ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02882.x

# Biofilm development by clinical strains of non-pigmented rapidly growing mycobacteria

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## Abstract

The relationship between clinical significance of non-pigmented, rapidly growing mycobacteria (NPRGM), *in vitro* biofilm development and sliding motility was evaluated in this study. One hundred and sixty-eight clinical strains of NPRGM were included. Forty-one of these were clinically significant isolates. Biofilm was formed by 123 strains. Seventy-six biofilm-positive and 25 biofilm-negative strains showed sliding motility. There was a relationship between clinical significance and biofilm development ($p < 0.000\,001$), sliding motility ($p\ 0.0037$) and species ($p < 0.000\,001$). No relationship was found between motility and biofilm development. The ability to develop biofilm is a characteristic that can have importance in the development of infections caused by NPRGM.

**Keywords:** Biofilm, clinical significance, microtitre, motility, rapidly growing mycobacteria

**Original Submission:** 24 January 2009; **Revised Submission:** 4 March 2009; **Accepted:** 13 March 2009

Editor: M. Drancourt

**Article published online:** 15 July 2009

*Clin Microbiol Infect* 2009; **15:** 931–936

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## Introduction

Since the development of the biofilm concept in the late 1970s, this structure has been recognized as an extremely important pathogenic factor in medicine. Biofilms have been described in a high number of human infections, especially those related to biomaterials [1,2].

Non-pigmented rapidly growing mycobacteria (NPRGM) are a group of mycobacteria that have been involved in a broad spectrum of human infections [3,4]. Among these diseases, biomaterial-related infections are an important group, ranging from extremely severe syndromes, such as prosthetic valve endocarditis, to relatively mild ones, such as surgical wound infections. Moreover, chronic syndromes caused by these mycobacteria, which have been considered biofilm-related infections (for example respiratory tract infections in patients with cystic fibrosis) have also been described [1]. A recent study showed that such biofilm-related infections represent more than two-thirds of all infections caused by these organisms [5].

There are several reports describing biofilm development by NPRGM [6,7]. However, only a few of them include clinically relevant species [8–12] and none of them includes a large number of clinical strains. From studies performed with *Mycobacterium smegmatis* culture collection strains, it has been claimed that there is a relationship between sliding motility and biofilm development [13,14]. However, there are data suggesting that these properties are not necessarily linked, at least in other species of slowly growing [15] or rapidly growing mycobacteria [12], but no study has included clinical strains of different species of NPRGM.

Recent studies performed with *M. abscessus* isogenic strains have shown a relationship between glycopeptidolipids and biofilm development [11]. Interestingly, in this report the strain that was able to develop biofilm did not show pathogenic potential in an *in vivo* model of infection [11,16,17]. *Mycobacterium abscessus* is especially correlated with respiratory tract infections [5], although it can cause a broader group of syndromes [3]. It is possible that these experimental results represent only a partial view of the problem, and a broader study with a high number of strains would give different results. We have already shown that a *M. abscessus* type strain is able to form biofilm, and under

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some conditions the growth of the biofilm produced by this strain is faster than that of other species [12].

Here we report a study analysing the relationship between biofilm development, sliding motility, and clinical significance in a group of clinical strains of NPRGM.

## Materials and Methods

### Mycobacterial strains

Clinical strains of NPRGM isolates from Madrid (Spain) were included in the study. The strains were recovered from clinical isolates of the Fundación Jiménez Díaz hospital between 1990 and 2006 and from all public hospitals in Madrid (Fundación Jimenez Diaz, Hospital Universitario San Carlos, Clínica Puerta de Hierro, Hospital La Princesa, Hospital Universitario La Paz, Hospital Ramón y Cajal, Hospital 12 de Octubre, Hospital Universitario Gregorio Marañón) and the Hospital Universitario de Getafe during 2005. The strains were identified according to commonly recommended schemes, using both biochemical tests [3] and PCR-RFLP analysis (PRA) of the Telenti fragment of the *hps*65 gene [18]. When an unusual PRA pattern, or discrepancies between phenotypical and genotypical methods, was detected, the strains were sent to the Mycobacteria Laboratory from the Centro Nacional de Microbiologia (Majadahonda, Spain) to be identified using *16S rRNA* gene sequencing. After identification, the strains were stored in skimmed milk at −20°C. After thawing, mycobacteria were checked for purity before performing the experiment.

The clinical significance of the strains was assessed by reviewing the clinical charts of the patients. The evaluation was performed according to the criteria of the American Thoracic Society for respiratory samples [19]. For non-respiratory samples we considered a strain as clinically significant if the strain was isolated from biopsy or from several exudates and/or characteristic pathology was found in the presence of associated physical signs or symptoms, with improvement after treatment. When the same organism was isolated from at least two different body sites, or if blood or bone marrow cultures were positive, cases were grouped as clinically significant disseminated NPRGM disease. Clinical data from these patients have been previously published elsewhere [5,20].

Culture collection strains of *M. fortuitum* ATCC 6841T, *M. chelonae* ATCC 35752T, *M. abscessus* DSM 44196T, *M. peregrinum* ATCC 14467T, *M. mucogenicum* DSM 44124, and *M. mageritense* ATCC 700351T were included in the study as controls [12].

### Sliding motility test

A motility assay was performed as described by Martinez *et al.* [13]. A colony of each strain was inoculated in the centre of a plate of motility medium (Middlebrook 7H9 (BD, Franklin Lakes, NJ, USA) with 0.3% agar (BD) and without supplements). The inoculated media were then incubated at 37°C in a 5% $CO_2$ atmosphere over 7 days, after which the diameter of the bacterial growth was measured using a digital caliper. We considered a motility test to be positive if the diameter of the colony was >7 mm (the diameter of the loop used to inoculate the plates), as defined previously [12].

### Biofilm development test

The experimental protocol was developed using the method previously described by us [12] with minimal modifications: only Middlebrook 7H9 was used as culture medium. The plates were incubated at ambient room temperature for 25 days. Medium was replaced on days 1, 4, 7, 11, 14, 18, 21 and 25. Photographs were obtained using a Leitz DM IL inverted microscope (Leica, Solms, Germany) with an attached Nikon Coolpix 8400 digital camera (Nikon, Tokyo, Japan), and analysed to evaluate the surface area covered by the biofilm using Image J software (National Institute of Health, Bethesda, MD, USA). The proportion of surface covered by biofilm at each time point was used to construct a growth curve of the biofilm. Each strain was tested at least two times in different experiments.

Strains that did not show biofilm development were re-tested using a more prolonged incubation time: photographs were taken on the same days plus days 28, 32, 35 and 39.

Twenty randomly selected clinical strains (four each of *M. abscessus*, *M. fortuitum*, *M. chelonae* and *M. peregrinum*, and two each of *M. mucogenicum* and *M. mageritense*) were also analysed using confocal laser scanning microscopy (CLSM), according to the previously described protocol [12].

### Statistical analysis

Logistic regression analysis was performed to evaluate the relationship between biofilm development, sliding motility and clinical significance. In our study, we considered the clinical significance as response variable, and as predictors we considered the species, sliding motility, biofilm development, and the corresponding percentages of area covered by biofilm at days 1, 4, 7, 11, 14, 18, 21 and 25 (the data of percentage of covered surface were grouped as one variable named percentage of covered surface). The sum of percentages can be considered as indicative of the speed at which the bacteria covered the surface of the well (i.e. the dynamics of biofilm growth). Biofilm development was indicated only if the strain was able to develop a biofilm during the study period.

We evaluated the null hypothesis of a null effect of each variable over the response (the clinical significance) given by

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the other variables. In order to study this, different models have been fitted: first, a model (hereafter referred to as the global model) including all the predictors, and corresponding simplified models removing: (i) the species, (ii) the sliding motility, (iii) the biofilm development and, finally, (iv) the percentage of covered surface. The global model with all predictors was compared with the simplified model where a variable (or a set of variables in the case of the percentages) is removed. The same analysis has been applied for certain species with a high number of strains (*M. chelonae* and *M. fortuitum*).

The relationship between colony phenotype, motility and biofilm development was investigated using chi-square and Fisher's exact tests.

## Results

One hundred and sixty-seven clinical strains (nine *M. abscessus*, two *M. alvei*, 30 *M. chelonae*, 90 *M. fortuitum*, five *M. mageritense*, eight *M. mucogenicum*, 21 *M. peregrinum*, one *M. porcinum* and one *M. septicum*) were included in the study. Forty-one of them were clinically significant (eight *M. abscessus*, 15 *M. chelonae*, 17 *M. fortuitum* and one *M. peregrinum*). Clinical data for these strains are shown in the Table 1.

Biofilm was formed by 122 strains (73.2%). Forty-five strains (26.8%) were unable to develop biofilm. These were: one *M. abscessus* (11.1% from this species), nine *M. chelonae* (30%), 22 *M. fortuitum* (24.4%), two *M. mucogenicum* (25%), ten *M. peregrinum* (47.6%) and the *M. septicum* strain (Fig. 1). Among species with five or more strains, all *M. mageritense* strains produced biofilm, although none of them was considered clinically significant. Among all other strains, percentages forming biofilm ranged between 52.4% (*M. peregrinum*) and 88.9% (*M. abscessus*) (Fig. 1). All *M. alvei* and *M. porcinum* strains were also positive for biofilm development.

Considering only the clinically significant strains, biofilm development was detected in 87.5% of *M. abscessus* strains, 73.3% of *M. chelonae* strains, 61.1% of *M. fortuitum* strains and the clinically significant strain of *M. peregrinum*.

Biofilm-producing strains showed a sigmoid growth curve, as previously described for collection strains (Fig. 2) (13). No species differences in growth curves were detected.

CLSM analysis showed the presence of channels inside the biofilm when it was fully developed, together with a variable amount of dead cells, higher at the end of the experiment.

Seventy-six of the biofilm-positive (61.8%) and 25 of the biofilm-negative strains (55.6%) showed sliding motility. No relationship between sliding motility and biofilm development was found (p 0.15).

**TABLE 1. Syndromes due to clinically significant non-pigmented, rapidly growing mycobacteria**

| No | Strain | Species | Syndrome | Biofilm production |
|---|---|---|---|---|
| 1 | FJD-4 | *Mycobacterium chelonae* | Skin and soft-tissue infection | Positive |
| 2 | FJD-5 | *M. chelonae* | Osteomyelitis | Negative |
| 3 | FJD-10 | *M. peregrinum* | Urinary tract infection | Positive |
| 4 | FJD-24 | *M. fortuitum* | Skin and soft-tissue infection | Negative |
| 5 | FJD-25 | *M. chelonae* | Catheter-related bacteraemia | Positive |
| 6 | FJD-29 | *M. chelonae* | Catheter-related bacteraemia | Positive |
| 7 | FJD-43 | *M. chelonae* | Endophthalmitis | Positive |
| 8 | FJD-51 | *M. chelonae* | Skin and soft-tissue infection | Negative |
| 9 | FJD-63 | *M. chelonae* | Skin and soft-tissue infection | Positive |
| 10 | FJD-64 | *M. abscessus* | Skin and soft-tissue infection | Positive |
| 11 | FJD-69 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 12 | FJD-84 | *M. chelonae* | Arthritis | Positive |
| 13 | FJD-85 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 14 | FJD-92 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 15 | FJD-95 | *M. abscessus* | Skin and soft-tissue infection | Positive |
| 16 | FJD-172 | *M. chelonae* | Bacteraemia | Negative |
| 17 | FJD-176 | *M. chelonae* | Catheter-related bacteraemia | Positive |
| 18 | FJD-193 | *M. abscessus* | Osteomyelitis | Positive |
| 19 | FJD-207 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 20 | FJD-211 | *M. chelonae* | Skin and soft-tissue infection | Positive |
| 21 | FJD-230 | *M. fortuitum* | Mammary prosthesis infection | Positive |
| 22 | FJD-233 | *M. fortuitum* | Hip prosthesis infection | Positive |
| 23 | FJD-241 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 24 | FJD-260 | *M. fortuitum* | Skin and soft-tissue infection | Positive |
| 25 | MCM-1 | *M. abscessus* | Respiratory tract infection | Positive |
| 26 | MCM-7 | *M. fortuitum* | Respiratory tract infection | Positive |
| 27 | MCM-8 | *M. fortuitum* | Neck abscess | Negative |
| 28 | MCM-13 | *M. fortuitum* | Canaliculitis | Negative |
| 29 | MCM-14 | *M. chelonae* | Catheter-related bacteraemia | Positive |
| 30 | MCM-15 | *M. fortuitum* | Surgical site infection | Positive |
| 31 | MCM-16 | *M. chelonae* | Endophthalmitis | Positive |
| 32 | MCM-24 | *M. abscessus* | Respiratory tract infection (Cystic fibrosis) | Positive |
| 33 | MCM-25 | *M. abscessus* | Respiratory tract infection (Cystic fibrosis) | Positive |
| 34 | MCM-28 | *M. fortuitum* | Bacteraemia | Positive |
| 35 | MCM-32 | *M. chelonae* | Skin and soft-tissue infection | Positive |
| 36 | MCM-53 | *M. fortuitum* | Catheter-related bacteraemia | Negative |
| 37 | MCM-65 | *M. fortuitum* | Catheter-related bacteraemia | Negative |
| 38 | MCM-66 | *M. fortuitum* | Catheter-related bacteraemia | Negative |
| 39 | MCM-80 | *M. chelonae* | Respiratory tract infection (Cystic fibrosis) | Negative |
| 40 | MCM-83 | *M. abscessus* | Respiratory tract infection (Cystic fibrosis) | Negative |
| 41 | MCM-86 | *M. abscessus* | Respiratory tract infection (Cystic fibrosis) | Positive |

In our study, we aimed to predict the clinical significance of the strains from the following variables: species, sliding motility, biofilm development and the values of the corresponding percentages of covered surface, measured at days 1, 4, 7, 11, 14, 18, 21 and 25. We fitted the model both with all the variables and with each variable removed individually. The p-values observed when we removed the following variables were: percentage covered surface 0.0232: biofilm, <0.000001: sliding motility 0.0037 and species <0.000001. Thus, the relationship between clinical significance and all these variables gave significant results. Next, a stepwise variable selection was applied to the global model using all the covered surface data. The final model gave as predictors of clinical significance the percentages of covered surface at days 1 and 21 (no statistically significant differences were

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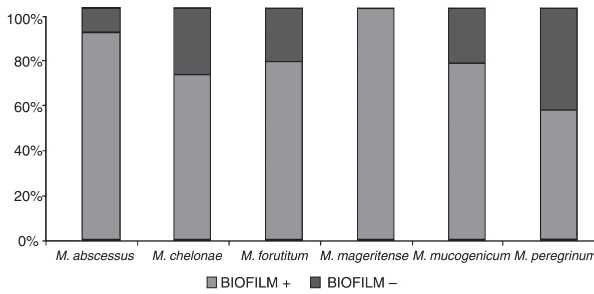


**FIG. 1.** Percentages of biofilm-developing strains among species with five or more isolates. Biofilm +, biofilm-producing strains; Biofilm –, biofilm-negative strains.

detected for all other days), biofilm development ability, sliding motility and the different species.

Additionally, for *M. chelonae* and *M. fortuitum* (the higher number of isolates), a stepwise variable selection was also applied. The final model showed the following results for the different variables as predictors of clinical significance: percentages of covered surface (p 0.0084 for *M. chelonae*, and p 0.066 for *M. fortuitum*), biofilm development ability (p 0.056 for *M. chelonae* and p 0.018 for *M. fortuitum*) and sliding motility (p 0.217 for *M. chelonae* and p 0.01 for *M. fortuitum*).

## Discussion

Despite their classical image of 'non-pathogenic' or 'colonizing bacteria', non-tuberculous mycobacteria have been

**FIG. 2.** Growth curves for biofilm-producing strains of species with five or more isolates (data represent the mean values from all isolates of a species). %, percentage of covered surface.

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recognized in recent years as emerging pathogens, mainly due to the increasing number of patients susceptible to these organisms, but also to the recognition of their importance as human pathogens. Among these organisms, NPRGM represent a group of bacteria with different characteristics, which make their spectrum of diseases broader than other mycobacteria [3,4]. Their true incidence is unknown, but is probably underestimated because of their potential identification as 'diphtheroids' [21] or simply because they are not included in the group of organisms that are looked for in many samples. Recently, we unexpectedly recovered NPRGM from retrieved orthopaedic prosthesis, even though the number of studied patients was low [5].

Almost all the cases of human infection caused by these organisms are due to three species: *M. abscessus*, *M. chelonae* and *M. fortuitum* [3,4]. One recent epidemiological study has confirmed this finding, although many other species were isolated [5]. In the present study, species was statistically confirmed as a predictor for infection.

Infections caused by these organisms were detected in biofilm-related syndromes, such as foreign-body infections or chronic respiratory tract infections. These data suggest that there could be a role for biofilm development as a pathogenic factor among NPRGM.

Several studies have analysed biofilm development among these species. Most studies analysed several aspects using *M. smegmatis* (an infrequent cause of human infections) collection strains [22–25], only a few of them included clinically relevant species [8–12], and none of them included a high number of clinical strains.

To the best of our knowledge, this is the first report analysing biofilm development among a high number of clinical strains that have also been evaluated for their role in human infections. In our study, a significant relationship between biofilm development ability and clinical infection has been demonstrated, indicating that biofilm development could be an important pathogenic factor for NPRGM. This fact is of great importance because biofilms are a well-known form of bacterial resistance against antibiotics [26,27], hence, the ability to develop these structures can explain treatment failures of some of these infections when foreign bodies were present [3,4]. Interestingly, all *M. mageritense* strains were able to develop biofilm (although the number of isolates is very low), in spite of the fact that none of them causes true human infection. Moreover, data for *M. chelonae* were just short of a significant relationship between biofilm developing ability and clinical significance (p 0.056), so we can deduce that biofilm formation is one factor that, in combination with others, may contribute to development of human infections.

Another interesting result of our study concerns the relation between sliding motility and biofilm development. Sliding motility has been defined as 'a kind of surface translocation produced by the expansive forces in a growing culture in combination with special surface properties of the cells resulting in reduced friction between cell and substrate' [13]. This property has been considered to be related to the glycopeptidolipid content of the mycobacterial cell wall and to other aspects of lipid metabolism [13,14,28]. There are several reports of a relationship between sliding motility and biofilm development ability in *M. smegmatis* [13,14] and *M. abscessus* [11]. However, other reports state that this relationship is not uniform [12,15,28]. Our study revealed biofilm-producing strains that were non-motile, as well as other strains that showed sliding motility but were unable to form biofilm in the test, with no relationship established between them. Moreover, in our report a statistical relationship between sliding motility and clinical significance was detected, for all strains including *M. fortuitum*. Despite the absence of a statistically significant relationship between biofilm and sliding motility, it is possible that a common factor could link both properties (probably related to lipid metabolism or the lipid composition of the mycobacterial cell wall, as previous reports have stated [13,14]) to the capacity of the strains for causing human infection.

In conclusion, sliding motility and biofilm-producing capacity were not invariably linked among clinical strains of NPRGM. Biofilm development ability is a property that is not uniformly present among clinical isolates of NPRGM. Nevertheless, it seems to be related to the capacity of the strains to cause human disease.

## Acknowledgements

This report was presented partially at the 29th Annual Congress of the European Society of Mycobacteriology (Plovdiv, Bulgaria, 2008).

## Transparency Declaration

This work was funded by grants from Capio Research Foundation (2006-1159 and 2007-1457) CICYR-MAT2006-12603-C02-02 and from the CONSOLIDER-INGENIO 2010 programme (FUNCOAT-CSD2008-00023). N.Z.M-d-H. was funded by the Fundación Conchita Rábago de Jiménez Díaz.
All authors declare no conflicts of interest.

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