (C) Morphology of the bacteria inside the inhibition zone. Scratches in the agar surface in (A) and (C) are artificial landmarks to facilitate microscopy. Magnification: (A) 40 ×; (B,C) 100 ×.

Scientific, USA) (data not shown). Bacterial growth at the border of the inhibition zone had therefore obtained properties characteristic of biofilm growth, which is observed in Fig. 7C,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 less than ca. 64 cells (after 5 h of incubation with a 2-h lag phase; Fig. 8B); they are later killed inside the early inhibition zone by 3.2–6.3 μg/mL tobramycin (Table 2; 7–24 h).

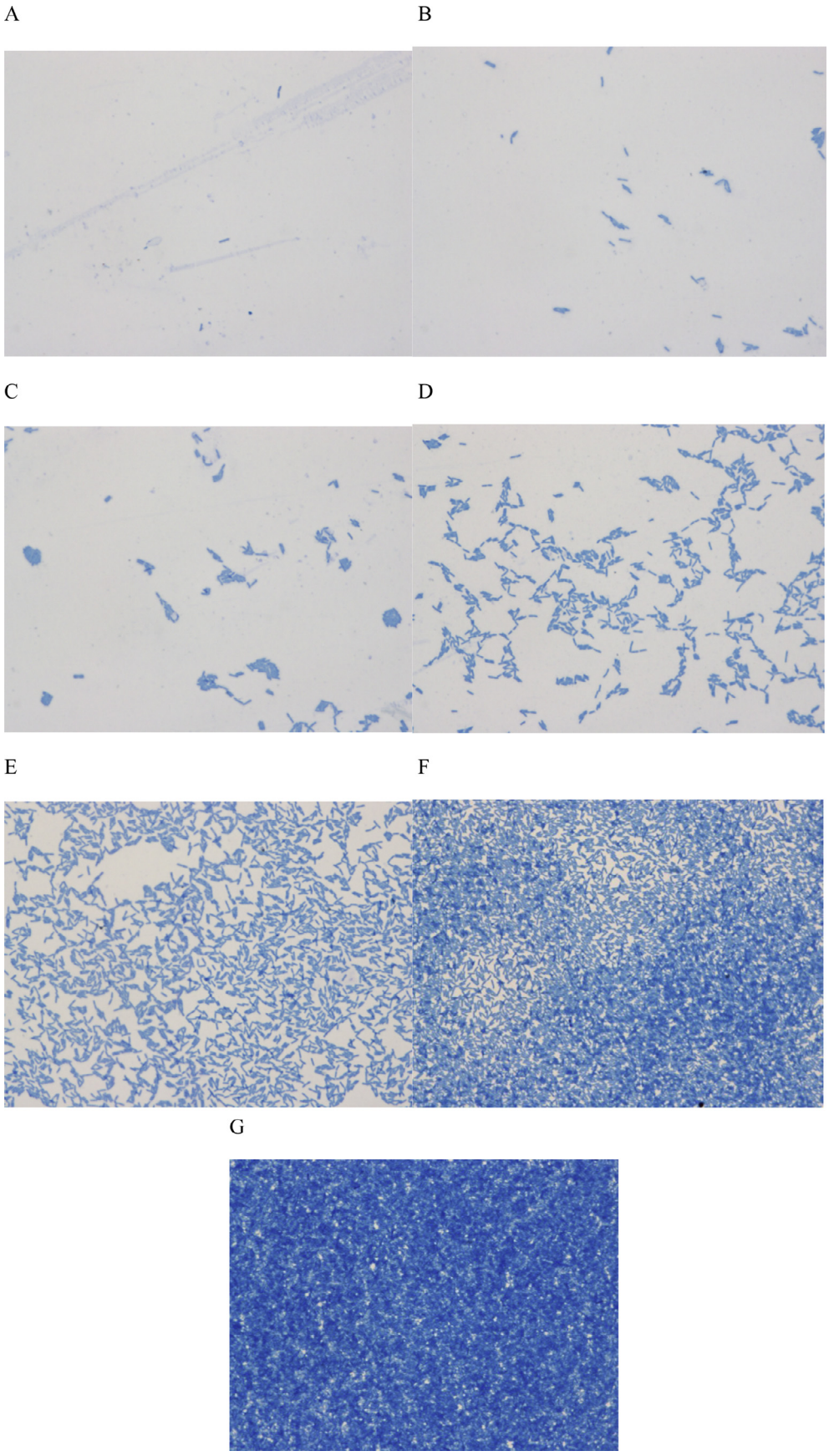


Fig. 6. Growth of *Pseudomonas aeruginosa* PAO1 (inoculum 10^7 CFU/mL) seeded on the agar surface with a tobramycin Neo-Sensitabs® tablet and incubated at 37°C for 24 h. The surface outside the area where the inhibition zone was expected was blotted with microscope slides, was fixed with methanol and was stained with methylene blue. 1000 × magnification. From top left, after the following incubation times: (A) 1 h; (B) 3 h; (C) 4 h; (D) 5 h; (E) 6 h; (F) 7 h; and (G) 24 h.

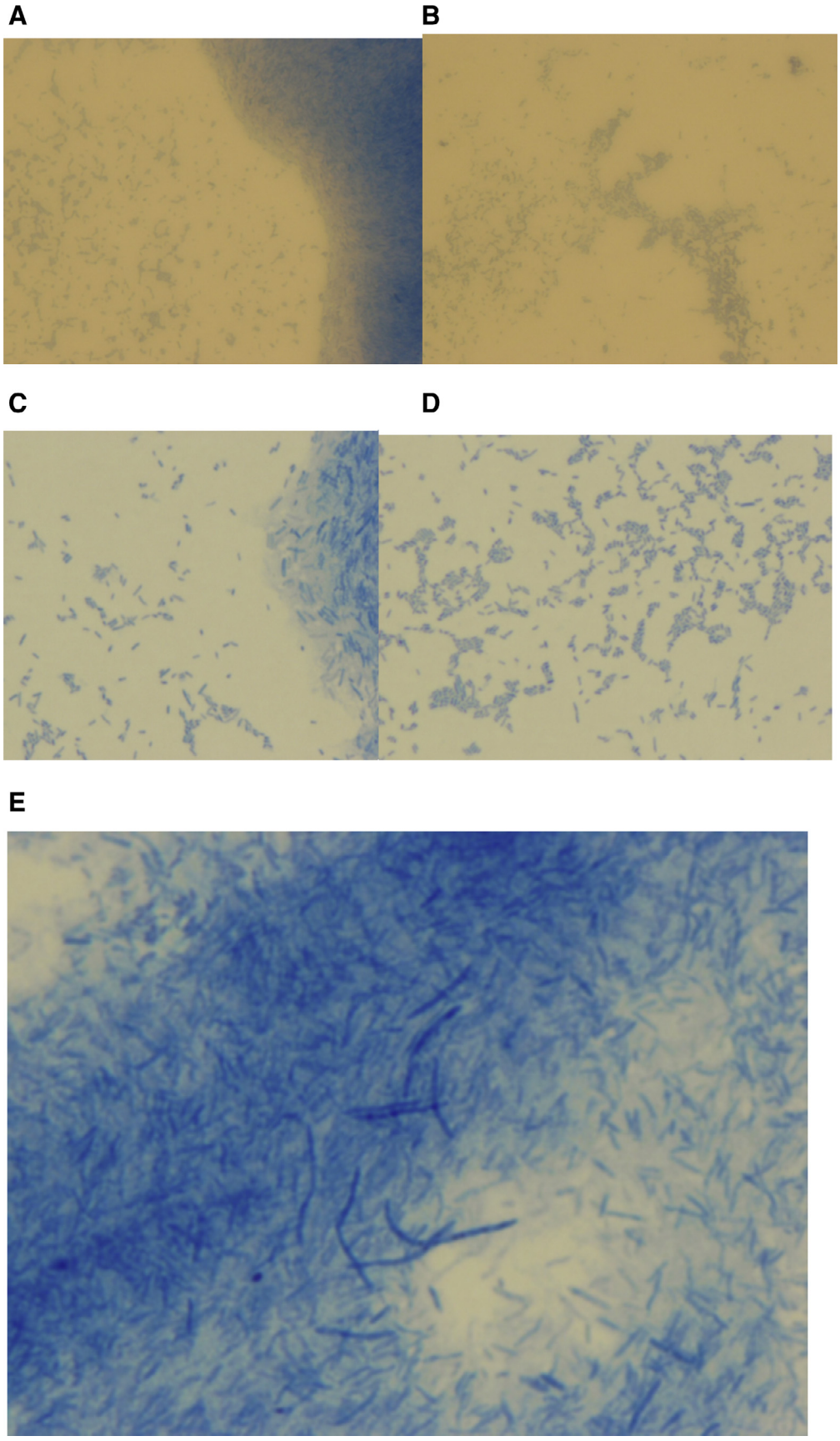


Fig. 7. Growth of *Pseudomonas aeruginosa* PAO1 (inoculum 10^7 CFU/mL) seeded on the agar surface with a tobramycin Neo-Sensitabs® tablet and incubated at 37°C for 24 h. The surface across the inhibition zone was blotted with microscope slides, was fixed with methanol and was stained with methylene blue. 1000 × magnification. (A,C) The border of the inhibition zone showing dense growth and a matrix between the bacterial cells. Bacterial aggregates inside the inhibition zone, also shown in (B) and (D), are dead as shown by stereomicroscopic-guided subculture. (E) Filamentous bacterial cells at the border of the inhibition zone probably due to the influence of tobramycin and the biofilm mode of growth. Bacteria inside the border of the inhibition zone are viable and showed dense growth following stereomicroscopic-guided subculture.

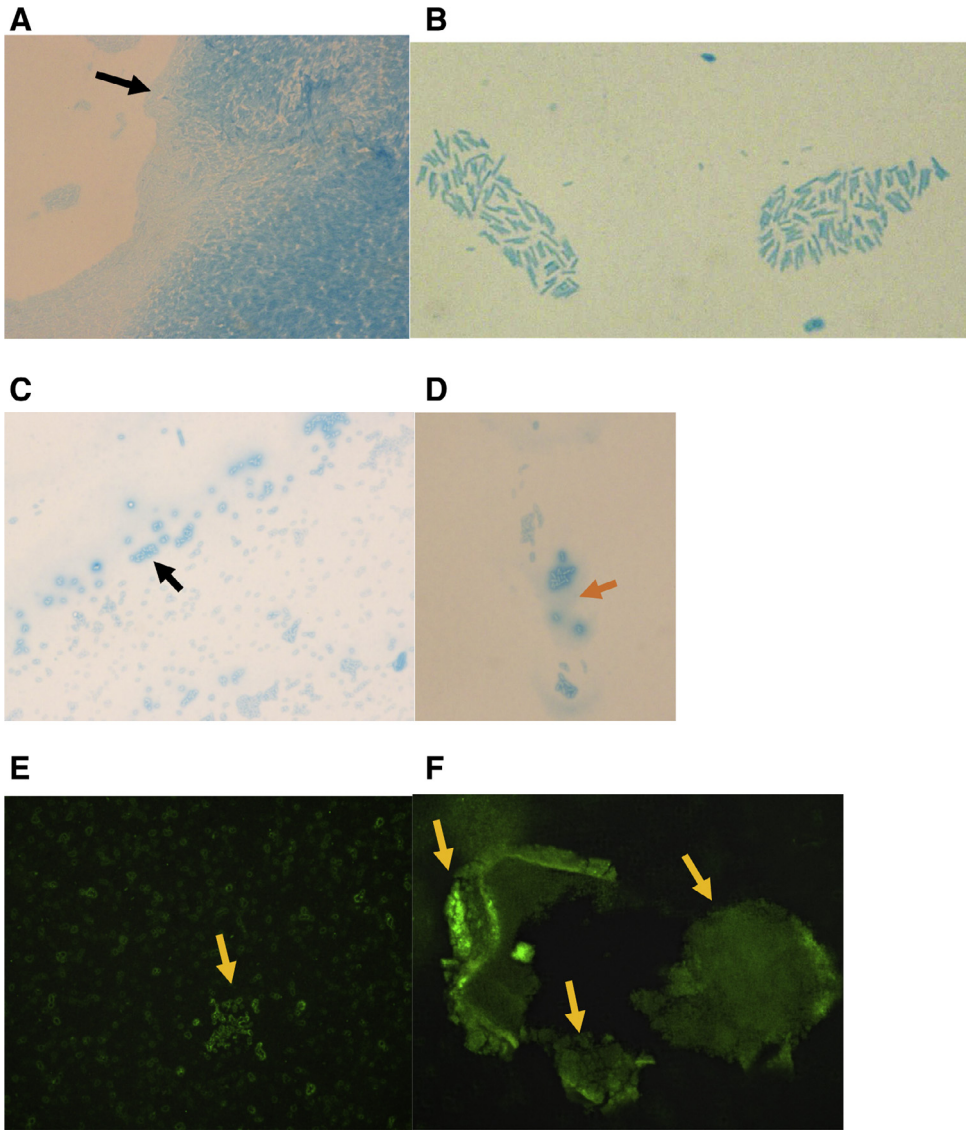


Fig. 8. Growth of *Pseudomonas aeruginosa* PAO1 (inoculum 10^7 CFU/mL) seeded on the agar surface with a tobramycin Neo-Sensitabs® tablet and incubated at 37°C for 24 h. The surface across the inhibition zone was blotted with microscope slides, was fixed with methanol and was stained with methylene blue. 1000 × magnification. (A–D) Polysaccharide production visualised 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) Confocal laser scanning microscopy of bacterial growth outside the inhibition zone, with *P. aeruginosa* PSL polysaccharide visualised by FITC-HHA lectin staining. Orange arrows, green fluorescence-stained PSL. FITC-HHA lectin, fluorescein isothiocyanate-conjugated *Hippeastrum* hybrid lectin (Amaryllis).

After 7 h (the final inhibition zone), when the bacterial aggregates reach ca. 256 cells, then they remain tolerant against 1.8–5.3 μg/mL tobramycin (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 a filamentous shape, were separated by a self-produced matrix, which is characteristic of biofilm growth (Figs 5B, 7C,E).

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

Fig. 8A shows that the matrix produced by bacterial growth at the border and especially outside the border of the inhibition zone contains polysaccharide that is visualised by the Alcian blue stain. It is seen in Fig. 8C,D 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 polysaccharide is identified as PSL (Fig. 8E,F). Fig. 8B shows two polysaccharide-containing bacterial aggregates that were located in the inhibition zone close to its border and that had stopped growing. Each of these aggregates contains ca. 64 cells, but stereomicroscopy-guided subculture from such regions inside the inhibition zone never showed growth, indicating that these small aggregates had been killed by tobramycin.

4. Discussion

Diffusion of tobramycin occurs in the water segment of the medium used for antimicrobial susceptibility testing. Generally the results are therefore independent of the medium used. Bacteria and fungi occur as individual (planktonic) cells or clustered together in aggregates (biofilms) that 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 transfer) 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 antimicrobial susceptibility by the agar diffusion method. In addition, the thicker the colony, the longer the diffusion time, which scales as the square of the diffusion distance [21]. Despite the much higher tolerance to antibiotics of biofilms measured by MBEC (analogous to the planktonic minimum bactericidal concentration), the PK/PD properties of antibiotics follow the same rules for planktonic and biofilm-growing bacteria [13,22,23].

Generally, in vitro formation of bacterial biofilms on a surface follows five steps: (i) reversible adhesion; (ii) irreversible adhesion; (iii) bacteria divide to form aggregates and extracellular matrix is produced, forming a young biofilm; (iv) the biofilm maturates and becomes structured; and (v) dispersal of single cells and sludging off of biofilm aggregates [24]. The current results from the tobramycin agar diffusion assay agree with the first four steps. Planktonic bacteria are seeded and start to grow and form aggregates, and when the aggregates become greater than ca. 64 cells after 5 h 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 with previously published results [25] showing in a surface-attached bacterial colony model that the bacterial cells are entering tolerant, stationary growth after 6–7 h. 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. [11]. The four steps of biofilm formation also correlate with the gradual increasing MBEC.

Calculation of the tobramycin concentration 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 microtitre plates as also reported by Abbanat et al. [20].

Formation of the biofilm matrix (steps 3–4) was also shown to occur in the experiments in the current study. 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 the 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 that are stained by Alcian blue are, e.g., lipopolysaccharide as well as 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 growth to biofilm mode of growth for the formation of the inhibition zone. Conventional interpretation of the diameter of the inhibition zone is that the susceptible category based on clinical breakpoints [8] indicates that it is *probably* successful to treat and eradicate infections caused by planktonically-growing bacteria. However, the biofilm mode of growth at the border of the inhibition zone and the corresponding tobramycin concentration in the agar offers a 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 regimen is used. This interpretation underlines that the conventional interpretation of agar diffusion, like microtitre dilution assays, does not provide key information to clinicians regarding the treatment of biofilm infections [17]. In contrast to the microtitre dilution assay, the agar diffusion assay gives visual information about the increased antimicrobial tolerance of the biofilm mode of growth. The Calgary Biofilm Device, which was used for determination of 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, formation of the inhibition zone 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 clinically 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 occurs, clinicians should suspect that the reason may be that biofilm-growing bacteria are causing the infection.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.12.015.

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