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
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 debilitating 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 practice as well [13, 14].”

The aim of this study was to address the extent of antimicrobial resistance in strains of NTM and other Corynebacteriaceae 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...
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).

### 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 atridis       | 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 amylolatum | Medicine A | Bed table               |
| 55AIII  | Gordonia spiti          | 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...
Fig. 1 (See legend on next page.)
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 to 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,
clarithromycin, imipenem and 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.

Fig. 2 Phylogenetic tree computed from the concatenated nucleotide sequences of 16S rRNA, *hsp65* and *spol* 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.
environment [9, 50]. Intraspecies 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 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 supplemented isolation medium.
Genomic DNA was extracted as previously described [57]. Identification of NTM and other actinobacterial isolates

μ (40 U/mL), amphotericin B (4 mg/mL), dextrose, catalase) and supplemented with polymyxin B medium enriched with 10% OADC (oleic acid, albumin, dextrose, catalase) and 7H10-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′-AGAAAGGAGGTGATCCAGGCC) [58]. PCR reactions were carried out with Supreme NZYTag 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′-ATCGACACTTCGGCAACGGCC), GrpoB2 (5′-GGTACCGGGCTCTCGATGAASCCG), and Tb 11 (5′-ACCCAGATGGTGTCCTCAT), Tb12 (5′-CTTG TCGAACCAGCATACCCT), 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, 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 direct 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 Moléculaar 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. mucigenicum* 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 5 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 phi 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: Healthcare-associated infection; 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 μg/mL) amphotericin B (4 μg/mL), nalidixic acid (15 μg/mL), trimethoprim (4 μg/mL) and acrifolin; PCR: Polymerase chain reaction; WHO: World Health Organization

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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
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References
1. WHO. Report on the Burden of Endemic Health Care-Associated Infection Worldwide. Geneva: World Health Organization; 2011. http://apps.who.int/iris/handle/10665/80135. Accessed 13 July 2017
2. Allegranzi B, Kilpatrick C, Storr J, Kelley E, Park BJ, Donaldson L, Global HAI Control office of the sampled tertiary hospital for their valuable collaboration.

Available in biology and develop high-quality tools for nontuberculous mycobacteria (NTM). Curr Environ Health Rep. 2016;3(2):161–8. doi: 10.1007/s40277-015-0112-7
3. ECDC. Healthcare-associated infections acquired in intensive care units - annual epidemiological report 2016 [2014 data]. In: Annual Epidemiological Report on Communicable Diseases in Europe. Stockholm: European Centre for Disease Prevention and Control; 2017. https://ecdc.europa.eu/en/publications-data/infections-acquired-intensive-care-units-annual-report-2016. Accessed 13 July 2017.
4. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. Bad Bugs, No Drugs: No ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(1):1–12.
5. WHO. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva: World Health Organization; 2017. https://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/
6. Fedrizzi T, Mehean CJ, Grottaola T, Giacobazzi E, Fregni Serpini G, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, et al. Genomic characterization of nontuberculous mycobacteria. Sci Rep. 2017;7:45258.
7. Falkingham JO III. Current epidemiologic trends of the nontuberculous mycobacteria (NTM). Curr Environ Health Rep. 2016;3(2):161–7.
8. Griffith DE, Akamit T, Brown-Elliott BA, Cataraizo A, Daley C, Gordin F, Holland SM, Horsburgh R, Hutt Infection Care Med. 2005;55(5):1871–6. doi: 10.1093/infec/dhi131
9. Spratt J, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, et al. Genomic characterization of nontuberculous mycobacteria. Sci Rep. 2017;7:45258.
10. Falkingham JO III. Current epidemiologic trends of the nontuberculous mycobacteria (NTM). Curr Environ Health Rep. 2016;3(2):161–7.
11. Griffith DE, Akamit T, Brown-Elliott BA, Cataraizo A, Daley C, Gordin F, Holland SM, Horsburgh R, Hutt Infection Care Med. 2005;55(5):1871–6. doi: 10.1093/infec/dhi131
12. Spratt J, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, et al. Genomic characterization of nontuberculous mycobacteria. Sci Rep. 2017;7:45258.
13. Falkingham JO III. Current epidemiologic trends of the nontuberculous mycobacteria (NTM). Curr Environ Health Rep. 2016;3(2):161–7.
14. Griffith DE, Akamit T, Brown-Elliott BA, Cataraizo A, Daley C, Gordin F, Holland SM, Horsburgh R, Hutt Infection Care Med. 2005;55(5):1871–6. doi: 10.1093/infec/dhi131
15. Spratt J, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, et al. Genomic characterization of nontuberculous mycobacteria. Sci Rep. 2017;7:45258.
55. Tauch A, Krieff S, Kalinowski J, Puhler A. The 51,409-bp R-plasmid pTP10 from the multiresistant clinical isolate Corynebacterium striatum M82B is composed of DNA segments initially identified in soil bacteria and in plant, animal, and human pathogens. Mol Gen Genet. 2000;263(1):1–11.

56. Radomski N, Cambau E, Moulin L, Haen R, Morlon R, Lucas F. Comparison of culture methods for isolation of nontuberculous mycobacteria from surface waters. Appl Environ Microbiol. 2010;76(11):3514–20.

57. Alarico S, Costa M, Sousa MS, Maranha A, Lourencco EC, Faria TQ, Ventura MR, Empadinhas N. Mycobacterium hassiacum recovers from nitrogen starvation with up-regulation of a novel glucosylglycerate hydrolase and depletion of the accumulated glucosylglycerate. Sci Rep. 2014;4:6766.

58. Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E. The genus Nocardiosphera represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiosphaerae fam. nov. Int J Syst Bacteriol. 1996;46(4):1088–92.

59. Devulder G, de Montclos MP, Flandrois JP. A multigene approach to phylogenetic analysis using the genus Mycobacterium as a model. Int J Syst Evol Microbiol. 2005;55(1):299–302.

60. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23(21):2947–8.

61. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press; 1969. p. 21–132.

62. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39(4):783–91.

63. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

64. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987(4):406–25.

65. Zuckerkandl E, Pauling L. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, editors. Evolving genes and proteins. New York: Academic Press; 1965. p. 97–166.

66. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455–77.

67. Woods GL, Lin S-YG, Desmond EP. Susceptibility test methods: mycobacteria, nocardia, and other actinomycetes. In: Jorgensen JH, Pfaller MA, Carroll KC, Funke G, Landry ML, Warnock DW, editors. Manual of clinical microbiology. 11th ed. Washington, DC: American Society of Microbiology; 2015. p. 1356–78.

68. CLSI. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. In., 3rd edn. Wayne: Clinical and Laboratory Standards Institute; 2015.

69. Brown-Elliott BA, Vasireddy S, Vasireddy R, Iakhiaeva E, Howard ST, Nash K, Parodi N, Strong A, Gee M, Smith T, et al. Utility of sequencing the erm (41) gene in isolates of Mycobacterium abscessus subsp. abscessus with low and intermediate clarithromycin MICs. J Clin Microbiol. 2015;53(4):1211–5.

70. Tortoli E, Fedrizzi T, Meehan CJ, Trovato A, Grottolia E, Giacobazzi E, Serpini GF, Tagliazucchi S, Fabio A, Bettua C, et al. The new phylogeny of the genus Mycobacterium: the old and the new. Infect Genet Evol. 2017;56:19–25.

71. Barry A, Bryskier A, Traczewski M, Brown S. Preparation of stock solutions of macrolide and ketolide compounds for antimicrobial susceptibility testing. Clin Microbiol Infect. 2004;10(1):78–83.

72. CLSI. Performance standards for antimicrobial susceptibility testing, vol. M100. 27th ed. Wayne: Clinical and Laboratory Standards Institute; 2017.