ORIGINAL PAPER

Temperature, pH and Trimethoprim-Sulfamethoxazole Are Potent Inhibitors of Biofilm Formation by *Stenotrophomonas maltophilia* Clinical Isolates

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Abstract

Stenotrophomonas maltophilia, an opportunistic pathogen usually connected with healthcare-associated infections, is an environmental bacterium. Intrinsic resistance to multiple antibiotics, with different virulence determinants in the last decade classified this bacterium in the group of global multiple drug resistant (MDR) organism. *S. maltophilia* clinical isolates, were collected from tertiary care pediatric hospital in Belgrade, Serbia to investigate influence of different factors on biofilm formation, kinetics of biofilm formation for strong biofilm producers and effect of trimethoprim-sulfamethoxazole (TMP/SMX) on formed biofilm. Most of the isolates (89.8%) were able to form a biofilm. Analysis of biofilm formation in different growth conditions showed that changing of temeperature and pH had the stronggest effect on biofilm formation almost equally in group of cystic fibrosis (CF) and non-CF strains. TMP/SMX in concentration of 50 μg/ml reduced completely 24 h old biofilms while concentration of 25 μg/ml effects formed biofilms in a strain dependent manner. Among strains able to form strong biofilm CF isolates formed biofilm slower than non-CF isolates, while shaking conditions did not affect biofilm formation. Swimming motility was detected in both CF and non-CF isolates, however more motile strain formed stronger biofilms. This study suggests that temperature, pH and TMP/SMX had the strongest influence on biofilm formation in analyzed collection of *S. maltophilia*. A positive correlation between motility and strength of formed biofilm was demonstrated.

K e y   w o r d s: *Stenotrophomonas maltophilia,* biofilm, cystic fibrosis, opportunistic pathogen, trimethoprim-sulfamethoxazole

Introduction

Biofilm as a multicellular aggregation of bacterial cells is one mode of their organization, which provides several benefits for its members. The most important are survival in the unfriendly environment due to increased resistance to antibiotics and other antimicrobial agents (Balcázar *et al.*, 2015). During biofilm formation, bacterial cells produce extracellular substances, which form a matrix as a single dynamic structure with different mutually interacting components (Payne and Boles, 2015). Although this extracellular matrix separates cells from their surroundings, bacterial cells could move freely from biofilm to planktonic form, which enables them to colonize surrounding biotic and abiotic surfaces. *Stenotrophomonas maltophilia* is a Gram-negative, multi-drug-resistant (MDR) opportunistic pathogen, which is usually connected with healthcare-associated infections (Brooke, 2012). Strains are usually isolated from water, soil, animals and plants and able to colonize different moist surfaces at homes and hospitals as well as surfaces of respiratory and urinary tracts. In addition, this pathogen possesses intrinsic and acquired resistance to various antibiotics (Sánchez, 2015). Recently it was classified in the emerging global opportunistic pathogens group (Brooke, 2014). The ability of *S. maltophilia* to form biofilm on different surfaces as well as its role in cystic fibrosis (CF) patients was analyzed previously (Pompilio *et al.*, 2008; Pompilio *et al.*, 2011). However, the real role of *S. maltophilia* in CF disease is still not clarified, although the latest data suggest that it comes to interspecies interactions between *S. maltophilia* and *Pseudomonas aeruginosa* as the main cause of CF infection (Sanders *et al.*, 2010; Pompilio *et al.*, 2015). In addition, the presence of *S. maltophilia* in the respiratory tract of CF patients forms specific conditions

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for *P. aeruginosa* chronic infection and/or increased virulence in mixed biofilm (Pompilio *et al.*, 2015).

In the last few years, *S. maltophilia* was frequently isolated at the Institute for Mother and Child Health Care "Dr Vukan Čupić", a University pediatric tertiary care hospital in Belgrade, Serbia mostly from the respiratory tracts of CF and non-cystic fibrosis (non-CF) patients. We investigated the ability of these strains to form biofilm under different growth conditions, kinetics of biofilm formation for strong biofilm producers and influence of trimethoprim-sulfamethoxazole (TMP/SMX) on formed biofilm of *S. maltophilia* isolates. The differences between CF and non-CF isolates are also assessed.

Experimental

Materials and Methods

Bacterial strains and media used. *S. maltophilia* clinical isolates were collected at The Institute for Mother and Child Health Care, a pediatric tertiary care hospital in Belgrade, Serbia. In total, 88 isolates were preliminarily identified using standard biochemical tests and automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France), and confirmed with molecular identification by sequencing PCR products for 16S rRNA gene. Isolates were grown on Luria Bertani (LB) overnight at 37°C with aeration. All isolates were stored in LB with 15% glycerol at −80°C. *P. aeruginosa* PAO1 and *Escherichia coli* DH5α were also grown in LB media. For testing biofilm formation assay Trypticase soy broth (TSB) was used.

Biofilm formation and motility assay. Biofilm formation assay was performed as described previously (Stepanović *et al.*, 2007) with the slight modification described previously (Madi *et al.*, 2016). Biofilm samples were treated with 200 µl of a solution containing 96% ethanol and acetone in ration 4:1 for 15 min and the optical density (OD) was read at 595 nm with Microplate Reader (Tecan, Italy). The low cut-off (ODc) was calculated as the three standard deviations $3 \times SD$ above the mean OD of control wells. Strains were classified according to the following criteria: no biofilm producer ($OD \leq ODc$), weak biofilm producer ($ODc < OD \leq 2 \times ODc$), moderate biofilm producer ($2 \times ODc < OD \leq 4 \times ODc$) and strong biofilm producer ($4 \times ODc < OD$). Motility was analyzed as described previously (Madi *et al.*, 2016).

Influence of different factors on biofilm formation by *S. maltophilia*. Strains, which formed strong (7), moderate (36) and weak (5) biofilm were chosen for further analyses. Briefly, overnight cultures of *S. maltophilia*, prepared in TSB broth were washed twice,

diluted with fresh TSB, and standardized to the density of 0.5 McFarland. Aliquots (200 µL) of standardized inoculums were added to the wells of sterile flat-bottom polystyrene 96 wells plates, incubation was performed in different conditions (pH 5.5 and 8.5), temperature (12, 30, 37, and 45°C), 10% saturation with CO_2 (Heracell 150, Thermo Fischer Scientific Inc., Walthman, MA, USA) biofilm formation was evaluated as described above in Biofilm formation assay paragraph. All experiments were performed in three independent repetitions.

Kinetics of biofilm formation. For seven strong biofilm producer strains kinetics of biofilm formation was determined. Aliquots (200 µl) of standardized inoculums were added to the wells of sterile flat-bottom polystyrene 96 wells plates and incubated at 37°C for 30 min, 1, 2, 4, 8, and 24 h. In addition, biofilm formation under dynamic conditions (shaking) was analyzed. Plates were rinsed, fixed and dyed as previously described, and the category of formed biofilm was calculated. Each assay was repeated three times.

The effect of trimethoprim-sulfamethoxazole on *S. maltophilia* formed biofilm. Biofilm formation by *S. maltophilia* was carried out as described above (Biofilm formation assay). After 24 h incubation at 37°C, the supernatant from each well was gently aspirated by micropipette; each well was then washed three times with 200 µl of 1X PBS and 200 µl of trimethoprim-sulfamethoxazole at two different concentration (25 and 50 µg/ml) were added to the wells. Controls were the formed biofilm by the strains without adding the antibiotic. Plates were incubated at 37°C for 6 h and biofilm was analyzed as described before. All experiments were performed in independent manner and repeated three times.

Statistical analysis. The statistical analysis and graph drawing were performed in R version 3.3.1. Heatmaps and cluster analysis was performed using R packages gtools, hclust and gplots. Statistical differences between the groups were assessed using t test.

Results and Discussion

The prevalence of *S. maltophilia* has increased in hospitals worldwide simultaneously with the appearance of a myriad of antibiotic resistant bacteria (Brooke, 2012, 2014). One of the usually present virulence factors in pathogenic bacteria and opportunistic pathogens is ability to form biofilm. Therefore, biofilm-associated infections substantially affect human health, increasing antibiotic resistance of bacteria and making it more challenging to combat such infections (Balcázar *et al.*, 2015). However, biofilm formation is influenced by different factors (Pompilio *et al.*, 2008; Di Bonaventura *et al.*, 2007).
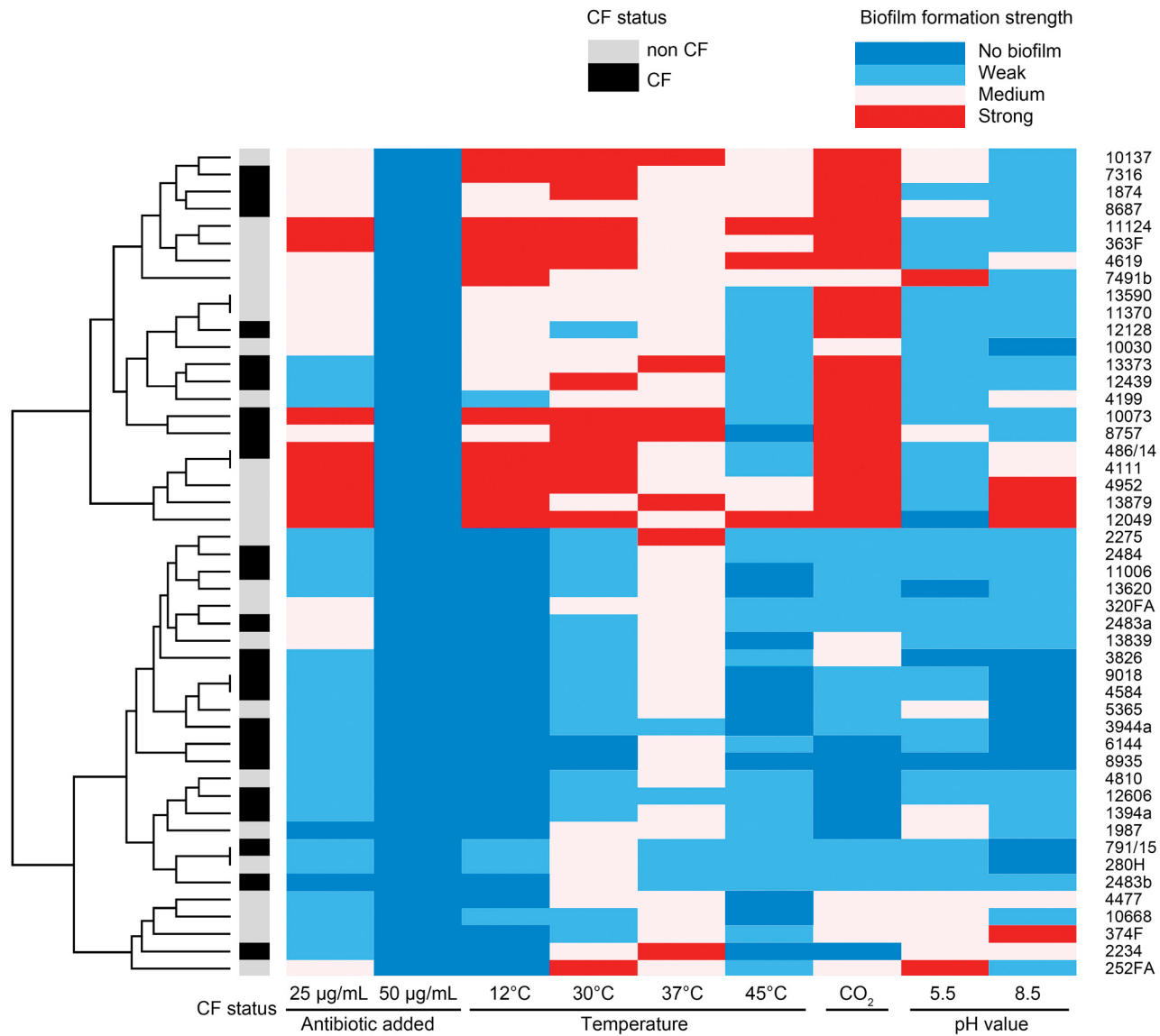
Fig. 1. Heatmap for biofilm formation under different growth conditions of *Stenotrophomonas maltophilia* clinical isolates. Conditions were grouped by antibiotic addition (first two), various temperature growth conditions, increased CO_2 and various environmental pH values.

Influence of different factors on biofilm formation by *S. maltophilia*. Previous results showed that among 88 *S. maltophilia* clinical isolates most of the strains were able to form a biofilm, both CF and non-CF isolates. Strong biofilm producers represented 7.95% and only nine strains (10.2%) did not form biofilm. For further analysis, we chose 48 strains (all strong and moderate biofilm producer as well as five weak biofilm producer). Results obtained for the influence of different factors on biofilm formation by selected *S. maltophilia* strains were present as a heatmap (Fig. 1). Additionally, hierarchical clustering was performed in order to acess the differences among the isolates abilities to form biofilm. Although isolate clusters slightly overlapped, all isolates are divided in four differentiated clusters in agreement with hierarchical clustering analysis. Clusters represent groups of isolates for which similar results in testing dif-

ferent factors on biofilm formation are obtained. Interestingly, both groups are present in the CF (black) and non-CF (gray) isolates suggesting that origin of strain did not influence the obtained results. Biofilm formation was the most affected with decrease or increase of temperature (12°C and 45°C) and changing pH to 8.5. The importance of the optimal temperature for biofilm formation was shown for the weak biofilm producer also (2483b, 791/15 and 280H), which formed a moderate biofilm at 30°C (Fig. 1). The temperature was showed to be the most relevant factor in biofilm formation by different strains not only in *S. maltophilia* but also in other bacterial species (Di Bonaventura *et al.*, 2007; Di Bonaventura *et al.*, 2008; The *et al.*, 2016). In addition, CF isolates were more sensitive to changes of temperature, pH and CO_2 concentration. Overproduction of thick and sticky mucus in patients with cystic

fibrosis forms a specific environment that certainly indirectly influences the characteristics of the bacteria that colonize it (Cantón and del Campo, 2010). This could be the reason for the slight difference in the char-

acteristics of CF *vs.* non-CF isolates, because of adaptation to specific environmental conditions.

Effect of trimethoprim-sulfamethoxazole on *S. maltophilia* formed biofilm. *S. maltophilia* is intrinsically
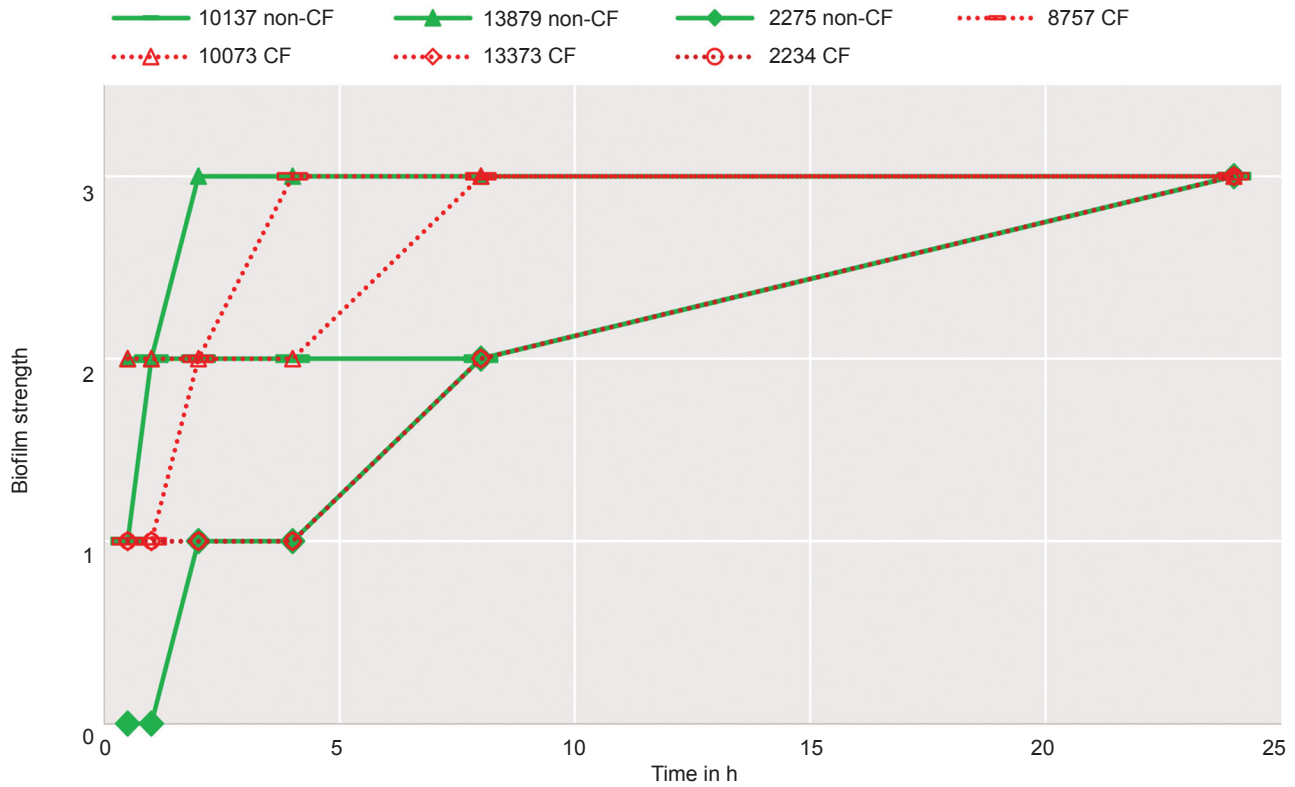

Fig. 2. Kinetics of biofilm formation by selected *Stenotrophomonas maltophilia* strains isolated from CF and non-CF patients. Biofilm strength was designated from 0 to 3 where 3 is strong, 2 is moderate, 1 is weak, and 0 is no biofilm.
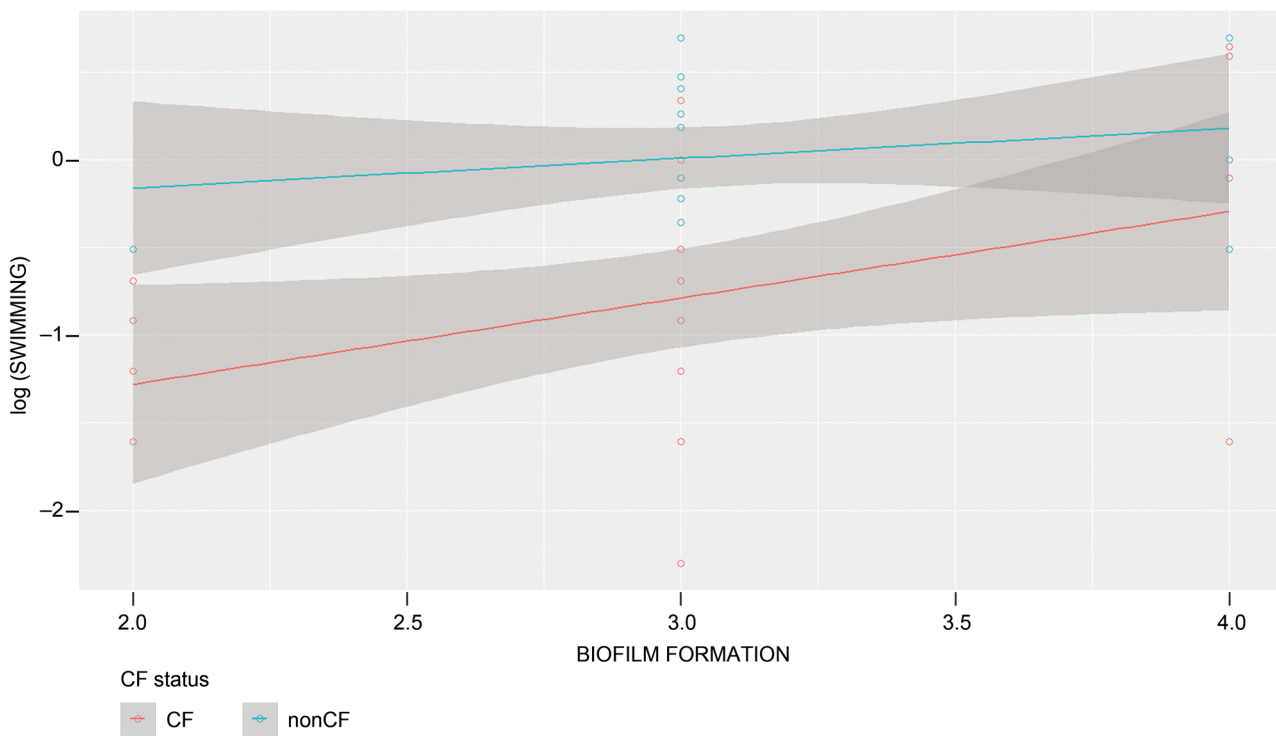

Fig. 3. Correlation between the strength of formed biofilm and motility of *S. maltophilia* strains isolated from CF and non-CF patients.

resistant to various antibiotics and so far, trimethoprim-sulfamethoxazole (TMP/MX) is recommended for the treatment of this bacterium (Abbott *et al.*, 2011). Although recently the number of reports of *S. maltophilia* resistance to TMP/SMX is increased (Hu *et al.*, 2016) we showed that in analyzed collection all isolates in their planctonic form were sensitive to it (Madi *et al.*, 2016). Once a biofilm has been formed, the bacterial cells become extremely robust against different antimicrobial agents. In this study, we further investigated the effects of TMP/SMX in two concentrations (25 µg/ml and 50 µg/ml) on biofilms formed by *S. maltophilia*. Both TMP/SMX concentrations were found to significantly contribute to the eradication of 24 h old biofilms (Fig. 1). However, cultivation of 24 h old biofilm with 50 µg/ml of TMP/SMX completely eradicated the formed biofilm in all tested strains. Lower applied concentration, 25 µg/ml of TMP/SMX affects the biofilm in a strain-dependent manner, from complete eradication to no, effect. Interestingly, strains more sensitive to other tested factors showed the higher sensitivity on 25 µg/ml of TMP/SMX.

Kinetics of biofilm formation. For selected strains, we determined the kinetics of biofilm formation (Fig. 2) which showed that non-CF isolates formed a biofilm faster than CF isolates. Although, they were all strong biofilm producers biofilm formation dynamic was significantly different between them correlating with the CF *vs.* non-CF phenotype. This might be, at least to some extent, attributed to the higher motility of non-CF isolates, which was shown previously (Madi *et al.*, 2016). In addition shaking conditions did not affect biofilm formation both by CF and non-CF isolates (data not shown).

Correlation between motility and strength of formed biofilm. All tested isolates showed swimming motility. We observed the same trend in changing motility and strength of formed biofilm (Fig. 3). Strains forming stronger biofilm showed higher motility with no statistically important differences in motility between CF and non-CF isolates (p = 0.78). Although, one study suggested that motility was important for biofilm formation in CF isolates (Pompilio *et al.*, 2015) in another lower motility in CF pathogens was described (Madi *et al.*, 2016). Thus, opposite results point to the complexity of the process of biofilm formation especially in the specific environment such as lung of CF patients.

Conclusion

The effect of analyzed factors on biofilm formation by *S. maltophilia* clinical isolates from CF and non-CF patents point out that there is no CF phenotype but we determined differences between these two groups of isolate. Complexity of this important virulence factor involves mutual influences of strains characteristics and environmental conditions. However, we could conclude that for the factors tested in this study temperature and pH had the strongest effect on strength of formed biofilm. A correlation between motility and biofilm formation was confirmed, a more motile strain formed stronger biofilm. In addition, TMP/SMX could easily eradicate the biofilm formed by the *S. matophila* clinical isolates tested in this study. Nevertheless, additional experiments are needed to completely evaluate mechanism of action of each factor on biofilm formation by this important opportunistic pathogen.

Conflict of interests

The authors declare no conflict of interest.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Ethics Committee of The Institute for Mother and Child Health Care (approval no. 8/6a) on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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