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Detection of *lfrA* and *tap* efflux pump genes among clinical isolates of non-pigmented rapidly growing mycobacteria

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ABSTRACT

This study was performed to detect *LfrA* and *Tap* efflux pumps among clinical isolates of non-pigmented rapidly growing mycobacteria (NPRGM). Gene detection was performed using polymerase chain reaction (PCR) with specific primers designed for each gene. Susceptibility of the strains to doxycycline, tigecycline and ciprofloxacin was analysed using the broth microdilution reference technique. In total, 166 clinical isolates were included in the study. The *lfrA* gene was detected in four strains (2.4%), comprising two strains of *Mycobacterium chelonae* (6.7% of this species), one *Mycobacterium fortuitum* (1.1%) and one *Mycobacterium mucogenicum* (14.3%). The *tap* gene was detected in 109 strains (65.7%), comprising 3 *Mycobacterium abscessus* (33.3%), 12 *M. chelonae* (40%), 75 *M. fortuitum* (84.3%), 2 *Mycobacterium mageritense* (40%), 15 *Mycobacterium peregrinum* (68.2%), 1 *Mycobacterium alvei* and 1 *Mycobacterium porcinum*; no strains of *M. mucogenicum* were *tap*-positive. No differences between *tap*-positive and -negative strains were observed for resistance to doxycycline (Fisher's exact test, $P = 0.055$). *lfrA* is rare among clinical isolates of NPRGM, whilst *tap* is found more commonly. No correlation was detected between the presence of the efflux pumps and resistance to quinolones or tetracyclines.

1. Introduction

Non-pigmented rapidly growing mycobacteria (NPRGM) are a group of species from the genus *Mycobacterium* that share common characteristics. All of them are environmental organisms that can be the cause of a broad range of human diseases [1]. Treatment of these infections is sometimes problematic because the susceptibility of individual strains to different antibiotics is variable, thus specific antimicrobial susceptibility testing must be performed for each case. It is known that these organisms can develop resistance against several families of antibiotics [2–4] and there are genes or gene families that are extended through members of this group, such as *erm* methylase genes [2]. In some cases these genes have been involved in phenotypic resistance, whilst in other cases their presence does not correlate with such in vitro resistance results [2].

The presence of efflux pumps is a resistance mechanism that is currently being considered of increasing interest. Many organisms share these genes and in some cases they have been considered an important resistance mechanism. Some of these genes have been described in different mycobacteria, including in NPRGM species [5,6]. Specifically, LfrA is an efflux pump that confers low-level resistance to quinolones, several dyes and anthracyclines [7]. Although it has been described in *Mycobacterium smegmatis*, no studies have been performed neither to evaluate its importance among clinically relevant species or among clinical isolates of NPRGM. The Tap efflux pump has been described in a clinically relevant species (*Mycobacterium fortuitum*) [5]. This protein is active against different antibiotics, especially tetracyclines, as well as some aminoglycosides and ethyl-netilmicin.

However, despite the reports related to these genes, no study has been performed in a large number of clinical strains that can inform us about the frequency of these resistance mechanisms among these organisms.

In this study, we evaluated the presence of the two efflux pump genes described in NPRGM in a group of clinical strains and correlate its detection with the appearance of phenotypic resistance.

2. Material and methods

Clinical isolates of NPRGM from Madrid, Spain, were included in the study. Species identification was achieved using a broad range of biochemical tests (nitrate reduction, 3-day arylsulphatase, growth on MacConkey agar without crystal violet, use of citrate, mannitol, inositol, sorbitol and rhamnose as carbon sources, and growth on 5% NaCl) and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the *hsp65* gene [8]. *Mycobacterium fortuitum* ATCC 6841^T was included as a positive control for *tap*, and *M. smegmatis* ATCC 19420^T was included as positive control for *lfrA*.

Susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. Tested antibiotics were tigecycline (Wyeth, Madison, NJ), ciprofloxacin and doxycycline (Sigma, St Louis, MO). CLSI breakpoints for ciprofloxacin and doxycycline were used.

Primers used in the study are shown in Table 1. PCR for the *tap* gene was performed as follows: 94 °C for 10 min followed by 30 cycles of 94 °C for 1 min, and 72 °C for 3 min. The reaction gave an amplification product of 363 bp. The PCR protocol for *lfrA* was as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 61 °C for 1 min and 72 °C for 45 s, followed by 15 min at 72 °C. The reaction gave an amplification product of 547 bp. A negative control (DNA-free sterile distilled water) (Sigma) was included for each 20 reactions.

The amplified fragments were purified using a Montage PCR Kit (Millipore, Billerica, MA) following the manufacturer's instructions. The purified fragments were sequenced in an automatic sequencer at Secugen (Madrid, Spain). Results were subsequently analysed using National Center for Biotechnology Information (NCBI) nucleotide BLAST software.

Statistical analysis was performed using Epi Info 3.4.1 software (CDC, Atlanta, GA).

3. Results

In total, 166 clinical strains were included in the study, comprising 9 *Mycobacterium abscessus*, 30 *Mycobacterium chelonae*, 89 *M. fortuitum*, 22 *Mycobacterium peregrinum*, 5 *Mycobacterium mageritense*, 7 *Mycobacterium mucogenicum*, 2 *Mycobacterium alvei*, 1 *Mycobacterium porcinum* and 1 *Mycobacterium septicum*.

Only four strains (2.4%) were positive for the *lfrA* gene, comprising two strains of *M. chelonae* (6.7% of this species), one *M. fortuitum* (1.1%) and one *M. mucogenicum* (14.3%). Only two of these strains (one *M. chelonae* and the *M. mucogenicum* strain) were phenotypically resistant to ciprofloxacin [minimum inhibitory concentration (MIC) = 4 mg/L], whereas the other two strains were in vitro susceptible (MIC = 0.12 mg/L). Among all other strains, eight were resistant (MIC \geq 4 mg/L) to ciprofloxacin (four *M. abscessus*, two *M. chelonae*, one *M. fortuitum* and one *M. septicum*) and five strains showed an MIC of 2 mg/L (two *M. abscessus* and three *M. chelonae*).

In total, 109 strains (65.7%) were positive for the *tap* gene, including 3 *M. abscessus* (33.3%), 12 *M. chelonae* (40%), 75 *M. fortuitum* (84.3%), 2 *M. mageritense* (40%), 15 *M. peregrinum* (68.2%), 1 *M. alvei* and 1 *M. porcinum*. MIC₉₀ values (MIC for 90% of the organisms) for doxycycline and tigecycline and the results of *tap* detection for species with five or more isolates are shown in Table 2. Forty of these strains were in vitro susceptible to doxycycline (MIC \leq 1 mg/L). Among the 55 *tap*-negative strains, 12 showed intermediate susceptibility and 32 were fully resistant to doxycycline. No statistically significant differences regarding susceptibility of the strains between *tap*-positive and -negative strains were found ($P = 0.055$, Fisher's exact test). No strain was positive for both genes simultaneously. Gene sequencing showed an 88–92% nucleotide homology of *lfrA* amplicons with the gene sequence of *lfrA* from *M. smegmatis*. For *tap* amplicons, the nucleotide homologies were 92–95% with the gene *tap* from *M. fortuitum*. Data were obtained from the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

4. Discussion

Treatment of infections caused by NPRGM is a complicated issue, not only due to susceptibility differences between species but also because of the presence of individual differences between strains of the same species, which make it necessary to know the individual susceptibility of each strain before a proper therapeutic scheme can be used [1]. These individual differences merge because of the ability of these mycobacteria to develop resistance against some antibiotics by different methods. The presence of modifying and/or hydrolytic enzymes active against macrolides [2], aminoglycosides [3] and β -lactams [10] has been described. Target modifications as the cause of resistance against quinolones [11] and macrolides [12] have also been described. The presence of multidrug-resistant mycobacterial strains has also focused scientific interest on multidrug resistance mechanisms such as efflux pumps, which have been described for other organisms. Among NPRGM, two of these pumps have been described: *LfrA* in *M. smegmatis* [7] and *Tap* in *M. fortuitum* [5].

In this study, we aimed to determine the presence of *lfrA* and *tap* genes among clinical isolates of NPRGM and to correlate the presence of these genes with phenotypic susceptibility against antibiotics supposedly affected by the presence of these genes, without further evaluation of the activity of efflux pumps in individual strains. In this study, the *lfrA* gene was detected in only four clinical strains, two of which were in vitro susceptible. This unexpected result could be due to problems in the expression of the gene or in their regulatory mechanisms, because it is known that expression of *lfrA* is regulated in the

operon by another gene, *lfrR*, and it could be affected by the presence of specific substrates [6]. The presence of quinolone-resistant strains with negative results for *lfrA* may be due to other mechanisms, probably target modifications. The low MIC values for the two *lfrA*-positive quinolone-resistant strains are consistent with those described for the Lfr efflux pump [13].

In this study, most intermediate and resistant strains were *tap*-positive. However, we have detected both *tap*-positive tetracycline-susceptible and *tap*-negative tetracycline-resistant strains, and no statistical relationship was found for the association of the presence of the *tap* gene and resistance to doxycycline. These results can be explained by the presence of other resistance mechanisms in the *tap*-negative resistant strains. The *tap*-positive susceptible strains may be due to the detection of inactive genes due to the presence of deletions, modifications in the regulatory system, or both. Some of the efflux pumps have been described as inducible enzymes, so it is also possible that some kind of induction is needed to develop phenotypic resistance.

No strain showed in vitro resistance against tigecycline, a new tetracycline-related antibiotic. This is probably due to the fact that tigecycline is a poor substrate for tetracycline-related efflux pumps [14].

Of interest, activation of transcription of the efflux pumps has recently been described as a potential resistance mechanism in biofilms [15]. In the biofilm, these genes can be activated through the bacterial quorum system, and

bacteria can develop phenotypic resistance even in strains that appeared to be antibiotic-susceptible in common antimicrobial susceptibility tests. Because these mycobacteria are able to develop biofilms [16], the presence of efflux pump genes in these strains could be a resistance mechanism that leads to clinical failures despite in vitro susceptibility of these agents.

In conclusion, the LfrA efflux pump is rare among NPRGM, whilst the Tap efflux pump is widely distributed among clinical strains of these organisms. However, other mechanisms must be involved in antibiotic resistance of these strains to quinolones and tetracyclines, and the actual role of these efflux pumps in clinical resistance against these antibiotics is unclear.

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Table 1

Primers used in the study

Primer (gene)	Sequence
RG 391 (<i>IfrA</i>)	5'-CGC CCC GAG CAC CGA GTT-3'
RG 392 (<i>IfrA</i>)	5'-GAT GAT CGA CAG GAA GTT-3'
Tap 1 (<i>tap</i>)	5'-GTC GCG TTC CCG TGG CTG GT-3'
Tap 2 (<i>tap</i>)	5'-CGA TAC CGG GGC CGA CGA TG-3'

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Table 2

Results of MIC₉₀ values (mg/L) and gene detection among clinical isolates of non-pigmented rapidly growing mycobacteria

Species	<i>tap+</i> strains			<i>tap-</i> strains		
	MIC ₉₀ DOX	% DOX-non- susceptible ^a	MIC ₉₀ TIG	MIC ₉₀ DOX	% DOX-non- susceptible ^a	MIC ₉₀ TIG
<i>M. abscessus</i>	>64	0	1	>64	100	0.5
<i>M. chelonae</i>	32	41.7	0.25	64	77.8	0.5
<i>M. fortuitum</i>	64	68	0.25	64	64.3	0.5
<i>M.</i> <i>mageritense</i>	8	50	0.12	16	66.7	0.06
<i>M.</i> <i>mucogenicu</i> <i>m</i>	–	–	–	64	57.1	0.5
<i>M.</i> <i>peregrinum</i>	32	53.3	0.06	64	100	0.12
All strains	64	63.3	0.25	64	77.2	0.5

MIC₉₀, minimum inhibitory concentration for 90% of the organisms; DOX, doxycycline; TIG, tigecycline.

^a Resistant + intermediate-susceptible.