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In-vitro evaluation of the adhesion to polypropylene sutures of non-pigmented, rapidly growing mycobacteria

N. Zamora[1], J. Esteban[1], T. J. Kinnari[1], A. Celdrán[2], J. J. Granizo[3] and C. Zafra[1]

[1]Department of Clinical Microbiology, [2]Department of Surgery and [3]Department of Epidemiology, Fundación Jiménez Díaz-UTE, Madrid, Spain

ABSTRACT

The ability of non-pigmented, rapidly growing mycobacteria (NPRGM) to attach to polypropylene sutures was evaluated using an in-vitro assay. Thirty clinical isolates and five culture collection strains of NPRGM, together with *Staphylococcus epidermidis* ATCC 35983, were tested. *Mycobacterium fortuitum* and *Mycobacterium chelonae* showed the highest attachment ability, which differed significantly from the results obtained with *Mycobacterium peregrinum*. According to these results, NPRGM are able to attach to polypropylene sutures, and the species implicated most frequently in human infection showed increased levels of attachment in comparison with the other mycobacteria studied.

Keywords Attachment, in-vitro assay, *Mycobacterium* spp., non-pigmented mycobacteria, polypropylene sutures

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INTRODUCTION

Non-pigmented rapidly growing mycobacteria (NPRGM) are interesting organisms, not only because they are isolated frequently in clinical laboratories, but also because they are well-documented human pathogens [1–4]. These organisms produce biofilm in several environmental settings, including soil and water systems [3,5]. They have also been isolated from other sources, e.g., ice machines, disinfectants and antibiotic solutions, all of which have been considered to be the source of outbreaks and pseudo-outbreaks caused by these organisms [2,3]. NPRGM are often described as a cause of nosocomial infections, especially those involving surgical complications related to foreign bodies [1,2], e.g., surgical site infections [6,7], bacteraemia [8,9], bone and joint infections [10–12], and prosthetic valve endocarditis [4,13–15]. The species isolated most frequently from such infections are *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus*, while other members of this group, e.g., *Mycobacterium peregrinum* and *Mycobacterium mucogenicum*, are found less frequently [1,3,11]. As disease caused by NPRGM is not subject to compulsory reporting, no data concerning their actual incidence have been published, but these species are isolated frequently in clinical microbiology laboratories [12], with one-third of isolates being considered to be clinically significant [16]. The fact that these organisms grow in commonly used culture media means that they can be misidentified as diphtheroids, so that their actual incidence could be even higher than that recorded by clinical mycobacteriology laboratories [14,15].

In the nosocomial setting, NPRGM infections have been associated frequently with foreign bodies, e.g., prosthetic heart valves or mammaplasty prostheses, as well as numerous surgical site infections [1–4]. Studies of skin and soft-tissue infections caused by NPRGM have identified trauma or previous surgery as risk-factors in >50% of cases [17,18]. In the Fundación Jiménez Díaz Hospital (Madrid, Spain), eight of 20 documented cases of infection caused by NPRGM have been associated with foreign bodies [16].

Corresponding author and reprint requests: J. Esteban, Department of Clinical Microbiology, Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain
E-mail: jesteban@fjd.es

Despite broad knowledge of the clinical, diagnostic and therapeutic aspects of human infections caused by NPRGM, only a few in-vitro studies of the pathogenic mechanisms involved have been published [19,20]. In particular, there has been only one previous in-vitro study of the ability of different species of NPRGM to adhere to various biomaterials [21], and only two species were tested. The present study aimed to evaluate the attachment of a wider range of species of NPRGM to suture materials used commonly in surgical procedures.

MATERIALS AND METHODS

Bacterial isolates

Thirty clinical isolates of NPRGM (two *M. abscessus*, nine *M. chelonae*, 11 *M. fortuitum*, two *M. mucogenicum* and six *M. peregrinum* (Table 1) were studied, together with the following strains from culture collections: *M. fortuitum* ATCC 13756; *M. chelonae* ATCC 19235; *M. peregrinum* ATCC 14467; *M. abscessus* DSM 44196; and *M. mucogenicum* DSM 44124. The glycocalyx-producing *Staphylococcus epidermidis* strain ATCC 35983 was used as a control.

Table 1. Isolates of non-pigmented rapidly growing *Mycobacterium* spp. included in the study

Isolate	Species	Sample	Clinical significance	Growth rate (days)
ATCC 13756	*M. fortuitum*	NA	NA	4
ATCC 19235	*M. chelonae*	NA	NA	4
FJD-24	*M. fortuitum*	Skin biopsy	Yes	4
FJD-26	*M. fortuitum*	Sputum	No	4
FJD-36	*M. fortuitum*	Sputum	No	5
FJD-37	*M. fortuitum*	Sputum	No	4
FJD-53	*M. fortuitum*	Sputum	No	5
FJD-55	*M. fortuitum*	Sputum	No	4
FJD-56	*M. fortuitum*	Sputum	No	4
FJD-58	*M. fortuitum*	Sputum	No	4
FJD-61	*M. fortuitum*	Bronchial washing	No	4
FJD-65	*M. fortuitum*	Sputum	No	5
FJD-69	*M. fortuitum*	Skin biopsy	Yes	4
FJD-25	*M. chelonae*	Blood culture	Yes	6
FJD-29	*M. chelonae*	Wound exudate	Yes	4
FJD-43	*M. chelonae*	Vitreous humour	Yes	5
FJD-44	*M. chelonae*	Ascitic fluid	No	4
FJD-50	*M. chelonae*	Wound exudate	Yes	6
FJD-51	*M. chelonae*	Wound exudate	No	5
FJD-83	*M. chelonae*	Pleural biopsy	No	5
FJD-40-1	*M. chelonae*	Pleural fluid	No	4
FJD-40-2	*M. chelonae*	Pleural fluid	No	4
FJD-64	*M. abscessus*	Wound exudate	Yes	4
FJD-95	*M. abscessus*	Wound exudate	Yes	4
DSM 44196	*M. abscessus*	NA	NA	4
FJD-18	*M. peregrinum*	Pericardial fluid	No	4
FJD-15	*M. peregrinum*	Blood culture	No	4
FJD-20	*M. peregrinum*	Renal biopsy	No	4
FJD-72	*M. peregrinum*	Bronchial aspirate	No	4
ATCC 14467	*M. peregrinum*	NA	NA	4
FJD-213	*M. peregrinum*	Lung biopsy	No	4
FJD-223	*M. peregrinum*	Urine	No	4
DSM 44124	*M. mucogenicum*	NA	NA	5
FJD-155	*M. mucogenicum*	Pleural fluid	No	4
FJD-187	*M. mucogenicum*	Blood culture	No	4

NA, not applicable.

Identification of bacterial isolates

All clinical isolates were identified initially by standard biochemical tests, including nitrate reductase, production of arylsulphatase, Tween-80 hydrolysis, growth on MacConkey agar without crystal violet, growth in the presence of NaCl 5% w/v, use of citrate, mannitol, inositol, sorbitol and rhamnose, and growth at 30°C, 37°C and 42°C. The tests were performed according to conventional protocols [22]. Identifications were confirmed by PCR restriction analysis, as described by Telenti *et al*. [23]. Bacteria were then stored in sterile skimmed milk at −20°C until further experiments were performed.

Evaluation of growth rate

Mycobacteria were harvested from growth on Tryptic Soy Agar containing sheep blood 5% v/v (bioMérieux, Marcy L'Etoile, France) and adjusted to 10^8 CFU/mL in sterile phosphate-buffered saline (PBS). This suspension was diluted to 10^6 CFU/mL, and 10 μL of this dilution was streaked on Tryptic Soy Agar containing sheep blood 5% v/v. The inoculated plates were incubated at 30°C for 1 week and growth of the isolates was recorded daily. The detection of colonies that were 0.5 mm in diameter was considered to be the baseline for evaluating the growth rate.

Growth media and incubation conditions

Mycobacterial isolates were grown at 30°C on Middlebrook 7H10 agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with glycerol for 7 days, and were later grown in Middlebrook 7H9 broth (Becton Dickinson) supplemented with Tween-80 0.01% v/v for 5 days at 30°C. No media changes were performed during the incubation period. *S. epidermidis* ATCC 35983 was grown overnight at 37°C on Tryptic Soy Agar containing sheep blood 5% v/v, and was grown subsequently in Tryptic Soy Broth (bioMérieux) at 37°C for 1 day.

Evaluation of attachment

To evaluate bacterial attachment, the protocol described by Yorganci *et al*. [24] was modified as follows. Sterile polypropylene monofilament suture (calibre 0) fragments (2 cm long) (Ethicon Inc., Sommerville, NJ, USA) were immersed separately in tubes containing 2.5 mL of PBS (Sigma, St Louis, MO, USA) and were incubated at 37°C for 24 h. The PBS was then replaced by 2.5 mL of bacterial inoculum, adjusted previously to a 0.5 × McFarland standard (10^8 CFU/mL). After incubation for 30 min at 37°C, the inoculum was removed and replaced by sterile PBS. The tubes were then incubated for 24 h at 37°C. Next, the suture fragments were washed 12 times with sterile distilled water to remove non-adherent bacteria, placed in 2.5 mL of sterile PBS, and sonicated at low power for 5 min in a Bandelin Sonorex TK-52 sonicator (Schalltec GmbH, Mörfelden-Walldorf, Germany). Attached bacteria were quantified by preparing 1:10 serial dilutions from the sonicate and streaking 100 μL of each dilution on Middlebrook 7H10 agar plates. The plates were incubated at 30°C for 7 days and the colonies were then counted. Attachment of *S. epidermidis* was quantified by the same procedure, except that the sonicate was streaked on Tryptic Soy Agar containing sheep blood 5% v/v and the plates were incubated for 24 h at 37°C. All experiments were performed in triplicate and repeated on two to four occasions.

Data analysis

The Bartlett test was used to confirm the normal distribution. The Kruskall–Wallis test was used for statistical multiple comparisons of the average total bacterial counts. Paired comparison between two species was performed using the Mann–Whitney test. In order to compare the attachment properties and growth rates of the individual isolates, the total bacterial count data were analysed by dividing the isolates into two groups (high or low bacterial count, with a breakpoint of 5×10^5 CFU). The groups were then compared, according to the day on which growth was detected for each isolate, using Fisher's exact test. All analyses were performed using SPSS v.10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

All species attached to the sutures. The average total count of attached bacteria was highest for *M. fortuitum* and lowest for *M. mucogenicum*. The average total counts were: *M. fortuitum*, 3.5×10^5 CFU (95% CI, 6.6×10^3 - 6.9×10^5; range, 5×10^2 - 3.6×10^6); *M. chelonae*, 1.9×10^5 CFU (95% CI, 2.5×10^4 - 3.5×10^5; range, 2.5×10^3 - 1.2×10^6); *M. abscessus*, 1.8×10^3 CFU (95% CI, 0 - 3.7×10^3; range, 2.5×10^3 - 6.25×10^3); *M. peregrinum*, 1.5×10^3 CFU (95% CI, 4.2×10^2 - 2.5×10^3; range, 5×10^2 - 7.5×10^3); *M. mucogenicum*, 1.1×10^3 CFU (95% CI, 8.5×10^2 - 1.3×10^3; range, 2.5×10^3 - 3×10^3); and *S. epidermidis*, 3.3×10^4 CFU (95% CI, 0 - 1.1×10^5; range, 6.75×10^4 - 9.75×10^4). The $\log_{10}$ values of the mean bacterial counts are shown in Fig. 1.

Among the isolates of *M. fortuitum* and *M. chelonae*, some isolates showed high values for adherence (five *M. fortuitum* and four *M. chelonae* isolates yielded total counts $>350 \times 10^3$ CFU), while other isolates yielded lower counts, similar to those observed for *M. peregrinum* and *M. mucogenicum*. One *M. fortuitum* isolate yielded extremely low counts of adherent bacteria (500 CFU) compared to all the other isolates of the same species.

The multiple comparison of the mean total bacterial counts among species did not indicate a statistically significant difference (p 0.0577). However, when paired data were analysed by the Mann–Whitney test, significant differences were found between *M. fortuitum* and *M. peregrinum* (p 0.017), and between *M. chelonae* and *M. peregrinum* (p 0.033). The difference between *S. epidermidis* and *M. peregrinum* almost reached significance (p 0.055).

Twenty-six isolates produced colonies 0.5 mm in diameter at day 4, and a further seven isolates at day 5. The two remaining isolates required 6 days to reach the required colony size (Table 1). Analysis of the growth rates showed no difference between the isolates with high or low attachment yields (Fisher's exact test, p 0.2).

DISCUSSION

Infections caused by NPRGM, although infrequent, can be devastating in the hospital setting [1,2,4], mainly because of the broad antimicrobial resistance of these organisms. Furthermore, such infections are associated mostly with biomaterials and require aggressive therapy, but a favourable outcome is unlikely in many cases of biomaterial-related infections caused by NPRGM, e.g., prosthetic valve endocarditis [1,4]. The difficulty in eradicating these organisms from a foreign body is probably associated with their ability to form biofilm [25].

Biofilm is a specialised mode of bacterial life that can be found in many environmental and clinical settings, including infections associated with foreign bodies [26]. The initial step in forming biofilm involves the adhesion of organisms to the surface of the biomaterial. This is a complex process that can be divided into two steps [27,28]. First, bacteria attach non-specifically to the surface as a result of, e.g., hydrophobicity, electrostatic attraction and van der Waals forces. Second, a more specific adherence occurs between the bacteria and the substratum; this includes the interaction of different structures and receptors located on the bacteria and the substratum.

Although the relationship between NPRGM and biomaterials has been known for decades [29], only a few studies concerning the ability of NPRGM to adhere to biomaterials have been reported. Vess *et al*. [21] studied the ability of several species of mycobacteria, including *M. abscessus* and *M. chelonae*, to adhere to polyvinyl chloride, and reported that both species were able to colonise the inner surface of polyvinyl chloride pipes and survive exposure to several disinfectants. Ridgway *et al*. [30] analysed the adherence of a *Mycobacterium* sp. to cellulose diacetate membranes, but the precise species was not identified. In addition, several studies have detected mycobacteria in biofilms [19,31–33], as well as

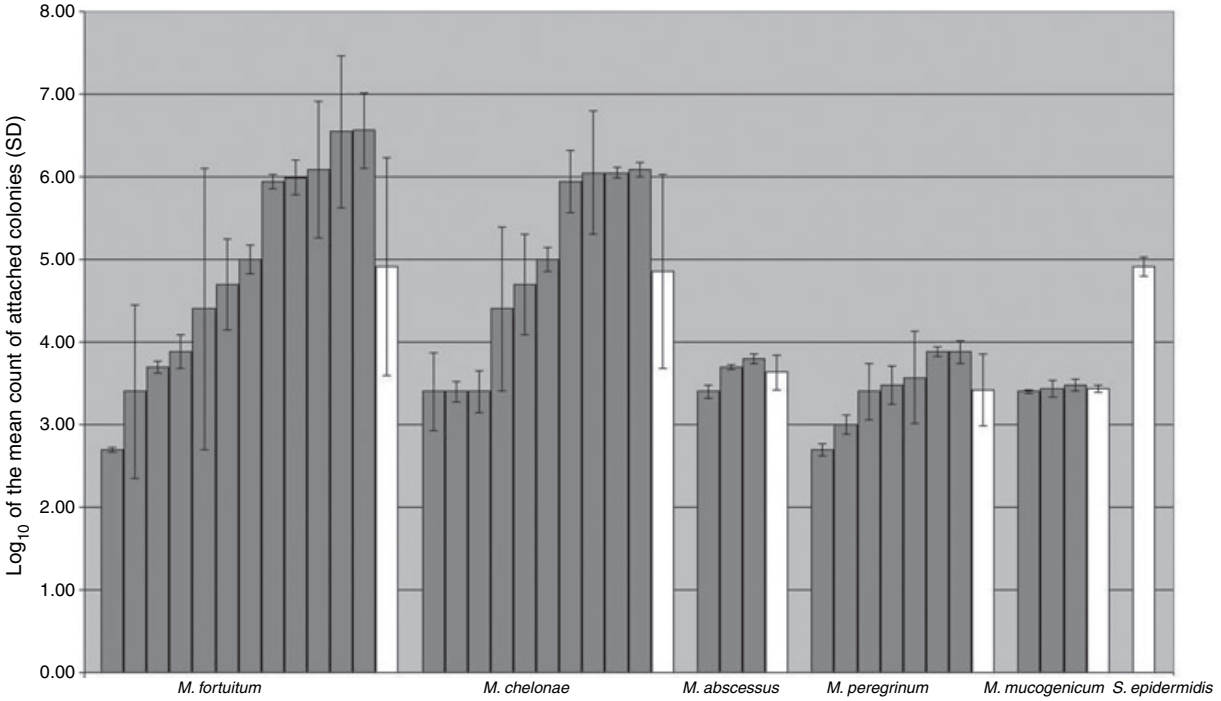

Fig. 1. Numbers (log_{10}) of bacteria attached to polypropylene sutures. Grey columns, values for individual strains; white columns, mean value for each species.

the in-vitro development of biofilm by standard strains of NPRGM [20,25,34]. Adherence ability, as the first part of the process, is confirmed implicitly by these studies.

Although the development of a biofilm was not investigated in the present study, it is probable that a biofilm could develop following the adherence of the isolates to the biomaterial. Raad *et al*. [8] detected biofilm caused by *M. chelonae* in an intravenous catheter from a patient with catheter-related bacteraemia, and several reports have shown that NPRGM are able to grow in biofilms in the most sterile hospital environments, including disinfectant trays and disinfected instruments [18,19,35].

The present study focused on polypropylene monofilament sutures as the test material because this biomaterial is used commonly in surgery, and many nosocomial diseases caused by NPRGM are related to surgical procedures and surgical site infections [1,2,15,17,18,29,36]. Indeed, two cases of NPRGM infections related to implantation of polypropylene monofilament mesh in hernia surgery have been diagnosed previously in the Fundación Jiménez Díaz hospital. In both of these cases, removal of the prosthesis was required to cure the patient. Mycobacteria were isolated in large numbers from the mesh, indicating the probable development of biofilm in both cases. *S. epidermidis*, the bacterium isolated most frequently from biomaterial-related infections, served as a control in the present study.

The present study included several isolates of the five most commonly described species of NPRGM. The statistically significant differences found could explain why the strongly adherent *M. fortuitum* and *M. chelonae* are described frequently as a cause of infections [1,2,4,29,36], while the less adherent *M. peregrinum* is rarely considered to be clinically significant. The limited number of isolates of other species tested means that further studies are needed to evaluate these species in more detail. No correlation was detected between growth rate and attachment, so another explanation may exist for the differences observed concerning attachment.

Although no specific adherence mechanism has been studied for mycobacteria, lipid metabolism has been implicated in the development of biofilm [20,37,38] and other related bacterial properties, e.g., sliding motility [39]. The mycobacterial cell wall includes a high proportion of lipid molecules,

which makes these bacteria more hydrophobic than other genera. Because hydrophobicity is an important mechanism for attachment to biomaterials [27,28], it can be speculated that differences in cell-wall lipids among strains or species of NPRGM could explain the differences detected in the present study. Other possible factors, e.g., the influence of plasma or serum on attachment, were not examined in the present study. Further studies are also required to investigate the differences found within individual species, e.g., *M. fortuitum* and *M. chelonae*.

In conclusion, the species of NPRGM tested were able to attach to polypropylene sutures. Differences among and within species were detected. The relationship between these findings and the ability to cause human disease requires further investigation.

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