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Phenotypic and molecular characterization of quinolone resistance in *Mycobacterium abscessus* subsp. *bolletii* recovered from postsurgical infections

Vinicius Calado Nogueira de Moura,[1] Marlei Gomes da Silva,[2] Karen Machado Gomes,[2] Fábrice Santana Coelho,[3] Jorge Luiz Mello Sampaio,[4] Fernanda Carvalho de Queiroz Mello,[5] Maria Cristina da Silva Lourenço,[6] Efigênia de Lourdes Teixeira Amorim[7] and Rafael Silva Duarte[2]

Correspondence
Rafael Silva Duarte
rsduarte@ufrj.br

[1]Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

[2]Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[3]Hospital Universitário Pedro Ernesto, Rio de Janeiro, Brazil

[4]Instituto Fleury de Ensino e Pesquisa, São Paulo, Brazil

[5]Instituto de Doenças do Tórax, Rio de Janeiro, Brazil

[6]Instituto de Pesquisa Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

[7]Sérgio Franco Medicina Diagnóstica, Rio de Janeiro, Brazil

Several outbreaks of infections caused by rapidly growing mycobacteria (RGM) were reported in many Brazilian states (2032 notified cases) from 2004 to 2010. Most of the confirmed cases were mainly associated with *Mycobacterium massiliense* (recently renamed as *Mycobacterium abscessus* subsp. *bolletii*) BRA100 clone, recovered from patients who had undergone invasive procedures in which medical instruments had not been properly sterilized and/or disinfected. Since quinolones have been an option for the treatment of general RGM infections and have been suggested for therapeutic schemes for these outbreaks, we evaluated the *in vitro* activities of all generations of quinolones for clinical and reference RGM by broth microdilution, and analysed the peptide sequences of the quinolone resistance determining regions (QRDRs) of GyrA and GyrB after DNA sequencing followed by amino acid translation. Fifty-four isolates of *M. abscessus* subsp. *bolletii*, including clone BRA100, recovered in different states of Brazil, and 19 reference strains of RGM species were characterized. All 54 *M. abscessus* subsp. *bolletii* isolates were resistant to all generations of quinolones and showed the same amino acids in the QRDRs, including the Ala-83 in GyrA, and Arg-447 and Asp-464 in GyrB, described as being responsible for an intrinsic low level of resistance to quinolones in mycobacteria. However, other RGM species showed distinct susceptibilities to this class of antimicrobials and patterns of mutations contrary to what has been traditionally defined, suggesting that other mechanisms of resistance, different from *gyrA* or *gyrB* mutations, may also be involved in resistance to high levels of quinolones.

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INTRODUCTION

Rapidly growing mycobacteria (RGM) are common in the natural environment, especially in water sources, and may contaminate medical solutions and equipment (Brown-Elliott & Wallace, 2002). RGM have emerged as important human pathogens, and have been responsible for an

Abbreviations: QRDR, quinolone resistance determining region; RGM, rapidly growing mycobacteria.

The accession numbers for the sequences determined in this study are GU831598, GU831599, HQ285728, HQ285729–HQ285760 and HQ324096–HQ324105.

increasing number of health-care-associated infections (Brown-Elliott & Wallace, 2002; Cardoso *et al.*, 2008; De Groote & Huitt, 2006; Duarte *et al.*, 2009; Phillips & von Reyn, 2001; Viana-Niero *et al.*, 2008; Wallace *et al.*, 1998).

The species *Mycobacterium massiliense* was first described in 2004 (Adékambi *et al.*, 2004) and it has been responsible for several outbreaks of wound infections related to medical procedures in many states of Brazil (Cardoso *et al.*, 2008; Duarte *et al.*, 2009; Viana-Niero *et al.*, 2008). A taxonomic note has been recently published by Leão *et al.* (2011) reclassifying *Mycobacterium massiliense* and *Mycobacterium bolletii* as belonging to the same subspecies of *M. abscessus*, namely *Mycobacterium abscessus* subsp. *bolletii* as suggested by Sampaio (2010). This taxonomic note also reclassifies the previous *Mycobacterium abscessus* as *M. abscessus* subsp. *abscessus*.

The largest epidemiological event associated with RGM in Brazil occurred between 2006 and 2007, when 1051 notified possible cases of postsurgical infections were spread around 63 hospitals in the state of Rio de Janeiro. During this epidemic, isolates belonging to a single clone of *M. abscessus* subsp. *bolletii* (previously named *M. massiliense*), designated BRA100, showed highly similar patterns by PFGE to those recovered in previous outbreaks in a few Brazilian states (Duarte *et al.*, 2009). Randomly selected isolates showed resistance to ciprofloxacin (a second-generation fluoroquinolone), cefoxitin and doxycycline (Duarte *et al.*, 2009). Futhermore, moxifloxacin (a fourth-generation fluoroquinolone with strong activity against *Mycobacterium tuberculosis*) was strongly suggested as a potential substitute for therapeutic schemes to treat *M. abscessus* subsp. *bolletii* infections, although not evaluated before.

The quinolones represent an option for antimicrobial treatment and are active against RGM, especially against the *Mycobacterium fortuitum* group (Brown-Elliott & Wallace, 2002). Fourth-generation quinolones are important drugs used in ophthalmology and are more effective against Gram-positive bacteria compared to the other generations (Scoper, 2008; Stroman *et al.*, 2005). This generation has also been demonstrated to be more active against mycobacteria and less affected by mutations in DNA gyrase (Brown-Elliott *et al.*, 2002). Previous studies have demonstrated the importance of susceptibility testing for this generation of quinolones (Brown-Elliott *et al.*, 2002; Höfling-Lima *et al.*, 2005; Reddy *et al.*, 2010).

Mycobacteria are naturally less susceptible to quinolones than other bacteria such as *Escherichia coli* (Wolfson & Hooper, 1985), but the level of susceptibility to these drugs may differ considerably according to the mycobacterial species (Guillemin *et al.*, 1995, 1998; Leysen *et al.*, 1989; Li *et al.*, 2004; Yew *et al.*, 1994). Since topoisomerase IV has not been identified in the *Mycobacterium tuberculosis* genome (Cole *et al.*, 1998), it is assumed that DNA gyrase is the only target for the quinolones in mycobacteria. The interaction between DNA gyrase and the quinolones involves conserved regions known as quinolone resistance determining regions (QRDRs) of the *gyrA* and *gyrB* genes (Yoshida *et al.*, 1990, 1991).

In the *E. coli* numbering system, amino acids at positions 83 and 87 (90 and 94 in the *M. tuberculosis* numbering system) in the A subunit and at positions 426, 447 and 464 (495, 516 and 533 in the *M. tuberculosis* numbering system) in the B subunit of DNA gyrase are frequently substituted in strains with acquired resistance to quinolones in mycobacteria (Cambau *et al.*, 1994; Von Groll *et al.*, 2009; Guillemin *et al.*, 1995, 1998; Revel *et al.*, 1994; Takiff *et al.*, 1994; Wang *et al.*, 2007) and seem to play a key role in the drug–enzyme interaction. Preliminary studies reported intrinsic resistance to quinolones in mycobacterial species due to the presence of the residues Ala-83 in GyrA, and Arg-447 and Asn-464 in GyrB (Guillemin *et al.*, 1998), which differ from the residues Ser-83 in GyrA, and Lys-447 and Ser-464 in GyrB, found in more susceptible species such as *E. coli* (Yoshida *et al.*, 1990, 1991).

Due to the lack of data on the susceptibility of *M. abscessus* subsp. *bolletii* and some other RGM species to drugs, the aims of this study were to perform the phenotypic and molecular characterization of quinolone susceptibility of clinical strains of *M. abscessus* subsp. *bolletii* and other RGM species by determining the MIC of six quinolones (comprising all quinolone generations), including moxifloxacin, and to correlate it with amino acid variations in the QRDRs of GyrA and GyrB.

METHODS

Strains and growth conditions. The bacteria studied comprised 52 randomly selected clinical isolates of *M. abscessus* subsp. *bolletii* recovered from surgically infected sites in the states of Rio de Janeiro (48), São Paulo (1) and Goiás (3) obtained from clinical laboratories between 2004 and 2008; two epidemiologically unrelated isolates of *M. abscessus* subsp. *bolletii* isolated from sputum in the city of Rio de Janeiro; and 19 reference strains of RGM, namely *M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* CCUG 48898, *Mycobacterium agri* ATCC 27406, *Mycobacterium aichiense* ATCC 27280, *Mycobacterium aurum* ATCC 23366, *Mycobacterium chelonae* ATCC 14472, *M. chelonae* ATCC 35752, *Mycobacterium chitae* ATCC 19627, *Mycobacterium chubuense* ATCC 27278, *Mycobacterium flavescens* ATCC 14474, *Mycobacterium neoaurum* ATCC 25795, *Mycobacterium fortuitum* ATCC 6841, *Mycobacterium gadium* ATCC 27726, *Mycobacterium parafortuitum* ATCC 19686, *Mycobacterium phlei* ATCC 11758, *Mycobacterium porcinum* ATCC 33776, *Mycobacterium rhodesiae* ATCC 27024, *Mycobacterium smegmatis* ATCC 14468 and *Mycobacterium vaccae* ATCC 15783. The clinical isolates of *M. abscessus* subsp. *bolletii* were selected and identified by PRA-*hsp65* and *rpoB* sequencing, and typed by PFGE as previously described (Duarte *et al.*, 2009). For the performance of the following assays, the isolates were inoculated on Löwenstein–Jensen (LJ) or Mueller–Hinton agar and incubated at 36 °C (±1 °C) for 5 to 7 days.

Quinolone susceptibility testing. The susceptibility of all isolates to six quinolones, nalidixic acid (first generation), ciprofloxacin, ofloxacin and levofloxacin (second generation), sparfloxacin (third generation) and moxifloxacin (fourth generation) (all from Sigma-Aldrich) was determined by broth microdilution as recommended by the Clinical Laboratory Standards Institutes (CLSI) (CLSI, 2011).

Table 1. Interpretative criteria for MICs for *Enterobacteriaceae*, *Staphylococcus* spp. and *Streptococcus pneumoniae* (CLSI, 2010)

Antimicrobial agent	MIC (μg ml^{-1})								
	Enterobacteriaceae			*Staphylococcus* spp.			*Streptococcus pneumoniae*		
	S*	I	R	S	I	R	S	I	R
Nalidixic acid	⩽16	–	⩾32	–	–	–	–	–	–
Ofloxacin	⩽2	4	⩾8	⩽1	2	⩾4	⩽2	4	⩾8
Ciprofloxacin	⩽1	2	⩾4	⩽1	2	⩾4	–	–	–
Levofloxacin	⩽2	4	⩾8	⩽1	2	⩾4	⩽2	4	⩾8
Sparfloxacin	–	–	–	⩽0.5	1	⩾2	⩽0.5	1	⩾2
Moxifloxacin	–	–	–	⩽0.5	1	⩾2	⩽1	2	⩾4

*S, susceptible; I, intermediate; R, resistant.

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality-control strains. The susceptibility patterns for ciprofloxacin and moxifloxacin were determined according to the MIC breakpoints defined by CLSI for RGM (CLSI, 2011). For the other quinolones the interpretative criteria for MIC were based on the breakpoints described for *Enterobacteriaceae*, *Staphylococcus* spp. and *Streptococcus pneumoniae* (Table 1, CLSI, 2010).

Sequencing of the QRDRs of the *gyrA* and *gyrB* genes. DNA was obtained from cell lysates by suspending a loopful of recent growth on LJ in sterile distilled water, followed by thermal lysis for 10 min and freezing at −20 °C. The PCR amplification of *gyrA* was performed as previously described (Guillemin *et al.*, 1995) using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) and the primers Pri9 (5′-CGCCGCGTGCTSATGCRATG-3′) and Pri8 (5′-YGGTGGRTCRTTRCCYGGCGA-3′). The amplification of *gyrB* was performed as previously described (Guillemin *et al.*, 1995) using the same polymerase and the primers GyrbD (5′-CCGAYTGCCGTT-CSACGGAT-3′) and GyrbE (5′-CGGCCATCARCACGATCTTG-3′).

The amplicons of *gyrA* (216 bp) and *gyrB* (268 bp) were purified with the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare). After purification, 5 ng of each purified PCR product was sequenced by using an ABI PRISM 3700 DNA Analyser and a BigDye Terminator cycle sequencing kit (Applied Biosystems) (Otto *et al.*, 2008).

Sequence analysis. The sequences obtained were analysed with BioEdit Sequence Alignment Editor version 7.0.5.1 and compared with those deposited in the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was constructed using MEGA version 4.1. *M. tuberculosis* strain ATCC 25618 (H37Rv) was included as reference for sequence alignment and phylogenetic analysis.

RESULTS

Quinolone susceptibility testing

All 52 clinical isolates showed >85 % similarity among the patterns obtained by PFGE, and were named as belonging to *M. abscessus* subsp. *bolletii* BRA100 clone based on the criteria of Duarte *et al.* (2009). These isolates, as well as the two non-clonal ones (obtained from sputa) showed high but not identical MICs for all quinolones tested (range from 8 to >2048 μg ml^{-1}). The highest MICs were for nalidixic acid (Tables 2 and 3).

The quinolone susceptibility testing among reference strains showed variable susceptibility patterns. Considering the

Table 2. MIC patterns of six quinolones for 54 *M. abscessus* subsp. *bolletii* clinical isolates

Antimicrobial agent	MIC (μg ml^{-1})			
	Range	Mode*	MIC_{50}†	MIC_{90}‡
Nalidixic acid	–	>2048	>2048	>2048
Ofloxacin	>128–32	128	128	>128
Ciprofloxacin	>32–8	16	16	>32
Levofloxacin	>128–16	64	64	>128
Sparfloxacin	>128–32	128	128	>128
Moxifloxacin	32–8	16	16	32

*The value that occurred most frequently among the isolates tested.
†The MIC capable of preventing the growth of 50 % of the isolates.
‡The MIC capable of preventing the growth of 90 % of the isolates.

Table 3. Correlation between the MICs of quinolones against 19 reference strains of RGM and the amino acid residue at position 83 in the QRDR in the A subunit of DNA gyrase

Species	Representative strain	MIC (μg ml^{-1})*						Amino acid 83† in GyrA QRDR
		NAL	OFX	CIP	LVX	SPX	MOX	
Susceptible‡								
M. aichiense	ATCC 27280	16	1	⩽0.06	⩽0.25	⩽0.25	⩽0.25	Ser
M. aurum	ATCC 23366	4	⩽0.25	⩽0.06	0.25	0.25	0.5	Ser
M. chelonae	ATCC 35752	256	1	0.25	0.5	⩽0.25	⩽0.5	Ser
M. chitae	ATCC 19627	1024	2	0.5	8	0.5	⩽0.5	Ser
M. chubuense	ATCC 27278	4	⩽0.25	⩽0.06	⩽0.25	⩽0.25	⩽0.5	Ser
M. flavescens	ATCC 14474	2	⩽0.25	0.06	⩽0.25	⩽0.25	⩽0.5	Ser
M. fortuitum	ATCC 6841	64	⩽0.25	⩽0.06	⩽0.25	⩽0.25	⩽0.5	Ser
M. neoaurum	ATCC 25795	2	⩽0.25	⩽0.06	⩽0.25	⩽0.25	⩽0,5	Ser
M. parafortuitum	ATCC 19686	16	⩽0.25	0.06	⩽0.25	⩽0.25	⩽0.5	Ser
M. phlei	ATCC 11758	256	0.25	0.06	⩽0.25	⩽0.25	⩽0.5	Ala
M. porcinum	ATCC 33776	>2048	1	1	⩽0.25	⩽0.25	⩽0.5	Ser
M. rhodesiae	ATCC 27024	32	⩽0.25	0.06	⩽0.25	⩽0.25	⩽0.5	Ser
M. smegmatis	ATCC 14468	512	1	0.5	⩽0.25	⩽0.25	⩽0.5	Ala
M. vaccae	ATCC 15483	128	0.5	0.5	⩽0.25	⩽0.25	⩽0.5	Ser
Resistant‡								
M. abscessus subsp. *abscessus*	ATCC 19977	>2048	>128	16	128	128	16	Ala
M. abscessus subsp. *bolletii*	CCUG 48898	>2048	128	16	32	128	16	Ala
M. chelonae	ATCC 14472	512	32	16	16	8	2	Ala
M. gadium	ATCC 27726	>2048	64	32	64	64	16	Ser
M. agri	ATCC 27406	>2048	32	>32	64	64	4	Ser

*NAL, nalidixic acid; OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; SPX, sparfloxacin; MOX, moxifloxacin.
†Numbering system used for *E. coli*.
‡Based on MIC breakpoints for ciprofloxacin.

MIC interpretations for ciprofloxacin, the RGM fell into two groups: susceptible and resistant (Table 3). The susceptible group comprised *M. aichiense* ATCC 27280, *M. aurum* ATCC 23366, *M. chelonae* ATCC 35752, *M. chitae* ATCC 19627, *M. chubuense* ATCC 27278, *M. flavescens* ATCC 14474, *M. fortuitum* ATCC 6841, *M. gadium* ATCC 27726, *M. neoaurum* ATCC 25795, *M. parafortuitum* ATCC 19686, *M. phlei* ATCC 11758, *M. porcinum* ATCC 33776, *M. rhodesiae* ATCC 27024), *M. smegmatis* ATCC 14468 and *M. vaccae* ATCC 15483. The resistant group comprised *M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* CCUG 48898, *M. agri* ATCC 27406 and *M. chelonae* ATCC 14472. The strain for which the MIC was highest was *M. abscessus* subsp. *abscessus* ATCC 19977, and the lowest MIC was for *M. neoaurum* ATCC 25725 (Table 3). The reference strain CCUG 48898 of *M. abscessus* subsp. *bolletii* showed resistance to high levels of all quinolones, similar to the clinical isolates.

Nucleotide sequences of the *gyrA* and *gyrB* QRDRs

The nucleotide sequences of *gyrA* and *gyrB* QRDR differed among the mycobacterial species (Fig. 1); however in *gyrA* they were identical for all clinical isolates of *M. abscessus* subsp. *bolletii* and the reference strain CCUG 48898. The homology analysis of the *gyrA* nucleotide sequences is shown in Fig. 2.

The nucleotide sequences of the *gyrB* QRDR of *M. abscessus* subsp. *bolletii* showed four different patterns. In pattern 1 (P1), consisting of 50 BRA100 isolates and the two non-clonal ones, sequences (GU831599) were identical and showed 95.7 % homology to the CCUG 48898 strain (HQ324105). Sequences in pattern 2 (P2, HQ285758), consisting of two BRA100 isolates from Rio de Janeiro, were identical to the CCUG 48898 strain. Patterns 3 (P3, one BRA100 isolate from Rio de Janeiro, HQ285759) and 4 (P4, one BRA100 isolate isolated from Goiás, HQ285760) showed, respectively, 99.1 and 97.4 % homology to the CCUG 48898 strain (Fig. 3).

Peptide sequences of the GyrA and GyrB QRDRs

The peptide sequences of the GyrA and GyrB QRDRs obtained for RGM were aligned with the peptide sequences of *M. tuberculosis*, *E. coli* and *Staphylococcus aureus*, using

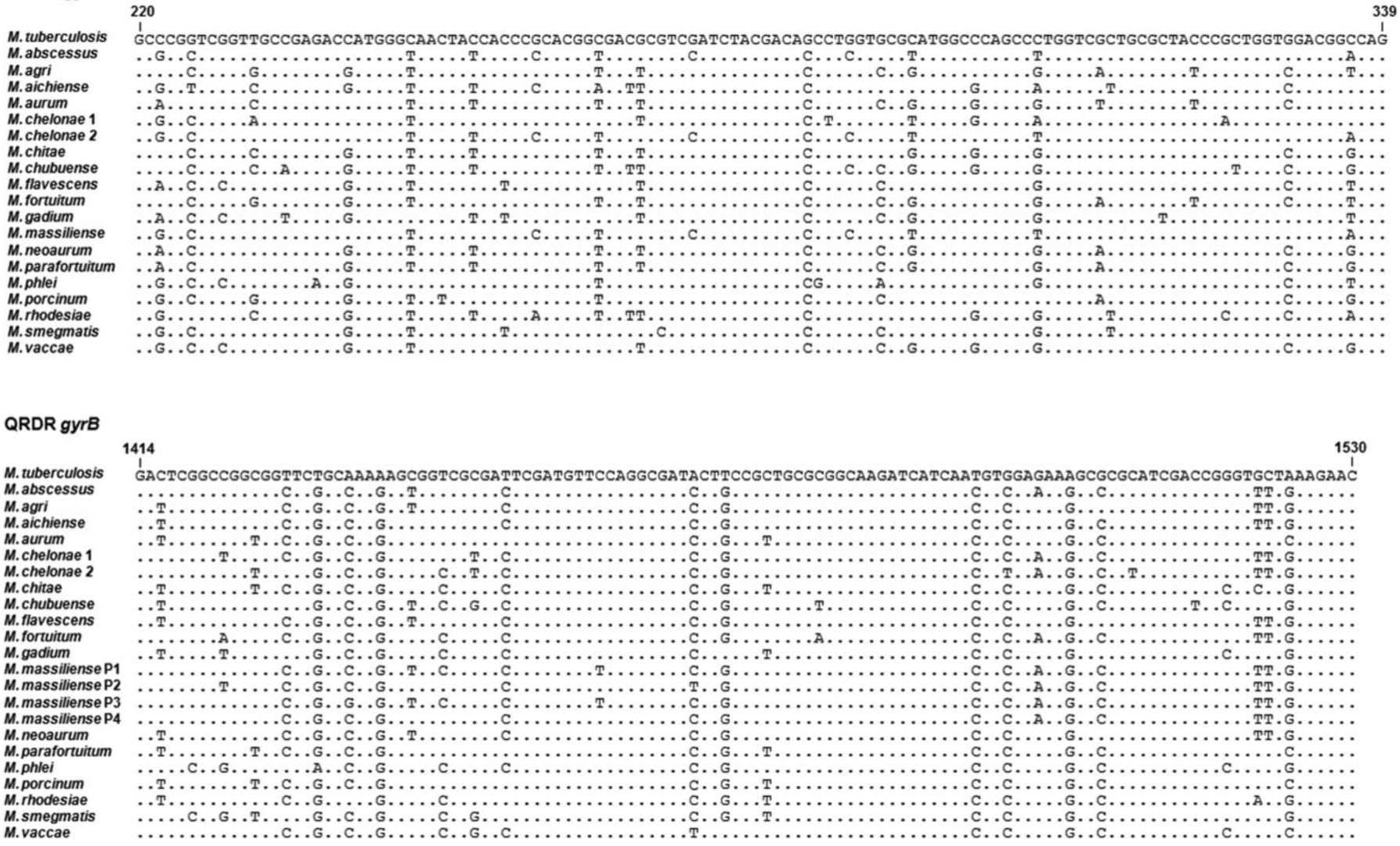

Fig. 1. Alignment of the nucleotide sequences of the QRDRs of *gyrA* and *gyrB* from species of RGM. Sequences extend from nucleotides 220 to 339 for *gyrA* and from nucleotides 1414 to 1530 for *gyrB*, in the numbering system used for *M. tuberculosis*. Sequences of *M. tuberculosis* were used as the reference and dots represent identical nucleotides. *M. chelonae* 1 represents ATCC 35752, *M. chelonae* 2 represents ATCC 14472; *M. abscessus* has been recently renamed as *M. abscessus* subsp. *abscessus*, and *M. massiliense* as *M. abscessus* subsp. *bolletii*.

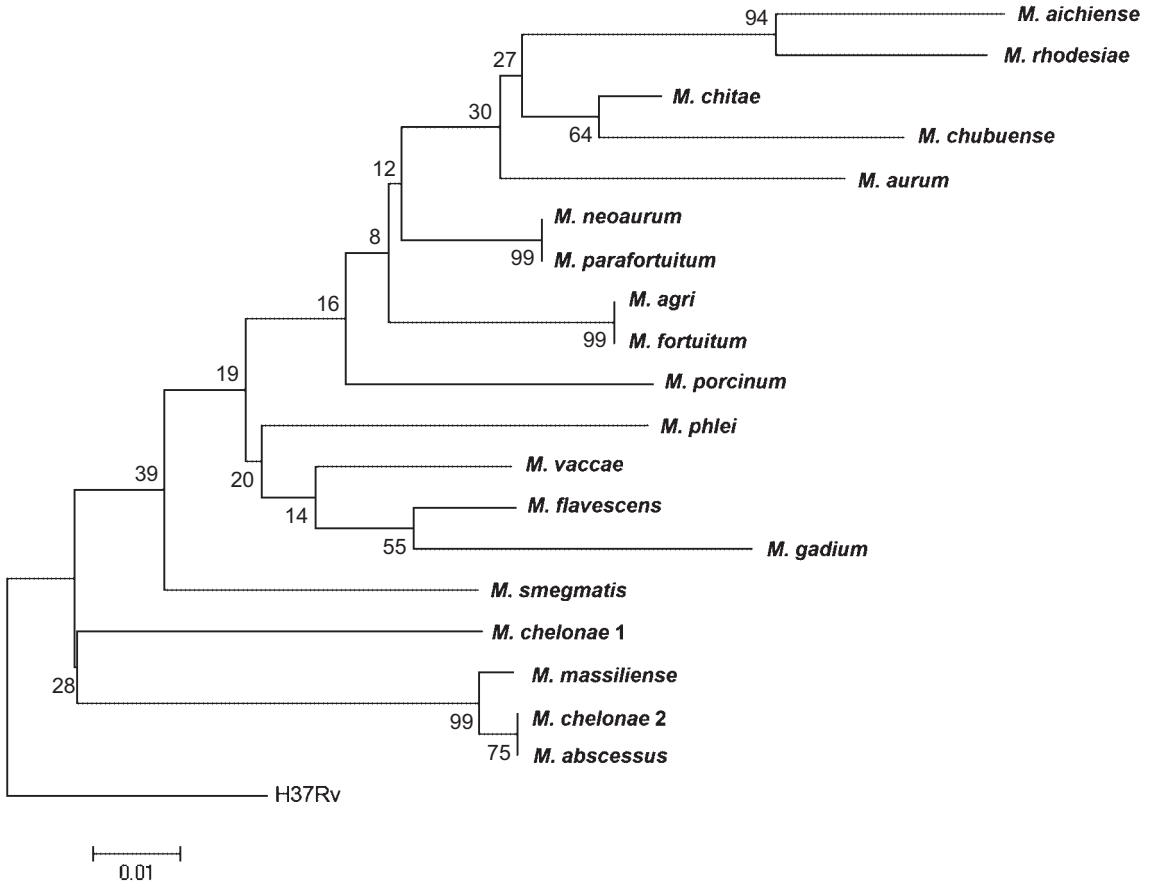

Fig. 2. Phylogenetic tree constructed by the neighbour-joining method using the nucleotide sequence of the *gyrA* QRDR. *M. chelonae* 1 represents ATCC 35752, *M. chelonae* 2 represents ATCC 14472; *M. abscessus* has been recently renamed as *M. abscessus* subsp. *abscessus*, and *M. massiliense* as *M. abscessus* subsp. *bolletii*. The numbers at the nodes are bootstrap values from 1000 resamplings.

the numbering system of the DNA gyrase of *E. coli* (Fig. 4). In all RGM strains, the peptide sequences were identical for the GyrB QRDR and showed Arg-447 and Asn-464, the same residues as found in *Staphylococcus aureus* and *Streptococcus pneumoniae*.

The peptide sequences of the GyrA QRDR were identical for all isolates of *M. abscessus* subsp. *bolletii* and showed Ala-83, also found in *Staphylococcus aureus* (Ito *et al.*, 1994) and *Streptococcus pneumoniae* (Pan *et al.*, 1996). Except for the Ser-83⟶Ala-83 variation, the peptide sequence of GyrA was identical for all RGM strains. Species that showed Ser-83 were *M. agri* ATCC 27406, *M. aichiense* ATCC 27280, *M. chubuense* ATCC 27278, *M. aurum* ATCC 23366, *M. chelonae* ATCC 35752, *M. chitae* ATCC 19627, *M. flavescens* ATCC 14474, *M. fortuitum* ATCC 6841, *M. gadium* ATCC 27726, *M. neoaurum* ATCC 25795, *M. parafortuitum* ATCC 19686, *M. porcinum* ATCC 33776, *M. rhodesiae* ATCC 27024 and *M. vaccae* ATCC 15483. Species that showed Ala-83 were *M. abscessus* subsp. *abscessus* ATCC 19977, *M. chelonae* ATCC 14472, *M. abscessus* subsp. *bolletii* CCUG 48898, *M. phlei* ATCC 11758 and *M. smegmatis* ATCC 14468.

The association between the MIC results and amino acid 83 in GyrA (Table 3) shows that the presence of Ser-83 and Ala-83 corresponded respectively to the susceptible and resistant profiles for most species. However, exceptions to this were observed in *M. phlei* and *M. smegmatis* strains, which contained Ala-83 and were classified as susceptible, and also in *M. agri* and *M. gadium*, which contained Ser-83 and were resistant to quinolones.

DISCUSSION

Over the past few years, RGM have played an important role as emerging health-care-associated pathogens (Brown-Elliott & Wallace, 2002; De Groote & Huitt, 2006; Phillips & von Reyn, 2001; Wallace *et al.*, 1998). An epidemic such as the one that occurred recently in different states of Brazil (see Introduction), had never been described before worldwide and it has been considered as an epidemiological emergency. Since RGM have been rising as a significant health problem, it is essential to evaluate their susceptibility patterns to current antimycobacterial drugs

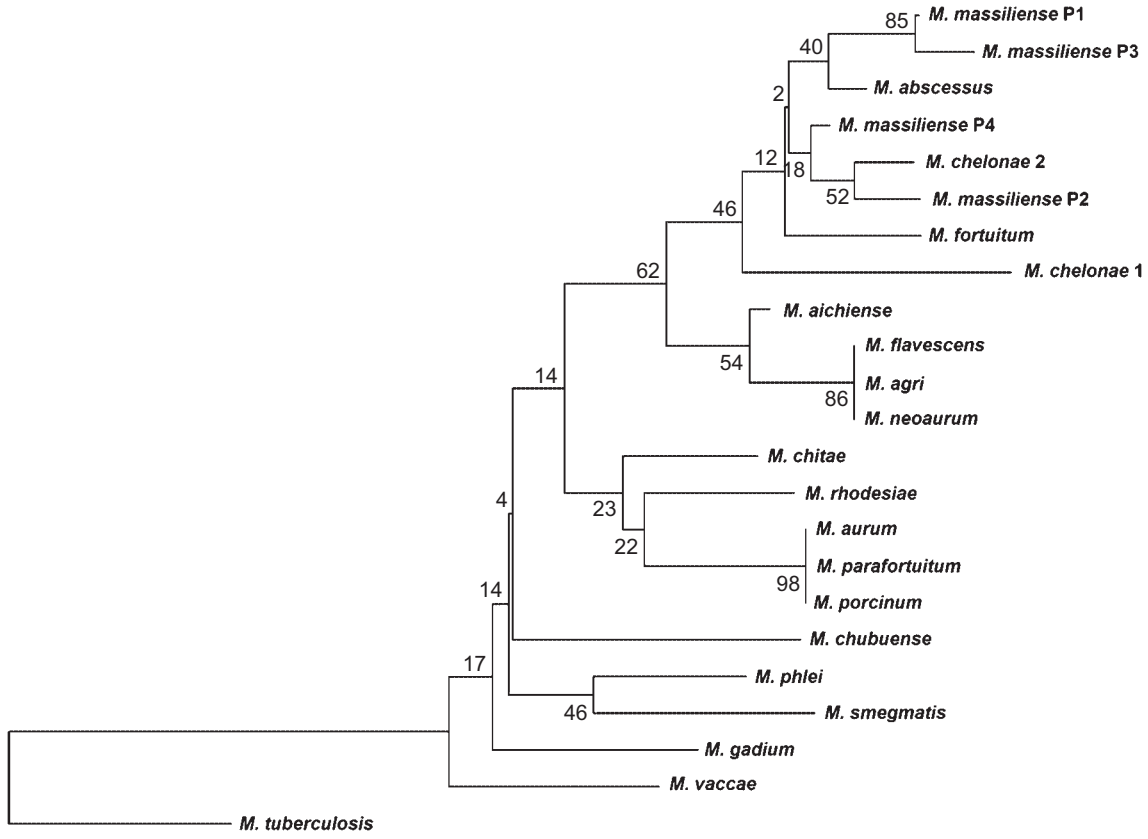

Fig. 3. Phylogenetic tree constructed by the neighbour-joining method using the nucleotide sequence of *gyrB* QRDR. *M. chelonae* 1 represents ATCC 35752, *M. chelonae* 2 represents ATCC 14472; *M. abscessus* has been recently renamed as *M. abscessus* subsp. *abscessus*, and *M. massiliense* as *M. abscessus* subsp. *bolletii*. The numbers at the nodes are bootstrap values from 1000 resamplings.

and to describe mechanisms of resistance to antimicrobial agents in order to improve treatment and drug choice.

In a previous study (Duarte *et al.*, 2009) resistance to ciprofloxacin was observed in some *M. abscessus* subsp. *bolletii* isolates recovered from postsurgical infections; however, the susceptibility of these strains to quinolones of other generations remained unknown until this present study. In the present study, most of the epidemic isolates were recovered from all hospitals with confirmed cases in the state of Rio de Janeiro, accounting for around 32 % of the total of isolates obtained (n=148), and additional representative strains from another two states with cases confirmed. During the characterization, these isolates exhibited clonal clustering by PFGE and were named as clone BRA100 (Duarte *et al.*, 2009; Leão *et al.*, 2010). Knowledge about potential emergence of *M. abscessus* subsp. *bolletii* and its antimicrobial resistance profile may contribute to systematic recovery of new isolates from different clinical sources and regions and improvement of their characterization.

The MIC results for nalidixic acid, ofloxacin, ciprofloxacin, levofloxacin, sparfloxacin and moxifloxacin demonstrated that quinolones of all generations were not inhibitory against any isolate of *M. abscessus* subsp. *bolletii*, including non-BRA100 isolates. The MIC results were similar to those found in previous studies (Guillemin *et al.*, 1995, 1998), and previously unknown quinolone susceptibility patterns of other reference strains were also characterized in the present study. Quinolones are widely used in Brazil for treatment of urinary tract, respiratory, gall bladder, skin and gonococcal infections, and also used prophylactically for urinary or general surgery. Additionally, moxifloxacin has been described as a possible alternative for treatment of tuberculosis, and *Mycobacterium avium* and *Bacteroides fragilis* infections. Incorrect prescription of these drugs in routine medical practice and their prophylactic use for gall-bladder surgery may occur. No data on prescriptions of quinolones for these purposes were available in this study.

It is important to mention that the CLSI have breakpoints for just two quinolones, ciprofloxacin and moxifloxacin, and the recommendations have been described only for *M. fortuitum* group (*M. fortuitum*, *M. peregrinum* and *M. fortuitum* third biovariant complex), *M. chelonae*, *M. abscessus* subsp. *abscessus*, *M. mucogenicum* and *M. smegmatis* group (*M. smegmatis*, *M. goodi* and *M. wolinskyi*). It was shown in this work that the breakpoints for the other

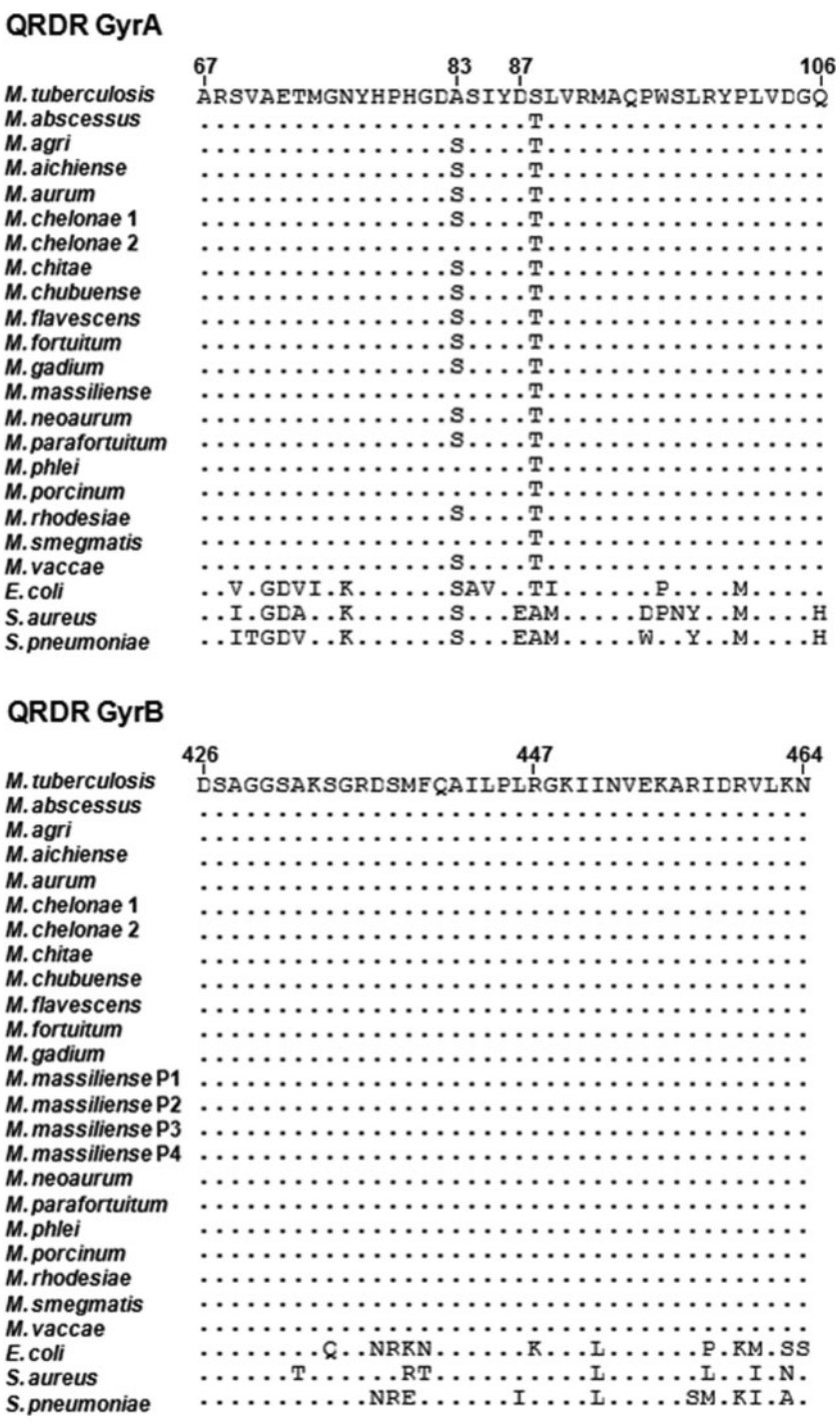

Fig. 4. Alignment of the peptide sequences of the QRDRs of GyrA and GyrB from species of RGM and from *E. coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Dots represent amino acids identical to those in *M. tuberculosis*. The GyrA QRDR extends from amino acid residues 67 to 106, and the GyrB QRDR extends from amino acid residues 426 to 464, in the numbering system used for *E. coli*. *M. chelonae* 1 represents ATCC 35752, *M. chelonae* 2 represents ATCC 14472; *M. abscessus* has been recently renamed as *M. abscessus* subsp. *abscessus*, and *M. massiliense* as *M. abscessus* subsp. *bolletii*.

quinolones described in the CLSI M100-S20 document for *E. coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (CLSI, 2010) were useful for determining the susceptibility pattern in mycobacterial species when compared to molecular results, suggesting a good applicability of these criteria to RGM. Since the previous CLSI M-24-A document for antimicrobial susceptibility testing in mycobacteria, published in 2003, new antimicrobial agents, including new generations of quinolones and additional mycobacterial species have been reported up to the newest version, published in 2011 (CLSI, 2011). So there is a need for continuous updating of this document, and information obtained in this study may be useful. In our study the susceptibility patterns for all generations of quinolones were precisely determined and reproducible for all RGM evaluated by using cation-adjusted Mueller–Hinton broth as recommended by CLSI.

The homology analysis demonstrated the high similarity between *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp.

abscessus for both *gyrA* and *gyrB*, but the RGM strains showed a different pattern for each tree. It is important to note that in *gyrA*, the sequences for clonal and non-clonal isolates were identical, whereas in *gyrB*, the clonal isolates had four different patterns (P1, P2, P3 and P4), even when comparing only the isolates belonging to the BRA100 clone. P2 was identical to the reference sequence of *M. abscessus* subsp. *bolletii* CCUG 48898, which may represent an intra- or inter-clone divergence between isolates. Possible reasons for this diversity being detected only in *gyrB* sequences are (i) different isolates from the same clone have suffered different evolutionary pressures, (ii) *gyrB* might play a different and possible minor role in the susceptibility of this species to quinolones or (iii) it represents a naturally hypervariable region of RGM genomes. Additionally, these results suggest that similar PFGE patterns of epidemiologically related isolates (BRA100, >85 % similarity) do not represent identical genome sequences, and even different phenotypic profiles may be observed in following studies evaluating distinct biological characteristics. Thus the concept of defining clonal strains for *M. abscessus* subsp. *bolletii* based only on PFGE patterns may be assumed with restrictions.

It was also observed that *M. chelonae* ATCC 14472 showed 100 % (*gyrA*) and 99 % (*gyrB*) homology to *M. abscessus* subsp. *bolletii* CCUG 50184 by using BLAST analysis. Furthermore, the *rpoB* sequence of this strain showed high similarity to *M. abscessus* subsp. *bolletii* (unpublished data). These results may suggest that this strain is in fact *M. abscessus* subsp. *bolletii* and not *M. chelonae*.

Considering the peptide sequences of GyrA, variation in residue 83 was found in the QRDR in the RGM species. Less susceptible species of RGM studied in this work, including *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, had Ala instead of Ser in this position, the same residues as found in less susceptible species such as *Staphylococcus aureus* (Ito *et al.*, 1994) and *Streptococcus pneumoniae* (Pan *et al.*, 1996). However, contrary to what has been traditionally described, *M. agri* and *M. gadium* were clear exceptions, since they were resistant to quinolones yet had Ser at this locus. This suggests that the variation between Ala and Ser in residue 83 may not be the unique determinant for a resistant or susceptible phenotype. The opposite was also observed and supports this hypothesis: strains phenotypically susceptible to quinolones, such as *M. phlei* and *M. smegmatis*, had Ala as residue 83 in the GyrA QRDR. Previous authors hypothesized that the differences in the structures between Ser and Ala should be related to intrinsic resistance in some species, such as *M. abscessus* subsp. *bolletii*. It is thought that Ala cannot provide a hydrogen bond because of its lack of a hydroxyl radical, providing the resistant profile (Guillemin *et al.*, 1995), but this does not explain the variations observed in the present study.

The peptide sequences of QRDR of GyrB were identical in all RGM tested and contained the residues Arg-447 and Asn-464, which also seem to be associated with lesser susceptibility to quinolones, compared to other bacteria. These residues differ from those found in *E. coli* (Lys-447 and Ser-464), a more susceptible species. Guillemin *et al.* (1998) argued that since Arg is bulkier than Lys and has an additional positive charge, and Asn is non-hydroxylated and bulkier than Ser, their presence could decrease the interaction between the DNA gyrase and the drug.

A recent study of *gyrA* and *gyrB* mutations in ciprofloxacin-resistant *M. abscessus* subsp. *bolletii* isolated from an outbreak in southern Brazil showed the substitution Ala-90→Val-90 (Ala-83→Val-83 using the *E. coli* numbering system) in the QRDR of GyrA in 88.6 % (31/35) of the isolates. This study also showed that 11.4 % (4/35) of the isolates did not show this substitution and were resistant to ciprofloxacin. The isolates from this outbreak had a PFGE profile characteristic of the BRA100 clone (Monego *et al.*, 2011). The BRA100 isolates studied in the present work did not show the substitution Ala-83→Val-83 and they were also resistant to quinolones of all generations, not only to ciprofloxacin, suggesting that (i) the BRA100 clone as defined by PFGE criteria does not consist of isolates with the same genomic sequence, as previously discussed in our work; (ii) resistance to quinolones may involve not only mutations in QRDR of *gyrA* and *gyrB* but also other mechanisms of resistance.

Mechanisms of antimicrobial resistance besides the classic low cell wall permeability have already been described for mycobacteria and they involve porins (Danilchanka *et al.*, 2008; Svetlíková *et al.*, 2009; Stephan *et al.*, 2004), efflux pumps (Li *et al.*, 2004; Liu *et al.*, 1996; Louw *et al.*, 2009) and a pentapeptide MfpA, which plays a role in quinolone resistance in *M. smegmatis* (Montero *et al.*, 2001). It has been recently proposed that a single mechanism which confers a low level of resistance to quinolones should not be underestimated and the combination of several mechanisms could explain the level of resistance achieved in clinical isolates (Hernández *et al.*, 2011). This might be the explanation for the high levels of resistance observed in *M. massiliense* and related RGM. Those mechanisms should also be studied and associated with the MIC results in order to complete the characterization of quinolone resistance.

In conclusion, even though quinolones of different generations may be effective against some species of RGM, they should not be used to treat *M. abscessus* subsp. *bolletii* infections due to the resistance to high levels found in this subspecies. The variation between Ser and Ala at position 83 may play a role in reduced susceptibility to quinolones in mycobacteria. However, this does not explain the high levels of resistance to all generations of quinolones in *M. abscessus* subsp. *bolletii* and related species, since the presence of Ala-83 in the GyrA QRDR is expected in some species of mycobacteria, including species susceptible to ciprofloxacin (Guillemin *et al.*, 1995, 1998), and no other amino acid change was observed in both

genes. Other mechanisms might be present in those species to explain such resistance. The present study represents an important aid to the knowledge about mechanisms of quinolone resistance in RGM and for future review of susceptibility criteria.

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