Enfermedades Infecciosas y Microbiología Clínica

Original article

Importance of antibiotic penetration in the antimicrobial resistance of biofilm formed by non-pigmented rapidly growing mycobacteria against amikacin, ciprofloxacin and clarithromycin

Alberto Ortíz-Pérez, Nieves Martín-de-Hijas, Noelia Alonso-Rodríguez, Diana Molina-Manso, Ricardo Fernández-Roblas, Jaime Esteban*

Department of Clinical Microbiology, Fundación Jiménez Díaz, Madrid, Spain

A R T I C L E   I N F O

A B S T R A C T

Objectives: To study the resistance of biofilms developed by non-pigmented rapidly growing mycobacteria (NPRGM) against amikacin, ciprofloxacin and clarithromycin in an *in vitro* model using clinical strains of different species.

Design: Antimicrobial susceptibilities of different clinical strains of *Mycobacterium abscessus, Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium peregrinum, Mycobacterium mucogenicum and Mycobacterium mageritense* using conventional techniques were measured. Biofilm resistance was measured by using the sandwich technique developed by Anderl et al. using a concentration of antibiotic of 50 mg/L. Penetration of antibiotics through biofilm was measured using the same technique with minimal modifications.

Results: NPRGM biofilms showed drug resistance (percentages of viable bacteria >1% of those of controls) against antibiotics that are commonly used for the treatment of infections caused by these organisms, although there are intraspecies differences between strains. We have detected differences in antibiotic penetration through biofilms with an important permeability barrier for ciprofloxacin. However, other mechanisms must be probably more important to explain the antimicrobial resistance of NPRGM biofilm.

Conclusions: Biofilms formed by NPRGM are resistant to amikacin, ciprofloxacin and clarithromycin. As no resistance differences between the tested antibiotics have been observed, it is likely that biofilm permeability of antibiotics is of low importance for antimicrobial resistance of biofilms.

© 2010 Elsevier España, S.L. All rights reserved.

Importancia de la penetración del antibiótico en a resistencia de biopelículas formadas por micobacterias no pigmentadas de crecimiento rápido frente a amicacina, ciprofloxacino y claritromicina

R E S U M E N

Objetivos: Estudiar la resistencia de biopelículas formadas por micobacterias no pigmentadas de crecimiento rápido (MNPCR) frente amicacina, ciprofloxacino y claritromicina en in modelo *in vitro* empleando aislamientos clínicos de diferentes especies.

Material y Métodos: Se estudiaron las sensibilidades de las diferentes cepas clínicas de *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium mucogenicum* y *Mycobacterium mageritense* mediante técnicas convencionales. La resistencia de dichas bacterias en la biopelícula fue estudiada mediante la técnica de sándwich descrita por Anderl et al. utilizando una concentración de antibiótico de 50 mg/L. La penetración de los antibióticos a través de la biopelícula fue estudiada mediante la misma técnica con mínimas modificaciones.

* Corresponding author.
  E-mail address: jestebanmoreno@gmail.com (J. Esteban).

Resultados: Las biopelículas de MNPCR presentaron resistencia (porcentajes de bacterias viables > 1% de los recuentos obtenidos en los controles) frente a todos los antibióticos que son empleados habitualmente en las infecciones causadas por estos organismos, si bien se detectaron diferencias dentro de la misma especie entre las diferentes cepas. Hemos detectado diferencias en la penetración de antibióticos a través de la biopelícula, especialmente con una importante disminución de la permeabilidad frente a ciprofloxacino. Sin embargo, otros mecanismos son, probablemente, más importantes para explicar la resistencia antimicrobiana de las biopelículas de MNPCR.

Conclusiones: Las biopelículas formadas por MNPCR son resistentes frente a amicacina, ciprofloxacino y claritromicina. Como no se demostraron diferencias importantes entre los distintos antibióticos, es probable que la permeabilidad de la biopelícula frente a los antibióticos tenga poca importancia en la resistencia antimicrobiana de las biopelículas.

Introduction

Non-pigmented rapidly growing mycobacteria (NPRGM) include different species of mycobacteria with common phenotypical characteristics[1] and some of them,, such as *Mycobacterium abscessus*, *Mycobacterium fortuitum* or *Mycobacterium chelonae*, have been known to be human pathogens for a long time.All the species involved in this group are environmental organisms that became human pathogens under special circumstances or risk factors.[2] Different studies have recovered NPRGM in biofilms associated with water systems,[3] and a recent study has shown that most human NPRGM infections are biofilm-related.[4] These infections include a broad spectrum of clinical syndromes, such as catheter-related bloodstream infections,[5] surgical site infections,[6] prosthetic joint-related infections,[7–14] respiratory tract infections in cystic fibrosis patients[12–14] and others.[1,2,4] *In vitro* studies have also demonstrated biofilm formation by almost all NPRGM, both in clinical and collection isolates, and a relationship between biofilm development ability and the production of human clinical disease.[15,16]

Biofilm development is an important factor in antimicrobial resistance. In this sense, different studies have shown the *in vitro* resistance of biofilms formed by NPRGM against disinfectants[17] or antibiotics,[18] including amikacin and clarithromycin resistance of *M. abscessus* embedded in biofilm.[19]

However, despite these observations, only a few reports have tried to determine the mechanisms of antimicrobial resistance among clinically relevant species of NPRGM. In this study, we have evaluated the permeability of different antimicrobials to cross over the biofilms developed by 6 different NPRGM species, as one of the factors that could be involved in the increased drug resistance of the biofilm.

Material and methods

Strains

Twenty biofilm-producing NPRGM clinical strains belonging to six different species (2 *M. abscessus*, 2 *M. chelonae*, 4 *M. fortuitum*, 3 *M. peregrinum*, 1 *M. mucogenicum* and 2 *M. mageritense*) were analyzed. These species were selected because they are the ones most commonly isolated NPRGM in human samples. These strains were identified by using a broad battery of biochemical tests (nitrate reduction, urease, 3-day arylsulphatase, Tween 80 hydrolysis, growth on McConkey agar without crystal violet, and use of citrate, mannitol, inositol, sorbitol and rhamnose) and PCR-restriction enzyme analysis (PRA) of hsp65 gene.[20] These strains were randomly selected from a collection of previously described biofilm-forming strains.[16] *M. fortuitum* ATCC 6841[T], *M. peregrinum* ATCC 14467[T], *M. abscessus* DSM 44196[T], *M. chelonae* ATCC 35752[T], *M. mucogenicum* DSM 44124, and *M. mageritense* ATCC 700351[T] were included in the studies as controls.

Antimicrobial susceptibility

The strains were tested for amikacin (SIGMA, St. Louis, Missouri, USA), clarithromycin (Abbott Park, Illinois, USA), and ciprofloxacin (SIGMA, St. Louis, Missouri, USA) susceptibility using the microdilution reference technique as previously described.[21] These antibiotics were selected because they are the ones most commonly used to treat NPRGM infections.

Antimicrobial susceptibility of biofilms

We obtain mature biofilms on sterile polycarbonate disks (ALBET-Hahnemuehle, Dassel, Germany) as described previously,[22] except that we used Tryptic soy-5% sheep blood agar plates instead of Müller-Hinton agar plates. After 5 days of incubation at 37 °C, disks were removed using sterile forceps and placed onto Müller-Hinton agar plates containing 50 μg/ml of amikacin, clarithromycin, and ciprofloxacin, respectively. After 24 hours at room temperature, disks were removed and introduced into 5 ml of sterile distilled water. We then dislodged the biofilm by vortexing for 1 minute and sonication (Ultrasons-H 3000840; J.P Selecta, Abrera, Spain) for 5 minutes. The number of colony forming units (CFU) of viable bacteria was quantified by streaking serial dilutions from the sonicate on Tryptic soy-5% sheep blood agar plates and incubating them for 5 days at 37 °C. All the strains were tested in triplicate.

Antibiotic permeability assay

We used the method described by Anderl et al.[22] with modifications. A 0.5 McFarland suspension of each strain was prepared in sterile PBS using glass beads and vortexing. Ten μl of each suspension were inoculated onto a sterile polycarbonate disk (ALBET-Hahnemuehle, Dassel, Germany), previously placed on Muller-Hinton agar plates (bioMérieux, Marcy-l'Etoile, France) and were incubated at 37 °C for 7 days. After this period, another polycarbonate disk was placed onto the developed biofilm and a 6-mm diameter sterile paper disk with a previously known amount of antibiotic (15 μg for clarithromycin and ciprofloxacin, and 30 μg for amikacin) was moistened with 15 μl of Muller-Hinton broth (bioMérieux, Marcy-l'Etoile, France) and placed on top of the membrane-biofilm-membrane "sandwich". The upper disks were removed after 3, 6, 24 and 48 hours at room temperature and the remaining antibiotic concentration was quantified by a disk-plate assay on Müller-Hinton agar plates (bioMérieux, Marcy l'Etoile, France) using *Kocuria rizophyla* ATCC 9341. Previously, we established a relationship between the inhibition diameter of *K. rhizophila* ATCC 9341 and different concentrations of antibiotics. The measurements of the inhibition diameter were taken with a digital calliper after 24 hour incubation at 37 °C in normal atmosphere.

We used identical "sandwich" structures of polycarbonate disks without any biofilm as positive controls for antimicrobial permeability, and a Parafilm© membrane instead the biofilm as a control

Table 1
Results obtained with the different strains tested.

Species	Number of the strain	MIC			% of viable bacteria in biofilm after antibiotic exposure		
		CLA	CIP	AK	CLA	CIP	AK
M. abscessus	T	1	2	8	68.42	66.67	40.35
M. abscessus	193	≤0.03	≤0.03	0.25	100.00	66.67	66.67
M. abscessus	25	≤0.03	8	0.5	72.72	81.74	48.89
M. chelonae	4	2	0.25	0.06	68.41	75.95	39.31
M. chelonae	T	0.06	0.06	16	100.00	92.00	75.08
M. chelonae	52	0.06	4	>64	100.00	100.00	100.00
M. fortuitum	22	0.12	0.12	0.5	16.67	0.33	33.65
M. fortuitum	T	2	≤0.03	1	72.22	41.67	66.67
M. fortuitum	206	0.12	0.25	1	68.63	68.63	70.59
M. fortuitum	207	0.25	≤0.03	2	21.02	62.92	27.67
M. fortuitum	75	0.12	≤0.03	0.5	100.00	96.67	100.00
M. mageritense	T	16	0.12	0.5	66.67	48.85	49.69
M. mageritense	45	>64	0.12	>64	100.00	77.78	100.00
M. mageritense	69	2	0.25	1	40.85	42.48	47.08
M. mucogenicum	T	0.25	1	2	100.00	100.00	100.00
M. mucogenicum	18	≤0.03	0.12	1	30.19	21.83	14.20
M. peregrinum	15	0.25	0.06	0.06	34.04	53.82	35.50
M. peregrinum	T	2	≤0.03	0.12	100.00	40.00	80.00
M. peregrinum	213	0.06	≤0.03	0.06	76.34	33.33	33.33
M. peregrinum	12	0.12	≤0.03	1	70.37	70.37	66.67

CLA: Clarithromycin; CIP: Ciprofloxacin, AK: Amikacin; T: Type strain.

for non-permeability (negative control). The negative control measurements were used to calculate the percentage of antibiotic concentration in the disk in order to avoid false results due to antibiotic degradation.

All experiments (including standard bioassays) were performed in triplicate.

Statistical analysis

To analyze the antimicrobial resistance of biofilms, we calculated the percentage of viable bacteria recovered after exposure, using the mean of controls without antibiotics as the 100% value.

We checked the normality of the series using the Bartlett test. For comparison of the mean values, ANOVA and Mann-Whitney/Wilcoxon tests were performed when indicated. All calculations were performed with the EPI-INFO 3.4.1 software (Center for Disease Control and Prevention, Atlanta, USA).

Results

The number of viable bacteria recovered from the biofilms after antimicrobial exposure ranged between 3.2×10^6 CFU/ml and 4.0×10^{14} CFU/ml (Table 1).

All antibiotic-treated biofilms showed a decrease in the percentage of viable bacteria, and no differences could be found between the different antibiotics tested. If we use the criterion for bactericidal concentration of biofilm[23] previously described, all biofilms produced would be considered resistant against all tested antibiotics. However, despite there being no overall differences observed between mycobacteria species and different antibiotics, a high intraspecies variation was observed (Table 1). No studies regarding antimicrobial susceptibility of released viable bacteria or development of antimicrobial resistance among them were performed.

The penetration curves in the biofilm for the three tested antibiotics were different (Figures 1 and 2). Amikacin was the antibiotic with the highest penetrability into biofilm, showing the smallest percentage of antibiotic in the disk at 24 hours (10.56%). Clarithromycin showed an intermediate penetration (23.96%) and Ciprofloxacin had a poor penetration in the biofilm, with a mean amount of 80.39% at 24 hours in the disk. This pattern was shared for all strains, in spite of the *in vitro* susceptibility differences of biofilms.

When we analysed the antimicrobial penetration with time of exposure, we observed a rapid penetration of amikacin and clarithromycin for all species, except for *M. abscessus* and *M. mageritense*. For clarithromycin, while the majority of species showed percentages of antibiotic remaining after 6 hours of exposure between 26.2-31.8%, *M. mucogenicum* showed 39.3%, *M. mageritense* 71.6% and *M. abscessus* 84.9%. For amikacin, *M. abscessus* showed a percentage of 67.2% of antibiotic after 6 hours of exposure, in comparison with 22.8-36.4% for the rest of the remaining species. These data suggest a more rapid penetration of antibiotic in some species, and a slower one in others. Such differences did not appear for ciprofloxacin.

Discussion

One of the reports that have evaluated the antimicrobial effect against biofilms formed by different species of mycobacteria showed an antimicrobial resistance increase of *Mycobacterium avium* biofilms against clarithromycin and rifampin, although their authors stated that these results were probably not due to permeability problems as the biofilm was extremely immature.[24] A more recent study reported the resistance of *Mycobacterium tuberculosis* biofilms against isoniazid and rifampin, suggesting that metabolic changes were probably responsible.[25] Among rapidly growing mycobacteria, some studies have shown the resistance to a broad spectrum of biocides.[17,26] A study of *Mycobacterium smegmatis* biofilm reported its isoniazid resistance and ruled out the slower biofilm growth as cause, with the authors suggesting that permeability features and/or other unknown mechanisms were responsible.[18] Another report showed that *M. abscessus* biofilms were resistant to cefoxitin, amikacin and clarithromycin, the nutrient limitations and a latent metabolic state being proposed as responsible.[19] Recent studies have shown that extracellular matrix in mycobacterial biofilm is formed mainly by lipids, including mycolic acids, but the actual role of these components in antimicrobial penetration is unknown.[27,28]

Our study is the first to evaluate biofilm resistance for different clinical species of mycobacteria. Of interest, despite the lack of differences observed in the percentages of viable bacteria obtained after exposure to antibiotics, important variations in the individual susceptibility of different strains biofilms were reported, as well as the individual variations previously observed for adherence,[29] the
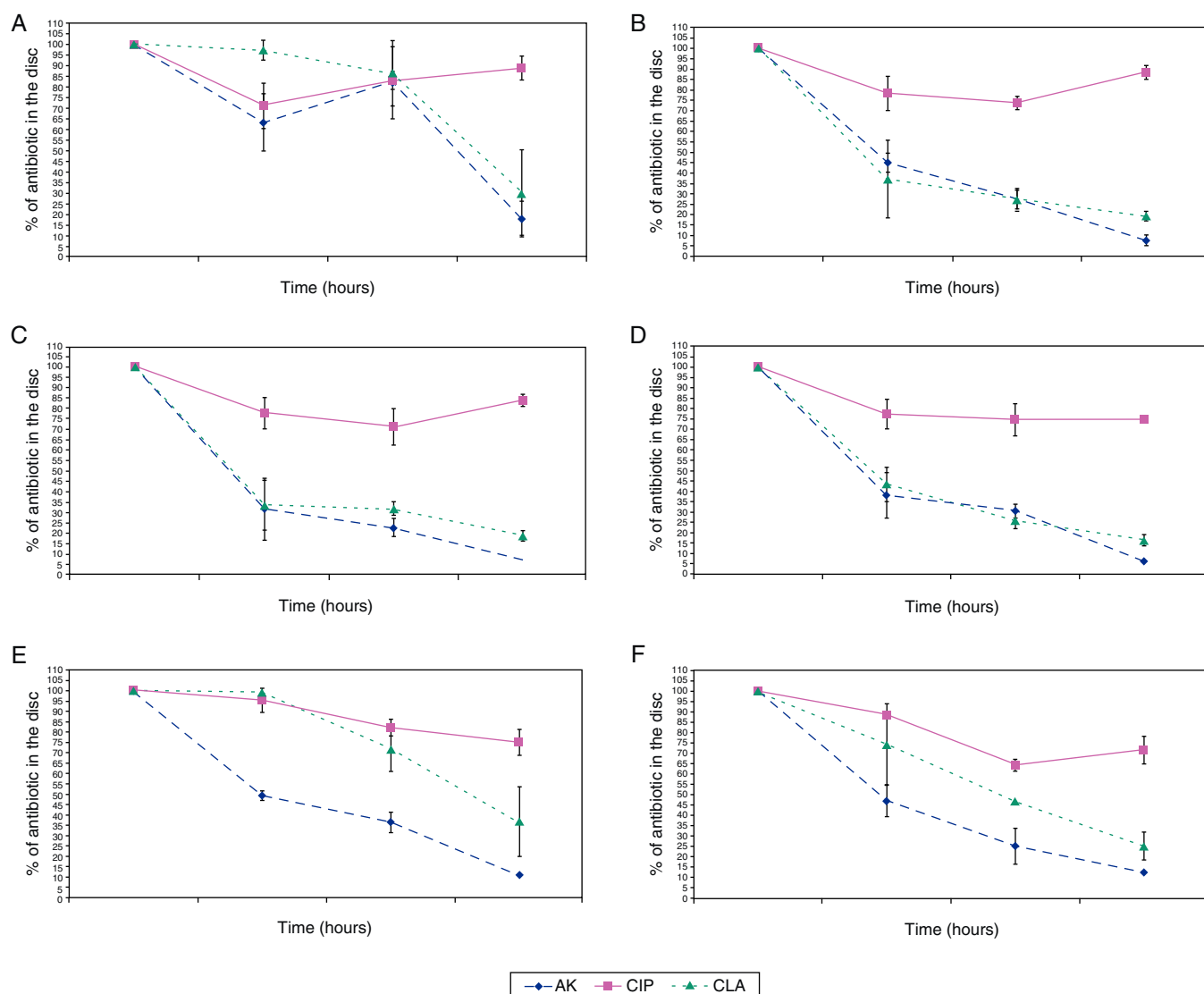
Figure 1. Penetration of antibiotics in the different species. Percentage of antibiotic that remains in the disk after 0, 3, 6, and 24 hours. 1A: *M. abscessus*, 1B: *M. chelonae*, 1C: *M. fortuitum*, 1D: *M. peregrinum*, 1E: *M. mageritense*, 1F: *M. mucogenicum*.
Footnote: AK: Amikacin, CIP: Ciprofloxacin, CLA: Clarithromycin.

first crucial step for biofilm development. Due to these observation, we studied one of the mechanisms claimed as important in biofilm resistance, the antibiotic penetration through biofilm.

Until now, no studies have analyzed the antimicrobial permeability for biofilms of different species of mycobacteria. In our analysis, amikacin showed better penetration than clarithromycin, but no statistically significant differences could be found between the activities of both antibiotics. However, a different pattern of penetration could be observed for ciprofloxacin, showing the minimal penetration through biofilm for all strains. These results could indicate, that despite of differences in antibiotic penetration, this phenomenon could have a minimal role (if any) in antibiotic resistance of NPRGM biofilms, because mature biofilms of all strains analyzed are resistant to tested antibiotics independently of the antibiotic penetration pattern.

These results are different to those obtained in other studies, where ciprofloxacin seemed to penetrate the biofilm formed by *Pseudomonas aeruginosa*,[30] *Klebsiella pneumoniae*[22] or *Staphylococcus epidermidis*[31] better than other antibiotics. In the study performed by Walters et al.[30] the penetration of ciprofloxacin through a *P. aeruginosa* biofilm was very much higher than

tobramycin. In our study, the comparison of ciprofloxacin and amikacin (the aminoglycoside used in the treatment of NPRGM infections) showed that the penetration of ciprofloxacin is very much lower than amikacin. Differences in the composition of the extracellular matrix could be the reason for these differences, as the matrix mainly consists of different polysaccharides in *P. aeruginosa*[32], while recent studies have showed that mycobacteria produce a lipid-rich matrix with a high content of mycolic acids[25] and glycopeptidolipids.[33]

In contrast to others studies, we have analyzed not only collection strains, but also clinical isolates. Genetic differences between wild strains and laboratory ones have been reported,[34] and these differences should be considered in the evaluation of antibiotic resistance of biofilms. In our study, no differences were detected in antimicrobial permeability in biofilms of different strains, although they do have different antimicrobial susceptibility (Table 1). These observations could indicate that antimicrobial permeability features are not species-dependent nor related to drug resistance.

It would be of interest to explore the effect of a combination of antibiotics on mycobacterial biofilms, especially because therapy of the infections caused by these organisms in usually
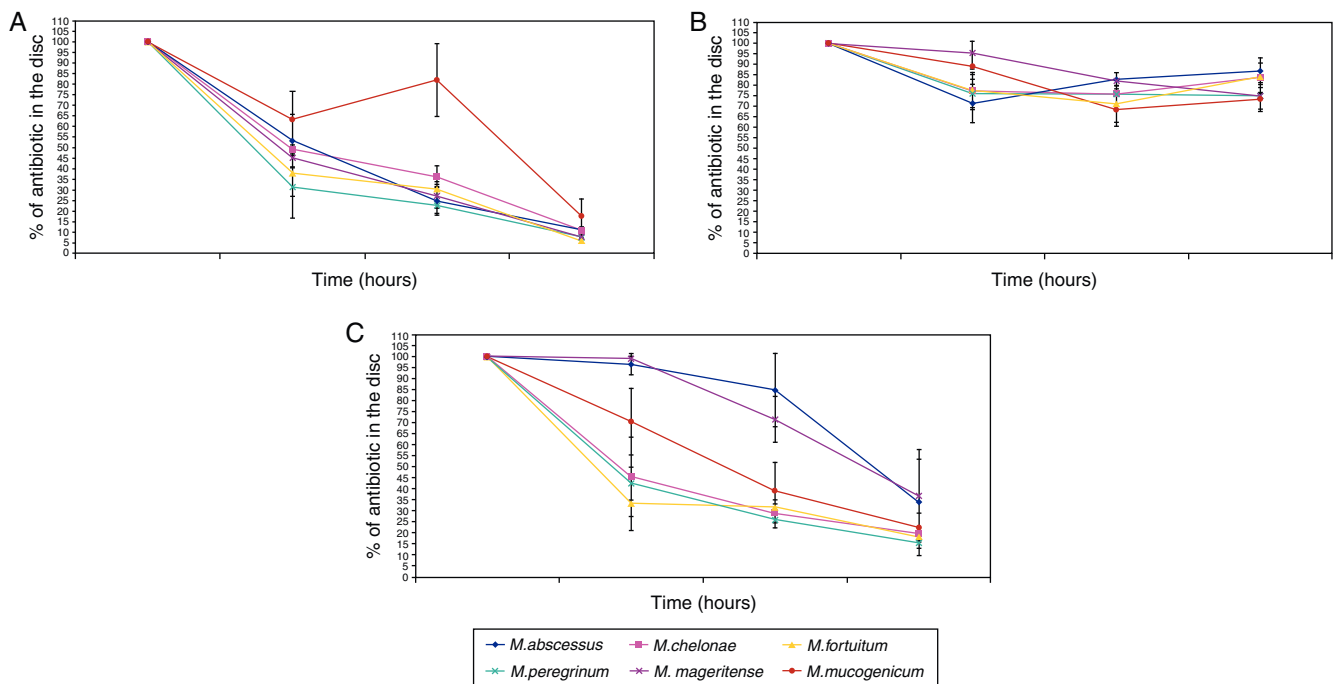
Figure 2. Penetration of antibiotics in the different species. Percentage of antibiotic that remains in the disk after 0, 3, 6, and 24 hours. 2A: Amikacin, 2B: Ciprofloxacin, 2C: Clarithromycin.

combined. Moreover, recent data suggest that macrolides have a *quorum sensing* inhibition effect in biofilms produced by *Pseudomonas aeruginosa*.[28] Further studies are necessary to evaluate this effect of macrolides in NPRGM biofilms, both alone or combined with other antimicrobials. More studies are also needed to confirm whether NPRGM biofilms are resistant to other antimicrobials.

In conclusion, NPRGM biofilms showed drug resistance against antibiotics that are commonly used for the treatment of infections caused by these organisms. We have detected differences in antibiotic penetration through biofilms with an important permeability barrier for ciprofloxacin. However, as there were no differences observed in the resistance for the tested antibiotics, it is likely that this mechanism could have a low importance for antimicrobial resistance of biofilms. Other mechanisms, such as metabolic latency, are probably more important in explaining the antimicrobial resistance of NPRGM biofilm.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

References

1. Brown-Elliott BA, Wallace Jr RJ. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev. 2002 Oct;15:716–46.

2. Howard ST, Byrd TF. The rapidly growing mycobacteria: saprophytes and parasites. Microbes Infect. 2000 Dec;2:1845–53.

3. Vaerewijck MJ, Huys G, Palomino JC, Swings J, Portaels F. Mycobacteria in drinking water distribution systems: ecology and significance for human health. FEMS Microbiol Rev. 2005 Nov;29:911–34.

4. Esteban J, Martin-de-Hijas NZ, Fernandez AI, Fernandez-Roblas R, Gadea I. Epidemiology of infections due to nonpigmented rapidly growing mycobacteria diagnosed in an urban area. Eur J Clin Microbiol Infect Dis. 2008 May 6.

5. Hawkins C, Qi C, Warren J, Stosor V. Catheter-related bloodstream infections caused by rapidly growing nontuberculous mycobacteria: a case series including rare species. Diagn Microbiol Infect Dis. 2008 Jun;61:187–91.

6. Kalita JB, Rahman H, Baruah KC. Delayed post-operative wound infections due to non-tuberculous *Mycobacterium*. Indian J Med Res. 2005 Dec;122: 535–9.

7. Eid AJ, Berbari EF, Sia IG, Wengenack NL, Osmon DR, Razonable RR. Prosthetic joint infection due to rapidly growing mycobacteria: report of 8 cases and review of the literature. Clin Infect Dis. 2007 Sep 15;45:687–94.

8. Tam WO, Yew WW, Yam WC, Yuen KY, Wong PC, Tse TF. Pacemaker infections due to rapidly growing mycobacteria: further experience. Int J Tuberc Lung Dis. 2007 Jan;11:118.

9. Rahav G, Pitlik S, Amitai Z, Lavy A, Blech M, Keller N, et al. An outbreak of *Mycobacterium jacuzzii* infection following insertion of breast implants. Clin Infect Dis. 2006 Oct 1;43:823–30.

10. Herold RC, Lotke PA, MacGregor RR. Prosthetic joint infections secondary to rapidly growing *Mycobacterium fortuitum*. Clin Orthop Relat Res. 1987 Mar:183–6.

11. Clegg HW, Foster MT, Sanders Jr WE, Baine WB. Infection due to organisms of the *Mycobacterium fortuitum* complex after augmentation mammaplasty: clinical and epidemiologic features. J Infect Dis. 1983;147:427–33.

12. Jonsson BE, Gilljam M, Lindblad A, Ridell M, Wold AE, Welinder-Olsson C. Molecular epidemiology of *Mycobacterium abscessus*, with focus on cystic fibrosis. J Clin Microbiol. 2007 May;45:1497–504.

13. Olivier KN, Weber DJ, Wallace Jr RJ, Faiz AR, Lee JH, Zhang Y, et al. Nontuberculous mycobacteria. I: multicenter prevalence study in cystic fibrosis. Am J Respir Crit Care Med. 2003 Mar 15;167:828–34.

14. Ebert DL, Olivier KN. Nontuberculous mycobacteria in cystic fibrosis. Infect Dis Clin North Am. 2002 Mar;16:221–33.

15. Esteban J, Martin-de-Hijas NZ, Kinnari TJ, Ayala G, Fernandez-Roblas R, Gadea I. Biofilm development by potentially pathogenic non-pigmented rapidly growing mycobacteria. BMC Microbiol. 2008;8:184.

16. Martín-de-Hijas NZ, García-Almeida D, Ayala G, Fernández-Roblas R, Gadea I, Celdrán A, et al. Biofilm development by clinical strains of non-pigmented rapidly growing mycobacteria. Clin Microbiol Infect. 2009;15:931–6.

17. Bardouniotis E, Huddleston W, Ceri H, Olson ME. Characterization of biofilm growth and biocide susceptibility testing of *Mycobacterium phlei* using the MBEC assay system. FEMS Microbiol Lett. 2001 Sep 25;203:263–7.

18. Teng R, Dick T. Isoniazid resistance of exponentially growing *Mycobacterium smegmatis* biofilm culture. FEMS Microbiol Lett. 2003 Oct 24;227: 171–4.

19. Greendyke R, Byrd TF. Differential antibiotic susceptibility of *Mycobacterium abscessus* variants In biofilms and macrophages compared to planktonic bacteria. Antimicrob Agents Chemother. 2008;52:2019–26.
20. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol. 1993 Feb;31:175–8.
21. NCCLS. Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes; approved standard. NCCLS document M24-A. 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA: NCCLS; 2003.
22. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother. 2000;44:1818–24.
23. Ceri H, Olson ME, Stremick C, Read RR, Morck DW, Buret A. The Calgary biofilm device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 1999;37:1771–6.
24. Falkinham 3rd JO. Growth in catheter biofilms and antibiotic resistance of Mycobacterium avium. J Med Microbiol. 2007 Feb;56(Pt 2):250–4.
25. Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel Y, et al. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol Microbiol. 2008;69:164–74.
26. Bardouniotis E, Ceri H, Olson ME. Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. Curr Microbiol. 2003 Jan;46:28–32.
27. Zambrano MM, Kolter R. Mycobacterial biofilms: a greasy way to hold it together. Cell. 2005;123:762–4.
28. Ojha A, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel Y, et al. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol Microbiol. 2008;69: 164–74.
29. Zamora N, Esteban J, Kinnari TJ, Celdran A, Granizo JJ, Zafra C. In-vitro evaluation of the adhesion to polypropylene sutures of non-pigmented, rapidly growing mycobacteria. Clin Microbiol Infect. 2007 Sep;13:902–7.
30. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother. 2003;47:317–23.
31. Yasuda H, Ajiki Y, Koga T, Yokota T. Interaction between clarithromycin and biofilms formed by *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 1994;38:138–41.
32. Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P, O'Toole GA, et al. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PA01 *Pseudomonas aeruginosa* biofilms. Proc Natl Acad Sci U S A. 2003;100:7907–12.
33. Howard ST, Rhoades E, Recht J, Pang X, Alsup A, Kolter R, et al. Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. Microbiology. 2006 Jun;152(Pt 6): 1581–90.
34. Costerton JW. The biofilm primer. 1 ed. Costerton JW, editor. Berlin: Springer-Verlag; 2007.