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Brief report

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Biofilm prevention concentration of clarithromycin against clinically relevant species of nontuberculous mycobacteria

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ABSTRACT

Introduction. *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* are a group of nontuberculous mycobacteria (NTM) that have been described as human pathogens. Their ability to develop biofilms in tissues and medical devices is one of the most important pathogenicity factors, with important implications in diagnosis and treatment. Macrolides are usually considered one of the bases of this treatment.

Methods. Here we have studied the biofilm prevention concentration (BPC) of 16 strains (n=16) with clarithromycin to avoid the biofilm development by these NTM.

Results. In this study, all *M. abscessus* strains have similar BPC, while MAC strains showed different values. For MAC the concentrations ranged between 1-16 mg/L, while for *M. abscessus* the concentration was 32 mg/L for all strains except one that was 64 mg/L.

Conclusions. These results open the possibility of using macrolides for the prevention of biofilm development in patients with a risk of developing NTM disease.

Keywords: nontuberculous mycobacteria, BPC, MIC, biofilm prevention, *Mycobacterium abscessus*, *Mycobacterium avium*, clarithromycin.

Eficacia de la claritromicina contra el biofilm de especies clínicamente relevantes de micobacterias no tuberculosas

RESUMEN

Introducción. *Mycobacterium avium* complex (MAC) y *Mycobacterium abscessus* son un grupo de micobacterias no tuberculosas (NTM) que han sido descritas como patógenos humanos. Entre los factores de patogenicidad más importantes se encuentra su capacidad para desarrollar biopelículas en tejidos y dispositivos médicos, con importantes implicaciones en el diagnóstico y tratamiento. Los macrólidos suelen considerarse una de las bases de este tratamiento.

Métodos. En este estudio hemos estudiado la concentración para la prevención de biopelículas (BPC) de 16 cepas (n=16) con claritromicina para varias de estas NTM.

Resultados. Todas las cepas de *M. abscessus* tienen BPC similares, mientras que las cepas de MAC mostraron valores diferentes. Para MAC las concentraciones presentaron un rango entre 1-16 mg/L, mientras que para *M. abscessus* la concentración fue de 32 mg/L para todas las cepas excepto una, que fue de 64 mg/L.

Conclusiones. Estos resultados abren la posibilidad de utilizar macrólidos para la prevención del desarrollo de biopelículas en pacientes con riesgo de desarrollar enfermedad por NTM.

Palabras clave: micobacterias no tuberculosas, BPC, CMI, prevención de biopelículas, *Mycobacterium abscessus*, *Mycobacterium avium*, claritromicina.

INTRODUCTION

Nontuberculous mycobacteria (NTM) include the majority of the species of the genus *Mycobacterium*, including human pathogens such as *Mycobacterium abscessus* and *M. avium* complex. The *Mycobacterium avium* complex (MAC) includes three species that are a cause of human infections (*Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium chimaera*), and are considered the commonest NTM isolated in humans throughout the world [1]. Infections caused by MAC are usually respiratory infections among patients with different comorbidities, while they can cause many different syndromes, including disseminated disease [2].

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On the other hand, *Mycobacterium abscessus* is included among rapidly growing mycobacteria and is found with increasing frequency as an opportunistic human pathogen [3]. Although its taxonomy remains under debate, a widespread taxonomic classification divided this species into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* [4]. Infections caused by these species are usually chronic and associated with immunological deficiencies, such as cystic fibrosis (CF), in which morbidity and mortality are associated with both infections and unusual immune responses [5].

NTM infections are currently considered biofilm-related infections, and this fact is of clinical importance because of the higher resistance against antibiotics of sessile bacteria compared with that of planktonic ones [6]. Therefore, avoiding biofilm formation could be extremely important for the management of the patients because it potentially can avoid the development of the disease or, at least, facilitate their treatment.

This study aimed to know the biofilm prevention concentration (BPC) of clarithromycin (an antibiotic that is considered a keystone in the treatment of NTM diseases) and compare it with the minimal inhibitory concentration of planktonic cells and antimicrobial susceptibility testing using *M. avium* complex and *M. abscessus* collection and clinical strains (n=16) isolated from patients with and without respiratory disease.

MATERIAL AND METHODS

Bacterial strains. A total of 16 strains (n=16) were used in this study. MAC strains used in this study were a total of nine including three type strains and two clinical strains of each species. *M. abscessus* strains used in this study were a total of seven including two type strains and five clinical strains (Table 1). The strains 330 and 624 showed a rough phenotype, while the strains 368, 611, and 531 have a smooth phenotype. The clinical significance of the clinical strains was determined according to the ATS-ERS-IDSA-ESCMID criteria [2], being clinically significant the strains *M. avium* 647, *M. intracellulare* 657, *M. chimaera* 655, and all the clinical strains of *M. abscessus*.

All strains were maintained at -80ºC and defrosted before performing the experiments, inoculated onto Middlebrook 7H10 agar (Difco™) plates supplemented with 10% Middlebrook OADC enrichment and 0.4% glycerol and incubated at 37ºC for 10-15 days. After 24h plates were checked for purity.

Antimicrobial susceptibility of planktonic bacteria. The studied clinical strains were tested for antimicrobial susceptibility using the CLSI recommendations for broth microdilution [7] and were interpreted following CLSI and [7] European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [8]. Minimal Inhibitory Concentration (MIC) of planktonic bacteria was obtained for each strain against clarithromycin (CHL) (Sigma-Aldrich, MA, USA) with a range of 1-256 mg/L. MAC strains were incubated for 10 days and *M. abscessus* strains were incubated for 5 days, all of them at 37ºC with 5% CO_2. After incubation, MIC values were read with the naked eye according to the CLSI/EUCAST recommendations. A positive control with bacteria without antibiotic and a negative control without microorganisms were included for each strain. This test was performed in triplicate for all strains.

Biofilm susceptibility assay. Susceptibility in biofilm was determined as proposed by Fernández-Olmos *et al.* with minor modifications [9]. Biofilm formation for all strains was reached by suspending the strains to 0.5±2 McFarland in liquid Middlebrook medium and 75 µL of each strain was placed onto a 96-well plate (ThermoFisher Scientific, MA, USA). Then, clarithromycin was added to the wells using concentrations between 0.25 and 256 mg/L and 75 µL of CHL serial dilutions to the 96-well plate to study a wide range of concentrations. The plates were incubated for 5 days for the *M. abscessus* strains and 10-15 days incubation for the MAC strains. After incubation, Biofilm Preventive Concentration (BPC) was determined by lack of biofilm formation by visual inspection using an inverted microscopy (Leica, LEITZ DM IL) and by measuring the plates in a spectrophotometer (TECAN, Switzerland) at OD_{595} nm. Positive controls with all ATCC strains were used in all the experiments. All strains were tested in triplicate. EUCAST criteria for MICs were adopted for BPCs.

RESULTS AND DISCUSSION

MICs and BPCs obtained for the *M. abscessus* strains and the MAC clinical strains are shown in Table 1. Interestingly, all but one *M. abscessus* strains showed identical BPC, while MAC isolates showed more variable concentrations, having all *M. avium* strains at the same concentration.

Biofilms are selective environments, placing demands on planktonic microbial cells. A pre-existing microbial biofilm may stimulate, inhibit, or have no effect on the adherence of a particular microbial species or type [10]. For instance, early studies of adherence relied upon having a surface covered with protein (e.g., bovine serum albumin). Without a conditioned surface a particular microorganism would not be able to adhere and form a biofilm [11]. In contrast, *M. avium* is capable of forming new biofilm thanks to its hydrophobic surface that can adhere to all types of surfaces [12].

Biofilm formation and surface adherence is an essential pathogenic factor for the *M. avium* complex because without adherence or biofilm formation cells would be washed away by any flowing force [13]. *M. avium* usually colonizes the respiratory tract of patients with chronic pulmonary disease which can evolve to an infection that is difficult to eradicate. Therefore, it is credible that biofilm formation might have a substantial effect on the maintenance of the infection. On the other hand, *M. abscessus* disease is considered a biofilm-related one, and this mycobacterium has been found forming biofilms in the lung tissue of patients with *M. abscessus* disease [14]. Moreover, many *in vitro* studies have shown that the biofilms formed by this species have an increased resistance against many antibiotics, including macrolides [15].

Table 1 **Minimum inhibitory and biofilm prevention concentration of Mycobacteria clinical strains.**

Strain	MIC (mg/L)	BPC (mg/L)	Number of dilutions between MIC and BPC
M. avium ATCC 25291	4	16	2
M. intracellulare ATCC 13950	8	8	0
M. chimaera DSM 44623	≤1	1	≥ 1
M. avium 717	≤1	16	> 4
M. avium 647	≤1	16	> 4
M. intracellulare 505	≤1	8	> 3
M. intracellulare 657	≤1	4	> 2
M. chimaera 575	≤1	1	≥ 1
M. chimaera 655	8	16	1
M. abscessus DSM 44196 rough	2	32	4
M. abscessus DSM 44196 smooth	2	32	4
M. abscessus subsp. *abscessus* 330	1	32	5
M. abscessus subsp. *abscessus* 368	4	32	3
M. abscessus subsp. *massiliense* 624	1	32	5
M. abscessus subsp. *massiliense* 611	0.25	64	8
M. abscessus subsp. *bolletii* 531	≥16	32	0

MIC, minimum inhibitory concentration; BPC, biofilm prevention concentration.

Active antibiotics against either planktonic cells or early attached cells might serve as a prevention method against biofilm formation and development. This can be confirmed with this study where the strains of all the species showed low BPCs for clarithromycin, and by the study of Carter et al, where subinhibitory concentrations of macrolides can inhibit partially the biofilm formation of several *M. avium* strains [16]. However, in our study, we looked for the concentration that inhibits completely the development of a biofilm, and this is a possible explanation of our results. Interestingly, MAC isolates showed different values, probably related to the differences detected in biofilm development among all these strains [17], differences that are not detected among *M. abscessus* strains, which are more uniform in biofilm development, as previously described [18]. The latter one is, in fact, very different from MAC, so we expected differences in the detection of BPC between both groups that were confirmed in the study. Former studies that showed the preventive value of macrolides against MAC infection in the first years of the AIDS pandemic [19] can be now explained (at least partially) for this property, because by avoiding biofilm formation we can potentially avoid the disease, and so improving the quality of life of the patients.

The main limitation of our study is the low number of tested strains. Future studies with a large sample size are needed as are studies that test other antibiotics that can be used in the treatment of nontuberculous mycobacteria. Another limitation is the lack of bovine serum in our culture medium, which can be a conditioning factor for biofilm development for mycobacteria. However, we have previously used this medium in previous studies with these species of mycobacteria with good results [17,18].

These results suggest the importance of developing an early aggressive treatment to prevent biofilm formation in this type of bacteria and open a possibility of preventive measures for these patients that potentially can change their management and outcome. However, how implementing this possibility is a matter for further research, because many issues (dosages, time, type of patients) need to be determined before introducing this type of prophylaxis in common clinical practice.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest

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