Pereira et al. BMC Microbiology (2019) 19:62
https://doi.org/10.1186/s12866-019-1428-4

BMC Microbiology

RESEARCH ARTICLE Open Access

Studies of antimicrobial resistance in rare mycobacteria from a nosocomial environment

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Abstract

Background: Nontuberculous mycobacteria (NTM) are ubiquitous in nature and recognized agents of opportunistic infection, which is often aggravated by their intrinsic resistance to antimicrobials, poorly defined therapeutic strategies and by the lack of new drugs. However, evaluation of their prevalence in anthropogenic environments and the associated antimicrobial resistance profiles have been neglected. In this work, we sought to determine minimal inhibitory concentrations of 25 antimicrobials against 5 NTM isolates recovered from a tertiary-care hospital surfaces. Antimicrobial susceptibilities of 5 other *Corynebacterineae* isolated from the same hospital were also determined for their potential clinical relevance.

Results: Our phylogenetic study with each of the NTM isolates confirm they belong to *Mycobacterium obuense, Mycobacterium mucogenicum* and *Mycobacterium paragordonae* species, the latter initially misidentified as strains of *M. gordonae*, a species frequently isolated from patients with NTM disease in Portugal. In contrast to other strains, the *M. obuense* and *M. mucogenicum* examined here were resistant to several of the CLSI-recommended drugs, suggestive of multidrug-resistant profiles. Surprisingly, *M. obuense* was susceptible to vancomycin. Their genomes were sequenced allowing detection of gene *erm* (erythromycin resistance methylase) in *M. obuense*, explaining its resistance to clarithromycin. Remarkably, and unlike other strains of the genus, the *Corynebacterium* isolates were highly resistant to penicillin, ciprofloxacin and linezolid.

Conclusions: This study highlights the importance of implementing effective measures to screen, accurately identify and control viable NTM and closely related bacteria in hospital settings. Our report on the occurrence of rare NTM species with antibiotic susceptibility profiles that are distinct from those of the corresponding Type strains, along with unexpected resistance mechanisms detected seem to suggest that resistance may be more common than previously thought and also a potential threat to frail and otherwise vulnerable inpatients.

Keywords: Nontuberculous mycobacteria (NTM), *Mycobacterium mucogenicum*, *Mycobacterium obuense*, *Mycobacterium paragordonae*, *Corynebacterineae*, Antimicrobial resistance

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Background

Hospitals are major sources of infectious agents with 7 to 10% of all inpatients estimated to develop at least one hospital associated infection (HAI) during their admission [1–3]. In addition to the debilitated health conditions rendering patients more susceptible to infections, hospital environments represent added risks inflicted by antibiotic resistant opportunistic pathogens [3, 4]. The World Health Organization (WHO) recently issued its first ever list of antibiotic-resistant 'priority pathogens,' the most prevalent and antibiotic resistant bacterial pathogens associated with nosocomial infections [5]. WHO emphasized "Mycobacteria was not subjected to review for inclusion in this prioritization exercise as it is already a globally established priority for which innovative new treatments are urgently needed" [5]. Most *Mycobacterium* species are environmental saprophytes designated nontuberculous mycobacteria (NTM) to be distinguished from those that cause tuberculosis [6]. Their ability to infect humans and to colonize man-made environments including hospitals with inadequate disinfection of water distribution systems, as well as an overall resistance to antibiotics and high prevalence of risk factors in the population namely chronic diseases and advanced age, all merge into a potential and serious health threat [7]. The growing number of NTM infections in the lung, soft tissue or skin of susceptible individuals, combined with their known association with nosocomial infections and outbreaks, has brought up a general awareness towards this bacterial group [7]. While clinical laboratories routinely screen for the presence of some important pathogens others are often neglected. These include fastidious and slowly growing pathogens or opportunists. For example, the clinical significance of NTM isolation besides the most prominent pathogens of the group (such as MAC or *M. abscessus*) is not totally understood, especially in lung disease. Most NTM are non pathogenic for healthy people, but almost all can be responsible for opportunistic infections in susceptible individuals. In cases of isolation of NTM usually considering normal colonizers or contaminants, patients require careful clinical evaluation, taking into account factors such as the patient's immunologic status and the site of infection to determine the significance of the isolate [8, 9]. Potential pathogens can colonize hospital settings but the awareness of their presence is low because they are not routinely screened. In fact, there exists a bias in screening since only about 2% of microorganisms grow well in standard clinical culture media and are considered significant [10]. Some of the undetected pathogens emerge as cause of atypical infections frequently associated with relevant drug resistance, highlighting the necessity for urgent measures for their routine detection and control. For example, contamination of ICU inanimate surfaces and equipment has been identified as a contributing source of transmission of pathogens to ICU patients in outbreaks [11].

Members of the genus *Mycobacterium*, which in December 2018 comprised 198 valid species [12], are among a restricted gallery of the most resilient organisms we know of as they can withstand multiple stress conditions such as high temperature, oxidative stress, nutrient deprivation and prolonged desiccation [6]. Other related bacteria from the suborder *Corynebacterineae*, namely *Corynebacterium* spp., *Nocardia* spp., *Rhodococcus* spp. and *Gordonia* spp. can also be the cause of atypical infections in susceptible individuals but their routine identification has been neglected in the clinical practice as well [13, 14].

The aim of this study was to address the extent of antimicrobial resistance in strains of NTM and other *Corynebacterineae* isolated from a nosocomial environment. Although health authorities neglect the fact that the prevalence of NTM infection is seriously underestimated in the European Union in general [15] and in Portugal in particular [16], only a strong commitment to NTM research will allow proportional responses to this health threat.

Results

Distribution of NTM and other actinobacterial isolates, identification and phylogenetic studies

Samples were collected from different sites of 4 hospital wards in 3 sampling events as previously described [17]. All isolates were recovered after 2 or 4 weeks of incubation in Middlebrook 7H10-PANTA medium and none after 6 weeks incubation. Of the actinobacterial isolates 10 belonged to the suborder *Corynebacterineae,* their correct phylogenetic identification and antibiotic susceptibility pattern were the focus of the present study, to address the extent of antimicrobial resistance in *Corynebacterineae* isolated from a nosocomial environment. The other 24 isolates belonging to genera *Dermacoccus*, *Kocuria*, *Microbacterium* and *Micrococcus* (all non-*Corynebacterineae*) were not identified to the species level and their antibiotic susceptibilities were not examined in this study.

As inferred from 16S rRNA phylogenetic analyses, 3 isolates of the 10 *Corynebacterineae* were related to species *Corynebacterium jeikeium*, *C. amycolatum* and *C. imitans* and other 2 isolates were closely related to species *Gordonia otitidis* and *G. sputi* (Table 1, Fig. 1).

Five of the *Corynebacterineae* were classified as members of the genus *Mycobacterium*, as determined from a phylogenetic tree of concatenated 16S rRNA, *hsp65* and *rpoB* genes (Fig. 2). One isolate was closely related to the species *M. mucogenicum*, other to *M. obuense* and 3 isolates were closely affiliated to the slowly growing species *M. paragordonae*, all nontuberculous mycobacteria (NTM) (Table 1, Fig. 2). Since *M. avium* is a commonly isolated NTM but was not detected in this study, control growth experiments to assess possible inhibition of growth

Table 1 NTM and other *Corynebacterineae* members isolated from different hospital sites (adapted from [16]). Phylogenetic trees in the present study confirm that isolates 10AIII, 29AIII and 35AIII are probably *M. paragordonae*

Isolate	Closely related species	Ward	Amenity
1AIII	*Gordonia otitidis*	Hematology	Restroom light switch
6FIII	*Corynebacterium jeikeium*	Hematology	Bed table
10AIII	*Mycobacterium paragordonae*	Hematology	Therapy room bench
22DIII	*Mycobacterium obuense*	Urology	Restroom sink
24AIII	*Mycobacterium mucogenicum*	Urology	Restroom light switch
29AIII	*Mycobacterium paragordonae*	Renal Transplant Unit	Therapy room bench
35AIII	*Mycobacterium paragordonae*	Renal Transplant Unit	Bed
52AIII	*Corynebacterium amycolatum*	Medicine A	Bed table
55AIII	*Gordonia sputi*	Medicine A	Bed hand support
58FIII	*Corynebacterium imitans*	Medicine A	Bed table light switch

by Middlebrook 7H10-PANTA were performed with some clinical *M. avium* isolates available in our collection, which ruled out such possibility (results not shown). *Corynebacterium* and *Gordonia* isolates were recovered from the Hematology and Medicine A wards (Table 1). NTM isolates were recovered from Hematology (*M. paragordonae*, 10AIII), Urology (*M. obuense*, 22DIII and *M. mucogenicum*, 24AIII), and Renal Transplant Unit (*M. paragordonae*, 29AIII and 35AIII) wards as previously described [17]. All but the *M. obuense* isolate were collected from dry surfaces/equipment.

Determination of minimal inhibitory concentrations (MIC) of antimicrobials

MIC values of the 25 antimicrobials tested are indicated in Table 2. Considering the Clinical & Laboratory Standards Institute (CLSI) susceptibility interpretation for the antimicrobials included in the standards, the *M. obuense* isolate exhibited higher resistance levels and was the single isolate resistant to clarithromycin, which could be explained by the presence of the gene *erm* (erythromycin resistance methylase, accession number MG770427) in the draft genome sequenced in this study. On the other hand, *tet(V)* genes associated to tetracycline resistance were detected in the *M. obuense* and *M. mucogenicum* isolates (accession numbers MG770425 and MG770428, respectively) but only the latter was resistant to this drug (Table 2). *Mycobacterium paragordonae* isolates ($n = 3$) were susceptible to amikacin, ciprofloxacin, clarithromycin and linezolid, four of the antimicrobials recommended by CLSI to test drug susceptibility of slowly growing mycobacteria [18]. MICs for the other antimicrobials tested were not possible to interpret. All *Corynebacterium* isolates were resistant to ciprofloxacin, linezolid, penicillin and to imipenem, although the latter is recommended for *Corynebacterium* susceptibility testing despite the fact that no interpretative criteria are available (Table 2). Both *Gordonia* isolates were resistant to imipenem while only one of them (55AIII) was resistant to ciprofloxacin. These *Corynebacterineae* isolates were used in the susceptibility study to broaden the information about drug resistance in this particular phylogenetic group, as the information available is still extremely limited. High MIC values were observed for three of the PANTA antimicrobials tested against *Corynebacterium* and *Gordonia* isolates. On the other hand, azlocillin and polymyxin B showed the lowest MICs (Table 2).

All tested aminoglycosides inhibited the in vitro growth of NTM and of the other *Corynebacterineae*, even at their lowest tested concentrations. Tobramycin, one of the two aminoglycosides considered in CLSI standards for NTM susceptibility testing [18] was an exception, with the rapidly growing *M. obuense* and *M. mucogenicum* isolates displaying resistant profiles (Table 2). High MIC values were obtained for spectinomycin, with all isolates being able to grow in the range of 4 to 64 μg/mL with the exception of one *Gordonia* isolate (1AIII) (MIC < 2 μg/mL).

Although amphotericin B is an antifungal agent and no growth inhibition was expected for the bacterial isolates under study, the fact is that four of the NTM isolates were inhibited at the two highest amphotericin B concentrations tested (Table 2). Divergent results between NTM and other *Corynebacterineae* were also observed regarding chloramphenicol and azlocillin with all NTM appearing to be resistant to these antibiotics, while MIC values for *Corynebacterium* and *Gordonia* isolates were in general much lower. *Corynebacterium* isolates were susceptible to vancomycin and *Gordonia* isolates presented similarly low MIC values. As expected with vancomycin MIC values for the NTM isolates were high, except for *M. obuense* that was surprisingly susceptible to this antibiotic and also to polymyxin B (Table 2). All of the NTM and *Gordonia* isolates were susceptible to linezolid. *Corynebacterium* isolates 6FIII and 58FIII were extremely resistant to penicillin (> 512 and 32 μg/mL) (Table 2).

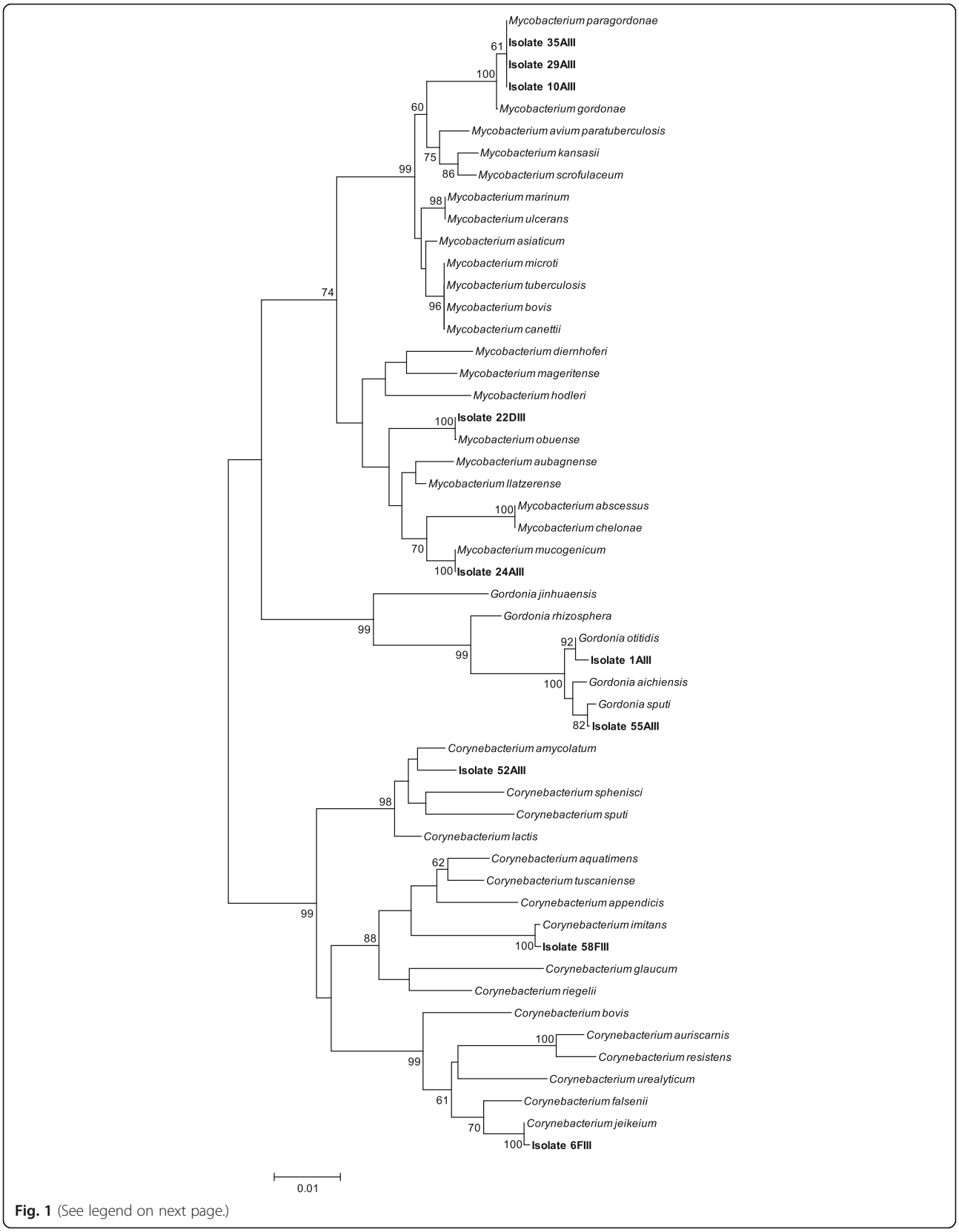

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of isolates and their closest phylogenetic relatives belonging to the *Corynebacterineae* genera *Mycobacterium*, *Corynebacterium* and *Gordonia* (16S rRNA gene sequences of type strains available from GeneBank) (also see Additional file 1: Figure S1, Additional file 2: Figure S2 and Additional file 3: Figure S3). The tree was created using the neighbor-joining method. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 1 inferred nucleotide substitutions per 100 nt

Results for ciprofloxacin, the only fluoroquinolone tested, ranged from high susceptibility in the 3 slowly growing NTM to the resistant phenotypes of the 2 rapidly growing NTM isolates (Table 2). Interestingly, high MIC values for ciprofloxacin were also obtained for the *Corynebacterium* isolates while the 2 *Gordonia* isolates had opposing results, one showed the lowest (< 0.125 μg/mL) and the other had the highest (16 μg/mL) MIC values measured.

Discussion

Nosocomial infections are a major concern worldwide and represent an increase in hospital stay and treatment costs, particularly if associated with drug resistant pathogens [1]. The literature refers that probable dissemination vehicles between the pathogen niche (water faucets, medical instruments, fomites) and patients are mainly the healthcare providers [19].

Different wards of one hospital were sampled [17] and one third of the Actinobacterial isolates found belonged to the suborder *Corynebacterineae*, namely *Corynebacterium amycolatum* and *C. jeikeium*, *Gordonia otitidis* and *G. sputi* or *Mycobacterium mucogenicum*, all of which include potentially pathogenic strains previously implicated in human infections [20–22]. Of note was the fact that all samples were collected from sites with frequent human contact, placing these opportunistic pathogens easily accessible to patients, visitors and to healthcare providers. The surfaces of hospital amenities are considered important sources of pathogenic agents transmission [23] and the prevailing consensus indicates NTM opportunistic infections have environmental origin, although human-to-human transmission of *M. abscessus* is a factor of dissemination in cystic fibrosis patients [24, 25]. Isolation of *M. mucogenicum* has been mainly associated with hospital water distribution systems [26–31]. However, the isolate belonging to this species was recovered from a dry surface as were all the NTM strains studied here except the *M. obuense* isolate. Although *M. avium* strains are frequently isolated from waters, plumbing and showerheads biofilms, they are not, to the best of our knowledge, recovered frequently from surfaces such as those sampled in this study also possibly because their tolerance to desiccation appears to be low [32, 33].

The *Mycobacterium* strains recovered appeared to be relatively rare [17]. However, because their identification was initially based on 16S rRNA sequences alone, the putative *M. gordonae* isolates were now confirmed to be more closely related to the species *M. paragordonae* after concatenation of partial sequences of genes 16S rRNA, *rpoB* and *hsp65*, which provided a stronger species association confirmed by the construction of the corresponding phylogenetic tree. The *M. paragordonae* species was originally described in 2014 based on a clinical isolate from a patient with a pulmonary infection [34]. Since then *M. paragordonae* was only isolated twice, both from healthcare settings [35, 36]. The NTM species more frequently isolated from patients in Portugal in the last years were those in the *M. avium* complex, *M. gordonae* and *M. kansasii* [37]. Interestingly, our phylogenetic study shows that the three presumptive isolates initially identified as *M. gordonae* based on 16S rRNA sequencing [17], are in fact members of the recently described and rarely isolated species *M. paragordonae*, which raises questions about the true identity of clinical *M. gordonae* isolates, one of the species often recovered from patients in Portugal [37].

Mycobacterium obuense on the other hand appears to be common in soils and plants [38–40], but has been only rarely isolated from clinical samples [41–44] and, to our knowledge, there has been only one report of its isolation from a hospital environment [35]. Still, both species have been isolated from sputum of patients with pulmonary infections but, as is often the case for rarely isolated NTM [45], their clinical relevance remains uncertain. In addition to its clinical relevance and ability to cause a range of infections, *M. mucogenicum* has also been commonly implicated in nosocomial outbreaks [21, 46]. Indeed, its presence in the healthcare environment, if persistent, may pose a risk for patients. Although more prevalent than *M. paragordonae* and *M. obuense*, *M. mucogenicum* is still a rarely isolated species [15, 44].

The *M. mucogenicum* isolate showed a multidrug resistance (MDR) profile, at least to 4 different classes of antimicrobials (fluoroquinolones, tetracyclines, aminoglycosides and sulfonamides). This differs from what has been reported for the type strain *M. mucogenicum* DSM44625, which was found to be susceptible to ciprofloxacin, doxycycline and sulfamethoxazole [47], unlike the isolate in this study which was resistant to these 3 antibiotics. Furthermore, van Ingen and colleagues tested 15 *M. mucogenicum* clinical strains against a panel of 11 antibiotics and found the majority to be susceptible to rifabutin, amikacin, ciprofloxacin and clarithromycin [48]. The *M. obuense* isolate, also exhibited a MDR profile namely to ciprofloxacin,

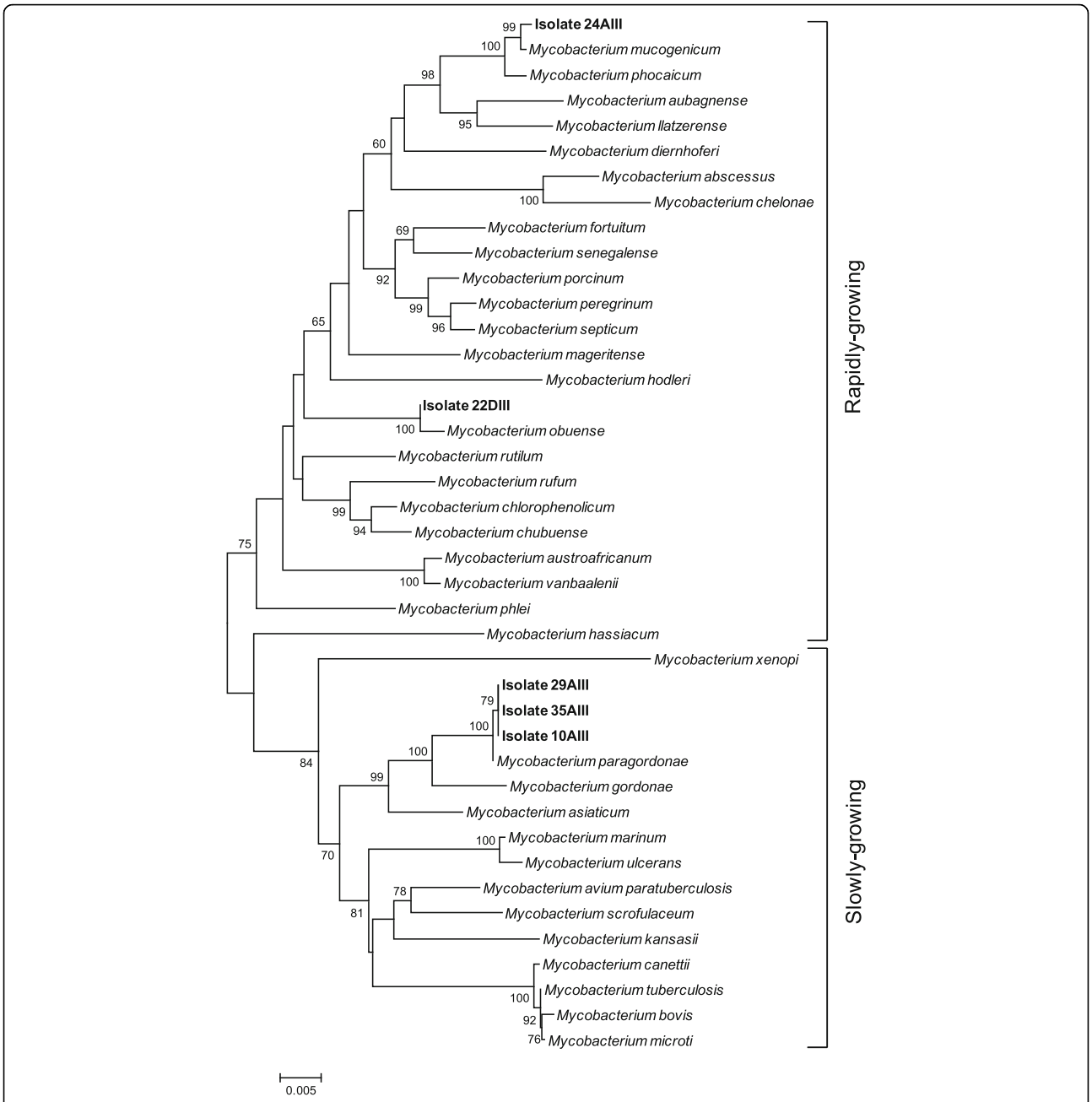

Fig. 2 Phylogenetic tree computed from the concatenated nucleotide sequences of 16S rRNA, *hsp65* and *rpoB* from *Mycobacterium* isolates and from strains selected from databases, using the neighbor-joining algorithm. The tree includes 41 strains after checking the congruence from each single-gene tree (see Additional file 1: Figure S1, Additional file 4: Figure S4 and Additional file 5: Figure S5). The evolutionary distances were calculated by the Jukes and Cantor method [61]. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 5 inferred nucleotide substitutions per 1000 nt

clarithromycin, imipenem and to tobramycin, unlike the *M. obuense* type strain ATCC27023 [47] and unlike a clinical isolate [48] both susceptible to ciprofloxacin, cefoxitin, tobramycin and clarithromycin. In our study, the *M. obuense* isolate was the only NTM resistant to clarithromycin. We have sequenced the genomes of the 5 NTM recovered (unpublished results), and *M. obuense* was the only to possess a classical *erm* gene [49]. To our knowledge, no *M. paragordonae* strains have been tested for antibiotic susceptibility prior to this work and the isolates tested here were susceptible to 4 of the CLSI antibiotics recommended for slowly growing mycobacteria. Thus, 2 of the 5 NTM isolated in this study presented MDR profile, and were more drug resistant than the previously isolated strains of the same species. Multidrug-resistant NTM have been described in the literature but not originating from the hospital

Table 2 MIC values [μg/mL (U/mL for polymyxin B)] of 25 antimicrobials used for susceptibility testing of NTM and other *Corynebacterineae*

Antibiotics	Concentration range (μg/mL)	*Mycobacterium* sp. isolates					*Gordonia* sp. isolates		*Corynebacterium* sp. isolates		
		Rapidly-growing		Slowly-growing							
		22DIII	24AIII	10AIII	29AIII	35AIII	1AIII	55AIII	6FIII	52AIII	58FIII
		M. obuense	*M. mucogenicum*	*M. paragordonae*							
Amikacin	2 – 256	8	<2	<2	<2	<2	<2	<2	<2	<2	<2
Cefoxitin	2 – 256	32	16	16	8	16	64	16	>256	8	32
Ciprofloxacin	0.125 – 16	8	4	0.25	<0.125	<0.125	<0.125	16	>16	4	8
Clarithromycin	0.0625 – 64	16	1	2	1	1	1	2	>64	2	>64
Doxycycline	0.5 – 64	1	>64	4	4	2	1	<0.5	<0.5	1	1
Imipenem*	1 – 128	32	2	ND	ND	ND	>128	>128	>128	>128	>128
Linezolid	0.5 – 64	4	4	8	4	2	8	4	16	4	8
Tobramycin	1 – 128	32	16	16	8	8	<1	<1	4	<1	<1
Amphotericin B	4 – 512	256	>512	64	256	64	>512	>512	>512	>512	>512
Azlocillin	4 – 512	64	64	32	16	64	8	8	8	8	8
Nalidixic acid	16 – 2048	64	64	128	>2048	64	128	512	1024	256	512
Polymyxin B	0.04 – 5.12	0.32	5.12	5.12	5.12	5.12	0.16	0.16	0.08	0.16	0.16
Trimethoprim	*4 – 512*	32	16	256	256	64	64	64	512	32	32
Ampicillin	2 – 256	>256	64	256	256	256	<2	<2	>256	<2	8
Chloramphenicol	*4 – 512*	16	8	256	32	8	<4	<4	<4	<4	16
Gentamicin	1 – 128	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Kanamycin	1 – 128	<1	<1	<1	<1	<1	<1	<1	4	<1	<1
Neomycin	2 – 256	<2	<2	<2	8	<2	<2	<2	<2	<2	<2
Penicillin	0.0156 - 512	ND	ND	ND	ND	ND	0.25	0.5	>512	0.25	32
Rifampicin	1 – 128	<1	<1	8	<1	<1	<1	<1	<1	2	<1
Spectinomycin	2 – 256	16	64	32	64	32	<2	4	16	8	16
Streptomycin*	1 – 128	8	4	2	2	<1	<1	<1	<1	2	<1
Sulfamethoxazole	1 – 128	16	>128	32	16	2	64	128	>128	32	>128
Tetracycline	*0.5 - 64*	<0.5	32	ND	ND	ND	<0.5	2	<0.5	<0.5	<0.5
Vancomycin	0.0625 - 512	<4	32	32	32	32	1	2	0.5	0.5	0.25

Resistant profile (red shading); intermediate profile (grey shading); susceptible profile (green shading), according to interpretative criteria [18, 68]. When no interpretive criteria are available only MIC values are reported. The dark line separates the antibiotics recommended by the CLSI to be tested for rapidly growing NTM (above the line) from the other 17 antimicrobials tested (below the line)

ND not determined

[a]Streptomycin is recommended for SGM susceptibility testing but no interpretative criteria are available. Imipenem is recommended for *Corynebacterium* susceptibility testing but no interpretative criteria are available. The 5 antibiotics with underlined names were utilized in the Middlebrook 7H10-PANTA supplemented isolation medium

environment [9, 50]. Intraspecific variability, infrequent isolation and lack of reports on drug resistance profiles all contribute to the difficulty in defining standard treatment guidelines for rare opportunistic NTM such as *M. mucogenicum* [51]. Remarkably, all NTM isolates in this study were susceptible to the aminoglycosides tested, except for the 2 rapidly growing NTM isolates that were resistant to tobramycin.

Corynebacterium are in general resistant to antimicrobial agents recommended for Gram-positive infections, including penicillins, cephalosporins, macrolides, fluoroquinolones, aminoglycosides and tetracycline, but they remain susceptible to vancomycin and linezolid [52]. The 3 *Corynebacterium* isolates in this study showed an unusual resistance pattern, since they were susceptible to aminoglycosides and tetracyclines, but were also resistant to linezolid. Riegel et al. tested the susceptibility of 13 nosocomial *C. jeikeium* isolates against gentamicin, with half presenting a MIC< 0.5 μg/mL and the other half a MIC> 16 μg/mL [53]. Scarce literature with low number of isolates hinders overall interpretation of results, which may be worth exploring for further awareness of antibiotic resistance in these increasingly detected potentially opportunistic pathogens.

One important observation from our study was the fact that all *Corynebacterium* and *Gordonia* isolates were highly resistant to imipenem. Although we did not assess the genetic background underlying this phenotype, it is possible to speculate that if the resistance is related to the presence of carbapenemases, these slowly growing bacteria can represent an unknown resistance pool against this important antibiotic. We found no literature reporting the presence of carbapenemases in species of the *Corynebacterineae*, but genetic mobile elements with different resistance genes have already been described in some of these species [54, 55], highlighting their ability to transfer antibiotic resistance features.

Only 5 of the 25 tested antimicrobials were those used in Middlebrook 7H10-PANTA culture medium for isolation although our results showed MICs much higher than the concentrations used in the selective medium. Possibly, increasing the concentration of these antimicrobials,

except azlocillin, could diminish the number of false positives for the selection of NTM from the hospital environment, as the presence of a higher number of colony forming units per plate observed in some samples could have affected the recovery rate (data not shown). This is worth testing to optimize present standard methodology for NTM isolation with PANTA-supplemented medium [56]. Surprisingly, while *Corynebacterium* and *Gordonia* isolates were highly resistant to the antifungal amphotericin B, the MICs for most NTM isolates were intermediate confirming that at higher concentrations amphotericin B seems to inhibit their growth.

Conclusions

The current study, although limited in number of isolates, revealed the poor knowledge we still have on the identity of viable NTM species present in hospital settings, as well as on their antibiotic resistance profiles and resistance mechanisms, raising relevant questions about the potential threat these and other potential opportunistic pathogens may represent for example to immunocompromised inpatients. Their presence in dry surfaces with which healthcare providers, visitors and patients themselves contact frequently, accompanied by their apparent multidrug resistance profiles, should be further investigated to comprehensively understand this potentially latent menace and help prevent dissemination through implementation of better disinfection strategies and enforcement of enhanced policies.

Materials and methods

Sample collection from hospital settings

Samples were collected from different surfaces and equipment located at three different wards of a tertiary care hospital, as previously described [17]. Suspensions without pre-treatment were directly plated in solid Middlebrook 7H10-PANTA supplemented medium [Middlebrook 7H10 medium enriched with 10% OADC (oleic acid, albumin, dextrose, catalase) and supplemented with polymyxin B (40 U/mL), amphotericin B (4 μg/mL), nalidixic acid (16 μg/mL), trimethoprim (4 μg/mL) and azlocillin (4 μg/mL)] [17, 56]. Plates were incubated at 30 °C between 1 and 6 weeks and colony growth was evaluated on a weekly basis. Isolation, plating and purification of colonies was performed in Middlebrook 7H10-PANTA, followed by cryopreservation at − 80 °C in Middlebrook 7H9 broth with 15% glycerol.

Identification of NTM and other actinobacterial isolates

Genomic DNA was extracted as previously described [57]. Amplification of the full-length 16S rRNA gene was performed by polymerase chain reaction (PCR) with universal primers 27F (5′-GAGTTTGATCCTGGCTCAG) and 1525R (5′–AGAAAGGAGGTGATCCAGCC) [58]. PCR reactions were carried out with Supreme NZYTaq DNA polymerase (NZYTech, Portugal) with 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. Products were purified using JET Quick PCR Purification Spin Kit (Genomed GmbH, Germany) according to manufacturer's instructions and sequenced at GATC Biotech (Germany). 16S rRNA gene sequences were compared with sequences at the NCBI database using the BLAST tool (http://blast.ncbi.nlm.nih.gov/) and assignment to species level considered nucleotide sequence identities of ≥99%. For species identity validation, DNA from *Mycobacterium* isolates was used for PCR amplification of partial sequences of *rpoB* and *hsp65* genes with mycobacterial-specific primers GrpoB1 (5′-ATCGACCACTTCGGCAACCGCC), GrpoB2 (5′-GGTACGGCGTCTCGATGAASCCG), and Tb 11 (5′-ACCACGATGGTGTGTCCAT), Tb12 (5′-CTTG TCGAACCGCATACCCT), respectively [59]. PCR reactions were carried out with KOD Hot-Start DNA polymerase (Novagen) according to manufacturer's instructions and PCR products were purified and sequenced, as described above.

Sequence analyses and phylogenetic trees

Phylogenetic analyses were performed after manually checking DNA quality using Sequence Scanner Software (Applied Biosystems). Sequence data was edited and assembled with BioEdit Sequence Alignment Editor. The 16S rRNA gene sequences of the isolates and type strains of the *Corynebacterineae* genera *Mycobacterium*, *Gordonia* and *Corynebacterium* were obtained from Genbank or ARB Silva database (https://www.arb-silva.de/) and aligned, each genus separately, with the Clustal X software package [60], visually examined and manually adjusted to allow maximal alignment. Jukes Cantor method was used to calculate evolutionary distances [61]. Phylogenetic dendrograms were constructed by the neighbor-joining method and evaluated by bootstrap analysis [62] of 500 resamplings of the data set, using MEGA6 software [63]. Three phylogenetic trees (Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4 and Additional file 5: Figure S5) were used to accurately determine the phylogenetic placement of the isolates for downstream selection of the Type strains to be used for the construction of the final tree (as described above) including the three genera belonging to *Corynebacterineae* (Fig. 1). The similarity values of the 16S rRNA gene sequences of the all isolates and the closest type strains were determined from the alignment used to construct the phylogenetic tree encompassing the three genera and are presented as Additional file 6: Table S1. The assignment to species level considered nucleotide sequence similarity value of ≥99% of the isolates towards the closest type strains. Amino acid sequences were deduced with the MEGA6 package from the 420- and 396-bp DNA

sequences of mycobacterial partial *hsp65* and *rpoB* gene sequences, respectively. Protein sequences were aligned with sequences of type strains obtained from the NCBI database using the Clustal X. Protein phylogenetic trees were constructed using the neighbor-joining [64]. Topology of trees were generated from evolutionary distances computed using the Poisson correction method [65], included in Mega6 and evaluated by bootstrap analysis [62] of 500 resamplings of the data set. All positions with less than 95% site coverage were eliminated. Protein alignments were used to determine the nucleotide position in the DNA sequences alignment and sequences from mycobacterial genes 16S rRNA, *hsp65* and *rpoB* were concatenated and further used for phylogenetic analyses as described above.

To search for the clarithromycin resistance gene *erm* and for the tetracycline resistance gene *tet(V),* chromosomal DNA from NTM isolates was used as template for draft genome sequencing at GATC Biotech (Konstanz, Germany) with 150 bp paired-end libraries on an Illumina HiSeq. Raw sequence reads were assembled de novo using SPAdes 3.11.1 [66] with specific parameters for 2 × 150 bp reads library de novo assembly, namely using BayesHammer module error correction and --careful option (our unpublished results).

Deposition of nucleic acid sequences in public databases

Partial 16S rRNA (1347–1378 bp) genes are available from [17] under accession numbers KT347497 and KT347499 to KT347502. Partial *rpoB* (371–398 bp) and *hsp65* (395–441 bp) genes sequences were deposited in European Molecular Biology Laboratory (EMBL) and GenBank databases under the accession numbers: KT992215 to KT992224 for the partial *rpoB* and *hsp65* sequences, respectively, and from KT832812 to KT832816 for the *Corynebacterineae* isolates partial 16S rRNA sequences. The clarithromycin resistance gene *erm* detected in the *M. obuense* genome and the tetracycline resistance genes *tet(V)* identified in *M. obuense* and *M. mucogenicum* draft genomes were deposited in GenBank database under accession numbers MG770427, MG770425 and MG770428, respectively.

Antimicrobial susceptibility testing and minimal inhibitory concentration (MIC)

Minimal inhibitory concentrations were determined after 5 days according to Clinical Laboratory Standards Institute (CLSI) recommendations for rapidly growing NTM and *Nocardia* [18, 67]. *Corynebacterium* isolates were incubated for 48 h according to CLSI recommendations [68]. The slowly growing *M. paragordonae* isolates 10AIII, 29AIII and 35AIII were incubated for 10 days. Clarithromycin susceptibility was determined after 14 days [69]. Classification of mycobacteria according to their growth rate is classically based on the time bacteria take to form colonies in solid media. Rapidly growing mycobacteria (RGM) are able to grow in under 7 days, whereas the ones that take more than 7 days are called slowly growing mycobacteria (SGM). Because phylogenetic studies of mycobacteria support this separation identification of SGM or RGM species was based on the phylogenetic tree constructed by Tortoli et al. [70] in addition to CLSI listing [18]. Briefly, a suspension of 0.5 McFarland density of each isolate was prepared in saline solution and diluted 1000-fold before testing in the next 30 min. A sterile 96-well microplate, previously prepared with Mueller Hinton (MH) medium supplemented with 0.5% OADC and containing decreasing concentrations of the tested antimicrobials, was inoculated with the diluted bacterial suspension and incubated for 5 days at 30 °C [18]. In addition to the antimicrobials considered for rapidly growing mycobacteria susceptibility testing in CLSI standards (cefoxitin, amikacin, imipenem, tobramycin, linezolid, doxycycline, clarithromycin and ciprofloxacin) also amphotericin B, azlocillin, nalidixic acid, trimethoprim, polymyxin B (these 5 used in Middlebrook 7H10-PANTA), rifampicin, chloramphenicol, tetracycline, penicillin, vancomycin and the aminoglycosides gentamicin, kanamycin, streptomycin, neomycin and spectinomycin were tested. Only antimicrobials considered in CLSI standards were interpreted for bacterial resistance levels [18, 68]. Diverse concentration ranges were used, with antimicrobials being diluted 8 times, in a 1:2 scaling (clarithromycin was diluted 12 times and penicillin was diluted 16 times). Stock solutions were prepared according CLSI guidelines [71, 72]. Appropriate controls were performed to ensure normal bacterial growth despite presence of diluted acetic acid, methanol or ethanol used to solubilize some antibiotics. All assays were performed in triplicate.

Additional files

Additional file 1: Figure S1. Phylogenetic dendrogram constructed by comparing 16S rRNA gene sequences of isolates 10AIII, 22DIII, 24AIII, 29AIII and 35AIII with *Mycobacterium* type strain sequences obtained from GenBank databases. Sequences were aligned using MEGA6. The tree topology was obtained by using neighbor-joining algorithm with Jukes–Cantor correction. All positions with less than 95% site coverage were eliminated. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 1 inferred nucleotide substitution per 100 nt. (PPTX 108 kb)

Additional file 2: Figure S2. Phylogenetic dendrogram constructed by comparing 16S rRNA gene sequences of isolates 1AIII and 55AIII with *Gordonia* type strain sequences obtained from databases. Sequences were aligned using MEGA6. The tree topology was obtained by using neighbor-joining algorithm with Jukes–Cantor correction. All positions with less than 95% site coverage were eliminated. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 5 inferred nucleotide substitution per 1000 nt. (PPTX 75 kb)

Additional file 3: Figure S3. Phylogenetic dendrogram constructed by comparing 16S rRNA gene sequences of isolates 6FIII, 52AIII and 58FIII

with *Corynebacterium* type strain sequences obtained from databases. Sequences were aligned using MEGA6. The tree topology was obtained by using neighbor-joining algorithm with Jukes–Cantor correction. All positions with less than 95% site coverage were eliminated. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 1 inferred nucleotide substitution per 100 nt. (PPTX 95 kb)

Additional file 4: Figure S4. Phylogenetic analysis of *rpoB* nucleotide sequences of mycobacterial isolates and 61 selected types strains of the genus *Mycobacterium*. The tree was created using the neighbor-joining algorithm and the evolutionary distances calculated by Jukes and Cantor method [61]. Bootstrap values above 60%, for 500 replicates, are given at branch points. Bar, 1 inferred nucleotide substitution per 100 nt. (PPTX 92 kb)

Additional file 5: Figure S5. Phylogenetic analysis of *hsp65* nucleotide sequences of mycobacterial isolates and the 55 selected type strains of the genus *Mycobacterium*. See Additional file 5: Figure S2 legend for further details. (PPTX 90 kb)

Additional file 6: Table S1. Pairwise similarity values (%) determined from the alignment used for the construction of the phylogenetic trees from: A. The concatenated nucleotide sequences of mycobacterial 16S rRNA, *hsp65* and *rpoB* of isolates and Type strains selected from the databases; B. 16S rRNA gene nucleotide sequences of isolates and Type strains of the genus Corynebacterium selected from the databases; C. 16S rRNA gene nucleotide sequences of isolates and Type strains of the genus *Gordonia* selected from the databases. (DOCX 48 kb)

Abbreviations

BLAST: Basic Local Alignment Search Tool; CFU: Colony forming units; CLSI: Clinical Laboratory Standards Institute; EMBL: European Molecular Biology Laboratory; HAI: Hospital-acquired infections; MDR: Multidrug resistance; MIC: Minimal inhibitory concentration; NCBI: National Center for Biotechnology Information; NTM: Nontuberculous mycobacteria; OADC: Oleic acid, albumin, dextrose, catalase; PANTA: polymyxin B (40 U/mL), amphotericin B (4 μg/mL), nalidixic acid (16 μg/mL), trimethoprim (4 μg/mL) and azlocillin; PCR: Polymerase chain reaction; WHO: World Health Organization

Acknowledgements

We thank Paula V. Morais (Life Sciences Department, University of Coimbra, Portugal), Ana M. Almeida (Instituto Piaget, Silves, Portugal) and the Infection Control office of the sampled tertiary hospital for their valuable collaboration.

Funding

We acknowledge Fundação para a Ciência e a Tecnologia (FCT) and COMPETE 2020, Operational Programme for Competitiveness and Internationalization (POCI) through FEDER projects UID/NEU/04539/2013 (POCI-01-0145-FEDER-007440) and PTDC/BTM-TEC/29221/2017 (POCI-01-0145-FEDER-029221), and for postdoctoral fellowship SFRH/BPD/108299/2015 to Susana Alarico, for PhD fellowship SFRH/BD/117777/2016 to Daniela Nunes-Costa, and for FCT Investigator contract (IF/01061/2014) to Igor Tiago. We also acknowledge the European Regional Development Fund, Centro2020 Regional Operational Programme (CENTRO-01-0145-FEDER-000012-HealthyAging2020) for postdoctoral grant to Ana Maranha. The funding bodies listed here did not participate in the design of the study, collection, analysis, interpretation of data or in manuscript writing.

Availability of data and materials

All data generated or analyzed during this study are included in this article. All nucleic acid sequences were deposited in GenBank and the corresponding accession numbers can be found under *"Deposition of nucleic acid sequences in public databases"* in the Materials and Methods section.

Authors' contributions

SGP and AM performed MIC determination, data analysis and interpretation and were involved in manuscript writing. SA and IT constructed phylogenetic trees and were involved in manuscript writing. DR cryopreserved the isolates and participated in MIC determination experiments. DN-C performed DNA extraction and PCR and was involved in manuscript writing. OC interpreted data and was involved in manuscript writing. OC and NE contributed material and reagents. NE and AM designed the study, analyzed and interpreted data, and wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 30 April 2018 Accepted: 26 February 2019
Published online: 19 March 2019

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