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Diagnostics, resistance and clinical relevance of non-tuberculous mycobacteria unidentified at the species level by line probe assays: a bi-national study

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Abstract

Objectives While the reported incidence of non-tuberculous mycobacterial (NTM) infections is increasing, the true prevalence remains uncertain due to limitations in diagnostics and surveillance. The emergence of rare and novel species underscores the need for characterization to improve surveillance, detection, and management.

Methods We performed whole-genome sequencing (WGS) and/or targeted deep-sequencing using the Deeplex Myc-TB assay on all NTM isolates collected in Slovakia and the Czech Republic between the years 2019 to 2023 that were unidentifiable at the species level by the routine diagnostic line probe assays (LPA) GenoType CM/AS and NTM-DR. Minimal inhibitory concentrations against amikacin, ciprofloxacin, moxifloxacin, clarithromycin, and linezolid were determined, and clinical data were collected.

Results Twenty-eight cultures from different patients were included, of which 9 (32.1%) met the clinically relevant NTM disease criteria. The majority of those had pulmonary involvement, while two children presented with lymphadenitis. Antimycobacterial resistance rates were low. In total, 15 different NTM species were identified, predominantly rare NTM like *M. neoaurum*, *M. kumamotonense* and *M. arupense*. Notably, clinically relevant *M. chimaera* variants were also identified with WGS and Deeplex-Myc TB, which, unlike other *M. chimaera* strains, appeared to be undetectable by LPA assays. Deeplex detected four mixed infections that were missed by WGS analysis. In contrast, WGS identified two novel species, *M. celatum* and *M. branderi*, which were not detected by Deeplex-Myc TB. Importantly, one of these novel species strains was associated with clinically relevant pulmonary disease.

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Discussion Our study demonstrates the clinical relevance of uncommon NTM and the effectiveness of targeted deep-sequencing combined with WGS in identifying rare and novel NTM species.

Keywords Non-tuberculous mycobacteria, Diagnostics, Novel species, Targeted next-generation sequencing, Whole-genome sequencing

Introduction

Non-tuberculous mycobacteria (NTM) are a diverse group of environmental organisms distinct from the *Mycobacterium* (*M.*) *tuberculosis* complex and *M. leprae*. Over 200 NTM species have been identified, with a subset known to cause a wide range of diseases, predominantly pulmonary infections in patients with pre-existing lung diseases, lymphadenitis, skin and soft tissue infections, and disseminated disease, particularly in immunocompromised individuals [1]. Based on recent reports, the prevalence of NTM infections is considered to be increasing, posing an emerging public health problem [2, 3]. This increase could be linked, amongst others, to advancements in diagnostics and increased awareness but also to an aging population, a higher prevalence of chronic diseases and the reduction of tuberculosis cases in developed countries [4].

Despite advances in diagnostic techniques, confirming NTM infection remains challenging due to slow growth, low sensitivity and time-consuming laboratory tests [5]. Accurate identification of NTM species in clinical specimens is essential for diagnosis and surveillance purposes and for selecting an effective treatment regimen, leading to shorter and more cost-effective therapy [6]. Acid-fast bacillus (AFB) smear microscopy, cultivation and sequencing of single genes like *16 S rRNA*, *rpoB*, *hsp65* or internal transcribed spacer (ITS) remain the gold standard for NTM diagnostics in many countries. However, they have long turnaround time and/or low discriminatory power [7–9]. Recently, line probe assays (LPA), such as the Genotype assays from Hain Lifescience, have become increasingly popular in diagnostic laboratories and have proven effective for accurate NTM species identification and resistance detection. These assays combine PCR-amplified DNA from solid or liquid culture to complementary (sub) species-specific probes on nitrocellulose strips [10, 11]. The Genotype Mycobacterium CM (common mycobacteria) assay enables the detection and differentiation of *M. tuberculosis* and 13 frequently isolated and/or clinically relevant NTM species or complexes, including *M. abscessus*, *M.* avium, *M. intracellulare*, *M. marinum/ulcerans*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, *M. fortuitum*, *M. scrofulaceum*, *M. interjectum*, *M. szulgai and M. malmoense*. The Genotype AS (additional species) allows for identifying another 14 species including *M. celatum* and *M. lentiflavum*. Lastly, the GenoType NTM-DR assay can differentiate between the three *M. abscessus* subspecies, allowing to identify *M. intracellulare* subsp. *chimaera*, further referred to as *M. chimaera*, and can detect resistance to macrolides and aminoglycosides for these species. Rare and novel NTM are typically identified only as *M.* species with these assays via the genus-specific probes without further identification on the species level. For these uncommon species, the clinical relevance remains unknown.

Targeted next-generation sequencing (tNGS) offers a promising alternative to line probe assays and whole genome sequencing (WGS), despite its higher cost. Amplification of specific gene regions lowers the input DNA requirements and mitigates the challenges associated with WGS in identifying NTM directly from clinical samples like sputum [12]. The Deeplex Myc-TB assay (GenoScreen, Lille, France) constitutes a state-of-the-art platform with target sequences that can differentiate more than 150 NTM species/subspecies based on *hsp65* and confirm mixed infections directly from clinical specimens [13]. Deeplex Myc-TB comes with an intuitive web application hosted on a secure cloud-based platform for analyzing and quickly interpreting results. This eliminates the need for advanced bioinformatics skills. This technique has proven to provide swift results in diagnosing *M. tuberculosis* and identifying resistance profiles, resulting in its incorporation into the latest WHO consolidated guidelines on tuberculosis [14]. The limitation of Deeplex Myc-TB is its exclusion of critical gene regions linked to NTM resistance, necessitating clinical laboratories to continue relying on results of phenotypic susceptibility testing or other molecular tests like the GenoType NTM-DR assay from Hain Lifescience. On the other hand, WGS enables the identification of all known and putative novel resistance-associated mutations and NTM species and facilitates the study of NTM transmission and phylogeny [15, 16].

This study aimed to evaluate the discriminatory power of tNGS and WGS in characterizing NTM species that were previously unidentified at the species level using GenoType Mycobacterium CM/AS and GenoType NTM-DR. Additionally, the study aims to investigate the clinical significance of these rare mycobacterial species and assess their in vitro drug susceptibility profiles.

Materials and methods

Patient isolates

This binational study included all mycobacterial isolates uncharacterized at the species level or identified as mixed NTM cultures using line probe assay (GenoType Mycobacterium CM ver 2.0/AS ver 1.0 and GenoType NTM-DR ver 1.0, Hain Lifescience GmbH, Nehren, Germany) and collected in Czech Republic (National Reference Laboratory for Mycobacteria, National Institute of Public Health, Prague) and Slovakia (National Reference Mycobacteriology Laboratory, National Institute for TB, Lung Diseases and Thoracic Surgery, Vyšné Hágy) between the years 2019 and 2023. Fully anonymized clinical data from the National Tuberculosis Register in the Czech Republic and Slovakia were retrieved, comprising age, sex, specimen type, diagnostic material, initial diagnosis, and clinical manifestations. Diagnostic criteria for relevant NTM-pulmonary disease were applied according to the American Thoracic Society (ATS), European Respiratory Society (ERS), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), and Infectious Diseases Society of America (IDSA) [17]. Repeated culture and GenoType testing for all isolates included in the study were performed at least 7 days apart for each patient to confirm the clinically relevant infection. However, only the initial isolate was archived if subsequent isolates were identified as the *M.* species. Negative sputum culture post-treatment was considered a successful treatment outcome.

Culture and determination of minimal inhibitory concentrations (MIC)

Cultures of unidentified Mycobacterial species were grown on Middlebrook Agar 7H9 at 37 °C until visible colonies were formed. MIC testing was performed using the broth microdilution method for amikacin, ciprofloxacin, clarithromycin, and linezolid at concentrations ranging from 0.5 to 32 mg/L and for moxifloxacin at concentrations ranging from 0.125 to 8 mg/L according to the instructions in the European Centre for Disease Prevention and Control handbook [18]. The breakpoints for interpreting susceptibilities followed the Clinical and Laboratory Standards Institute (CLSI) guidelines [19].

DNA extraction and sequencing

DNA for WGS and tNGS was isolated from 1 mL of heat-inactivated early-positive MGIT culture utilizing the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). 9 µL of extracted genomic DNA was amplified using the Deeplex Myc-TB following the manufacturer's instructions [13]. Sequencing libraries for both tNGS and WGS were prepared using 0.2 ng of amplicons and whole DNA, respectively, with the Nextera XT library preparation kit (Illumina, San Diego, CA) and sequenced on the MiSeq platform (Illumina, San Diego, CA) utilizing the Illumina MiSeq V2 to produce 150 basepair paired-end reads.

Sequencing data analysis

FASTQ files generated by tNGS were uploaded to the Deeplex web software (https://app.deeplex.fr) for automatic species identification analysis. FASTQ files generated by WGS were processed using the NTMseq pipeline v 1.0.2 (https://github.com/ngs-fzb/NTMtools/tree/main/scripts/NTMseq), which includes (i) sequence quality control (ii) contamination detection (iii) NTM species detection and antibiotic resistance prediction with NTMprofiler v.022, which is based on species-specific kmers, and v.040, which is based on average nucleotide identity (ANI) compared to reference NTM genomes (https://github.com/jodyphelan/NTM-Profiler), (iv) resistance prediction with AMRFinder + v.3.11.2 [20] and gene database v.2023-09-26.1 with default values as well as relaxed thresholds of 50% coverage and identity (v) pairwise average nucleotide identity calculation and (vi) assembly using shovill (https://github.com/tseemann/shovill) with down-sampling to 100x coverage and spades [21] v3.15.0 as assembly algorithm. The resulting draft assemblies were submitted to the type strain genome server (TYGS) (https://tygs.dsmz.de/) for species identification. Digital DNA: DNA hybridization (DDH) values (d0, d4 and d6) and confidence intervals were calculated using the recommended settings of the Genome-to-Genome Distance Calculator (GGDC) 4.0 [22, 23].

For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted via the TYGS web server using the Genome BLAST Distance Phylogeny approach (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d5 [22]. 100 distance replicates were calculated each. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1, including SPR postprocessing [24]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint and visualized with PhyD3 [25].

Results

Patients and isolates data

Overall, only 28 isolates were identified as *M. species* or mixed NTM by GenoType Mycobacterium CM/AS and GenoType NTM-DR (all three tests performed for each isolate) during the 5-year study period in the Czech Republic and Slovakia. The occurrence of NTM species that could not be identified using GenoType was thus

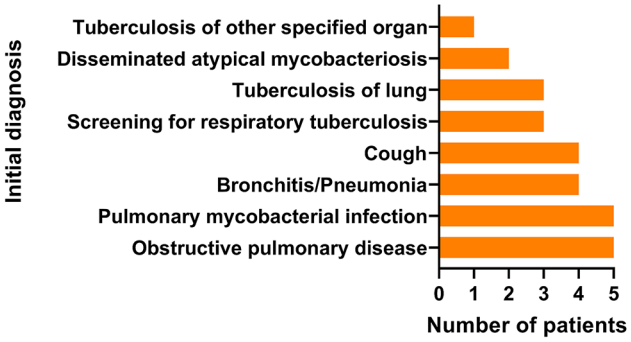

Fig. 1 A summary of the initial diagnostic assessment upon which sample collection was based

relatively low in both Slovakia and the Czech Republic. Slovakia recorded 9 such cases in 2023, while the Czech Republic reported 1 case in 2019, 4 in 2020, 6 in 2021, 1 in 2022, and 7 in 2023. In the Czech Republic, the proportion of this NTM species among all identified NTM (except *M. gordonae*) was 0.9% (1/106) in 2019, 3.6% (4/112) in 2020, 6.5% (6/92) in 2021, 1.1% (1/95) in 2022, and 3.8% (7/185) in 2023. In Slovakia, 7 isolates of M. species represent 4.4% of the total number of identified NTM (except *M. gordonae*; $n = 158$). Among the patients, 15 (53.6%) were females, and 13 (46.4%) were male. The median age of the patients was 65.4 ± 20.7 years (ranged 2 to 87). Out of 28 isolates, 25 (88.9%) were obtained from sputum, 2 (7.4%) from lymph node biopsy and 1 (3.7%) from urine. Most patients were diagnosed with lung diseases (Fig. 1, Supplementary Table 1).

NTM identification by Deeplex Myc-TB and WGS

The data obtained by Deeplex Myc-TB allowed species classification of 27 out of 28 (96.4%) isolates. In total, 13 different species were reported, with *M. neoaurum* the most prevalent species ($n = 8$), followed by *M. kumamotonense* ($n = 5$), *M. arupense* ($n = 5$) and *M. chimaera* ($n = 3$). In one isolate, no mycobacteria were detected (Fig. 2). Six samples contained mixed NTM species, two of which were also positive for multiple NTM species by GenoType NTM-DR (Supplementary Table 1). The other sample initially identified as mixed NTM species by GenoType NTM-DR was characterized solely as *M. chimaera* using Deeplex Myc-TB.

Due to insufficient DNA concentration and the inability to acquire additional culture material for further DNA extraction, WGS was only performed on 19 isolates. Among the 9 isolates without WGS data, 5 were identified as *M. neoaurum*, 2 as *M. arupense*, 1 as a mixed NTM species (*M. arupense*/*M. yongonense*), and 1 as *M. holsaticum* by Deeplex Myc-TB. A comparison of Deeplex Myc-TB and WGS results revealed discrepancies in 7 out of 19 isolates (Table 1). Four of these isolates were initially identified as mixed NTM species by Deeplex Myc-TB. However, WGS analysis did either not detect any NTM but instead *Tsukamurella tyrosinosolvens* ($n = 1$) or detect only 1 NTM species ($n = 3$) in these samples. Based on WGS, potential novel NTM species were found in 3 samples. However, Deeplex analysis identified these as mixed samples or as *M. arupense*. In the sample where no mycobacteria were detected with Deeplex Myc-TB, WGS identified *M. celatum*. In addition, WGS reported *M. branderi* in one sample, whereas Deeplex Myc-TB reported a mix of *M. chimaera* and *M. massiliense* (i.e. *M. abscessus* subsp. *massiliense*). Based on the above, the agreement rate of WGS and Deeplex Myc-TB was 63.2%. The agreement rate between TYGS and NTMprofiler was 100% for the 12 isolates where TYGS reported an NTM.

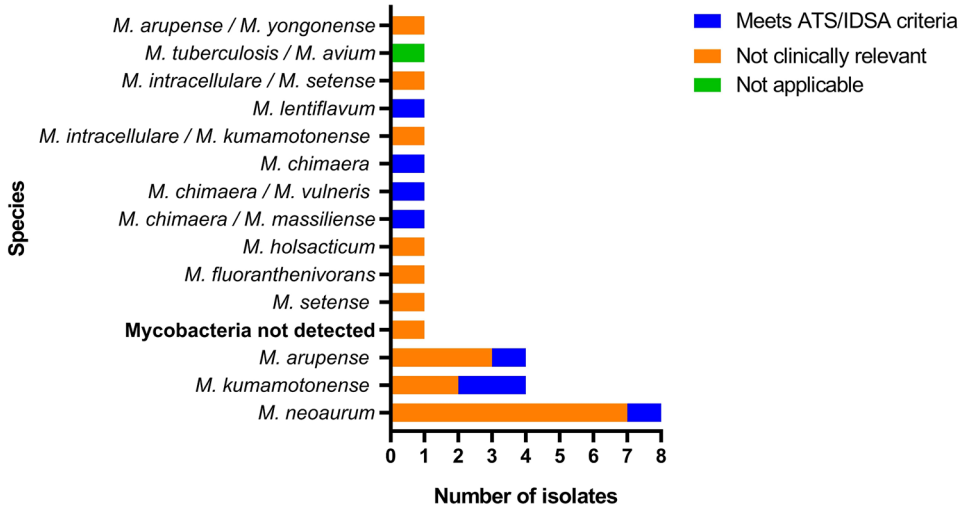

Fig. 2 NTM species identified by Deeplex Myc-TB

Table 1 List of strains with discordant results between Deeplex Myc-TB and WGS

Sample	GenoType NTM-DR	Deeplex Myc-TB	WGS
CZ118/21	unidentified Mycobacterial species	*M. intracellulare (80.7%) / M. kumamotonense (19.2%)*	*Tsukamurella tyrosinosolvens* (TYGS), NTM not detected (NTMprofiler)
CZ131/21	unidentified Mycobacterial species	*M. intracellulare (87.7%) / M. setense/houstonense (12.0%)*	Potential novel NTM species (TYGS), NTM not detected (NTMprofiler)
CZ468/21	unidentified Mycobacterial species	*M. arupense*	Potential novel NTM species (TYGS), NTM not detected (NTMprofiler)
CZ614/21	unidentified Mycobacterial species	*M. chimaera (84.6%) / M. massiliense (15.0%)*	*Streptococcus mitis* (TYGS), *M. branderi* (NTMprofiler)
CZ265/20	unidentified Mycobacterial species	*M. arupense*	Potential novel NTM species (TYGS), NTM not detected (NTMprofiler)
CZ356/20	Mixed	*M. chimaera (91%) / M. vulneris (8.8%)*	*M. intracellulare* (TYGS and NTMprofiler)
CZ118/23	unidentified Mycobacterial species	NTM not detected	*M. celatum* (TYGS and NTMprofiler)

TYGS - Type Strain Genome Server; NTM– non-tuberculous mycobacteria

Phylogenetic classification and characterization of potential novel NTM species

To further classify the potentially new species, a GBDP tree was calculated, incorporating all closely related *Mycobacterium* reference genomes (Fig. 3).

Genome-to-genome distances were computed based on dDDH and ANI to assess species-level boundaries. Based on dDDH formula d0 and d4, the closest reference genome to strain CZ13121 is *M. wolinskyi*, a rapidly growing mycobacterium (RGM). The ANI compared to the *M. wolinskyi* type strain ATCC700010 was only 86.3% with 75.0% alignment fraction (AF), indicating that CZ13121 represents a distinct species within the *Mycobacterium* genus.

For strain CZ26520, dDDH formula d0 suggests that the closest reference genome is *M. senuense*, with *M. heraklionense* as the second closest and not *M. arupense* as suggested by Deeplex Myc-TB. In addition, using the dDDH formula d4, which is considered more reliable for draft genomes, the closest related genome is *M. heraklionense*, a slow-growing mycobacterium (SGM) belonging to the *M. terrae* complex. Similarly, for strain CZ46821, both dDDH formulas d0 and d4 indicate that the closest reference genome is *M. heraklionense* but ANI with this strain is still < 95%. Moreover, as CZ26520 and CZ46821 show a pairwise ANI of 98.6% and AF of 94%, the two strains can be considered as belonging to the same novel species.

Based on experimental growth rate, CZ26520 and CZ46821 were classified as slow-growing NTM, while CZ13121 was categorized as a fast-growing NTM.

Resistance and clinical relevance of NTM

Most isolates (22/28) were susceptible to amikacin, ciprofloxacin, moxifloxacin, clarithromycin and linezolid (Table 2). The most prevalent resistance was observed to ciprofloxacin (4/28). Among the resistant isolates, the novel species CZ131/21 demonstrated exceptional inducible resistance to clarithromycin, with a MIC exceeding 32 µg/ml, which might be attributed to the presence of a methyltransferase (*erm*) gene as detected by AMRfinder + with relaxed thresholds (Supplementary Table 2). The isolate *M. branderi* (CZ614/21) demonstrated resistance to both linezolid and ciprofloxacin. This patient met the ATS/ERS/ESCMID/IDSA criteria and, despite resistance, administering the combination of ethambutol, ciprofloxacin, and clarithromycin led to successful sputum culture conversion (Tables 2 and 3). *M. kumamotonense* (CZ126/21) from a patient with confirmed pulmonary mycobacteriosis exhibited resistance to moxifloxacin and ciprofloxacin, and intermediate susceptibility to linezolid, but treatment and outcome data were not available for this patient (Tables 2 and 3).

Five additional patients met the ATS/ERS/ESCMID/IDSA criteria for NTM pulmonary disease, and two cases involved pediatric patients presenting with typical cervical lymphadenitis (extrapulmonary NTM infection) caused by *M. lentiflavum* and *M. celatum*, yielding a substantial rate of confirmed mycobacterial infections (9 out of 27 patients; 33.3%) (Table 3). All four patients who fulfilled ATS/ERS/ESCMID/IDSA criteria and received a combination therapy of rifampicin, ethambutol, and clarithromycin showed culture conversion and, thus, positive treatment outcomes. In addition, one patient with lymphadenitis was successfully treated by surgical removal of the lymph node. At the same time, first-line anti-tuberculosis drugs with clarithromycin were effective in treating mixed infections with *M. tuberculosis* and *M. avium* (Supplementary Table 1). Furthermore, no relapse was observed in patients who achieved a successful treatment outcome as of December 2023.

Discussion

To our knowledge, this is the first study comparing the discriminatory power of Deeplex Myc-TB and WGS in identifying NTM species previously unidentified by the commercially available line probe assays from Hain lifesciences. To enhance the clinical significance of this study, we conducted MIC testing for all isolates against

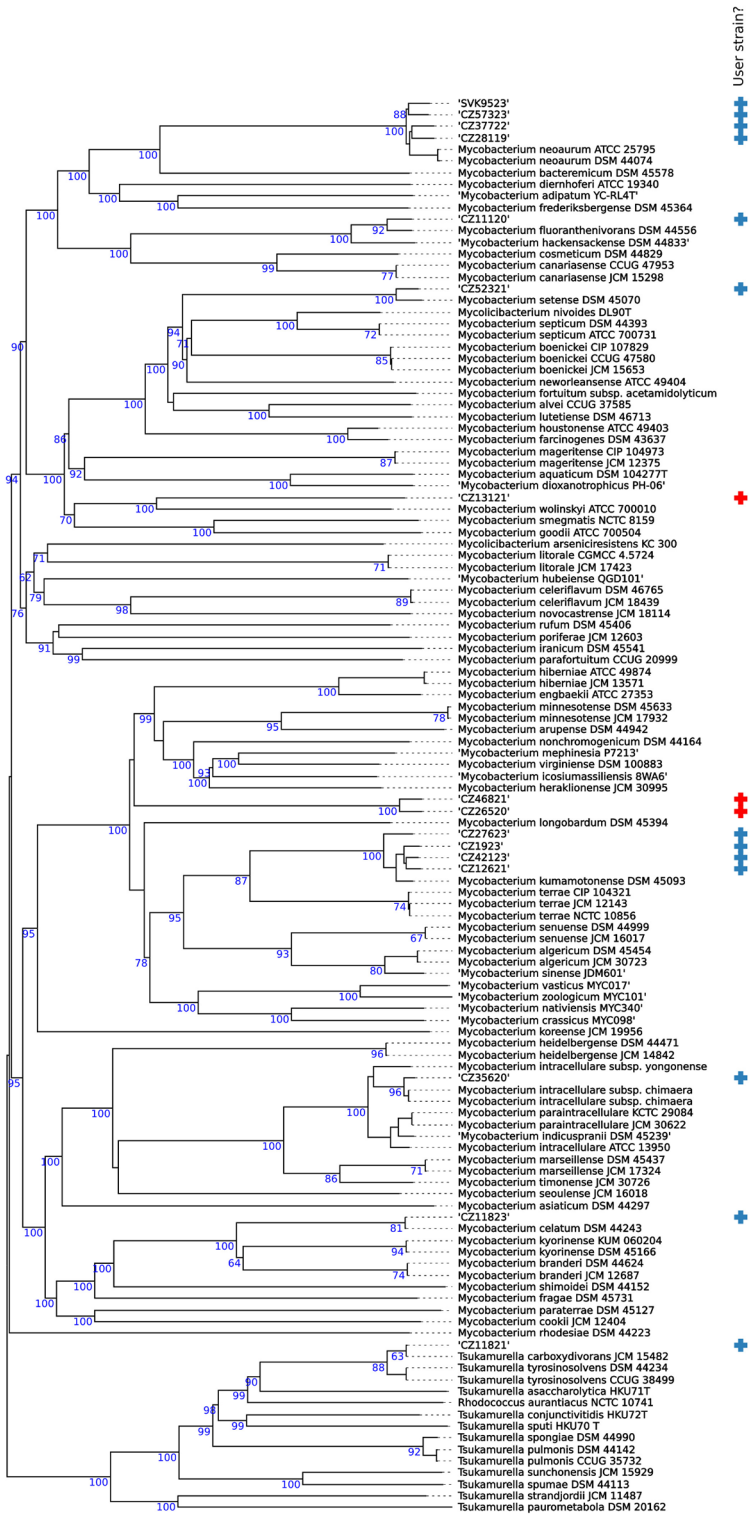

Fig. 3 Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudobootstrap support values > 60% from 100 replications, with an average branch support of 81.5%. The tree was rooted at the midpoint. Red crosses indicate novel NTM species. Three highly contaminated samples (CZ11223, CZ61421 and CZ45720) were not included. User strain refers to the isolates obtained in this study. The blue plus (+) symbol represents previously identified NTM species, while the red plus (+) symbol indicates novel NTM species

Table 2 Minimum inhibitory concentrations (MICs) of NTM against 5 antimycobacterial drugs

Patient	Species identified by tNGS/WGS	MIC (µg/ml)				
		AMI	CIP*	MXF	CLA	LZD
CZ281/19	*M. neoaurum*/ *M. neoaurum*	0.5 (S)	0.5 (S)	NE	0.25 (S)	NE
CZ377/22	*M. neoaurum*/ *M. neoaurum*	0.5 (S)	0.5 (S)	NE	NE	0.25 (S)
CZ573/23	*M. neoaurum*/ *M. neoaurum*	0.5 (S)	0.5 (S)	0.125 (S)	0.5 (S)	0.5 (S)
SVK95/23	*M. neoaurum*/ *M. neoaurum*	0.125 (S)	1 (S)	0.125 (S)	0.25 (S)	0.25 (S)
SVK407/23	*M. neoaurum*/NP	0.5 (S)	0.5 (S)	0.125 (S)	0.125 (S)	0.5 (S)
SVK408/23	*M. neoaurum*/NP	0.5 (S)	1 (S)	0.5 (S)	0.25 (S)	0.5 (S)
SVK432/23	*M. neoaurum*/NP	0.5 (S)	0.5 (S)	0.125 (S)	0.25 (S)	0.25 (S)
SVK231/23	*M. neoaurum*/NP	0.125 (S)	0.5 (S)	NE	0.5 (S)	0.25 (S)
CZ118/21	*M. intracellulare* - *M. kumamotonense*/ *Tsukamurella tyrosinosolvens*	0.5 (S)	0.5 (S)	0.125 (S)	1.0 (S)	4.0 (S)
CZ126/21	*M. kumamotonense*/ *M. kumamotonense*	32.0 (I)	**16.0 (R)**	**4.0** (**R**)	0.5 (S)	16.0 (I)
CZ19/23	*M. kumamotonense*/ *M. kumamotonense*	2.0 (S)	2.0	NE	NE	NE
CZ276/23	*M. kumamotonense*/ *M. kumamotonense*	1.0 (S)	1.0	0.5 (S)	1.0 (S)	4.0 (S)
CZ421/23	*M. kumamotonense*/ *M. kumamotonense*	4.0 (S)	4.0	2.0 (I)	4.0 (S)	**32.0** (**R**)
CZ131/21	*M. intracellulare* - *M. setense*/ potential novel species	4.0 (S)	0.5 (S)	0.125 (S)	**> 32.0** (**R**)	8.0 (S)
CZ614/21	*M. chimaera* - *M. massiliense*/ *Streptococcus mitis* (TYGS), *M. branderi* (NTMprofiler v0.2.2; 0.4.0)	32.0 (I)	**16.0** (**R**)	0.5 (S)	1.0 (S)	**32.0** (**R**)
CZ356/20	*M. chimaera* - *M. vulneris*/ *M. intracellulare*	4.0 (S)	**4.0** (**R**)	0.5 (S)	0.5 (S)	16.0 (I)
CZ457/20	*M. tuberculosis* - *M. avium*/ *M. tuberculosis* - *M. avium*	NE	NE	NE	NE	NE
CZ523/21	*M. setense*/ *M. setense*	0.5 (S)	0.5 (S)	0.125 (S)	4.0 (S)	4.0 (S)
CZ265/20	*M. arupense*/ potential novel species	2.0 (S)	1.0 (S)	0.5 (S)	0.125 (S)	0.25 (S)
CZ18/23	*M. arupense*/NP	8.0 (S)	**8.0** (**R**)	NE	NE	NE
SVK397/23	*M. arupense*/NP	4.0 (S)	1.0 (S)	0.25 (S)	0125 (S)	0.5 (S)
CZ118/23	NTM not detected/ *M. celatum*	0.5 (S)	2.0 (S)	0.25 (S)	0.5 (S)	**32.0** (**R**)
CZ468/21	NTM not detected/ potential novel species	1.0 (S)	1.0 (S)	1.0 (S)	0.5 (S)	0.5 (S)
SVK300/23	*M. arupense* - *M. yongonense*/NP	4.0 (S)	1.0 (S)	NE	NE	0.25 (S)
CZ111/20	*M. fluoranthenivorans*/ *M. fluoranthenivorans*	0.5 (S)	0.5 (S)	NE	0.25 (S)	NE
SVK403/23	*M. holsaticum*/NP	0.5 (S)	0.5 (S)	0.125 (S)	0.25 (S)	0.25 (S)
CZ112/23	*M. lentiflavum*/ *M. lentiflavum*	0.5 (S)	0.5 (S)	NE	NE	NE
SVK1921/23	*M. chimaera*/NP	4.0 (S)	0.5 (S)	0.125 (S)	0.5 (S)	8.0 (S)

*no recommended breakpoints for slow-growing mycobacteria by CLSI; NE– not evaluated; NP– not performed; R– resistant; I– intermediate; S– sensitive; AMI– amikacin; CIP– ciprofloxacin; MXF– moxifloxacin; CLA– clarithromycin; LZD– linezolid, tNGS– targeted next generation sequencing, boldface numbers indicate that isolate is resistant to specific drug

Table 3 Patients fulfilling the ATS/ERS/ESCMID/IDSA criteria/clinically relevant NTM disease

Patient	NTM species (WGS)	Sex	Age	Initial diagnosis	Clinical manifestation	Therapy	Outcome
CZ126/21	*M. kumamotonense*	F	74	COPD	Pulmonary	NA	NA
CZ19/23	*M. kumamotonense*	F	86	Cough	Pulmonary	NA	NA
CZ614/21	*M. branderi*	M	78	Pulmonary mycobacterial infection	Pulmonary	ETB, CIP, CLA	Culture negative
CZ265/20	potential novel species	F	73	Pulmonary mycobacterial infection	Pulmonary	NA	NA
CZ356/20	*M. intracellulare*	M	68	Pulmonary mycobacterial infection	Pulmonary	RIF, ETB, CLA	Culture negative
CZ112/23	*M. lentiflavum*	F	3	Disseminated atypical mycobacteriosis	Lymphadenitis	Surgical removal of the lymph node	Culture negative
CZ118/23	*M. celatum*	M	2	Disseminated atypical mycobacteriosis	Lymphadenitis	NA	NA
SVK95/23	*M. neoaurum*	F	70	Pulmonary mycobacterial infection	Pulmonary	RIF, ETB, CLA	Culture negative
SVK1921/23	**M. chimaera*	M	69	Pulmonary mycobacterial infection	Pulmonary	RIF, ETB, CLA	Culture negative

NA– not available; F– female; M– male; COPD - chronic obstructive pulmonary disease; ETB– ethambutol; RIF– rifampicin; CIP– ciprofloxacin; CLA– clarithromycin; INH– isoniazid; PZA– pyrazinamide, *identification by Deeplex Myc-TB only

five drugs commonly used in the treatment of NTM infections and retrieved outcome data for patients with extrapulmonary NTM infection or those meeting ATS/ERS/ESCMID/IDSA criteria for NTM pulmonary disease.

Deeplex Myc-TB identified NTM species in 27 (96.4%) isolates with the predominance of *M. neoaurum* and *M. kumamotonense*. Despite their widespread environmental presence, *M. neoaurum* and *M. kumamotonense* infections are rare [26, 27]. This study confirms the first three cases of pulmonary infections caused by these NTM in Slovakia and the Czech Republic. Notably, Deeplex Myc-TB and/or WGS identified *M. intracellulare (including M. chimaera)*, *M. (abscessus subsp.) massiliense*, *M. lentiflavu*m and *M. celatum* in a total of 5 samples. These species should have been detectable by the GenoType LPA, as they have species-specific probes for these species included, yet they were not identified by the tests. We speculate that the discrepancies in identification may be due to human error during the GenoType Mycobacterium assays or because the species-specific LPA probes are sequences that are not universally conserved across all strains of the respective species. We also observed a relatively high number of mixed NTM species identified by GenoType and Deeplex Myc-TB. However, WGS data detected the samepro mixed infection in only one sample (*M. tuberculosis*/*M. avium*), further highlighting the limitation of WGS combined with current bioinformatic tools and the greater sensitivity of Deeplex Myc-TB for detecting mixed and minority NTM populations in cultures and likely also primary specimen. On the other hand, Deeplex Myc-TB failed to detect two known NTM species in our dataset, i.e. *M. branderi* and *M. celatum*, possibly due to mismatches in the primer sequences. It also missed two potentially novel species, as Deeplex Myc-TB only relies on one gene (*hsp65*) for species identification and just reports the closest match for the detected allele(s). Based on its simplicity in data interpretation, high sensitivity for detecting mixed infections, and low probability of infections caused by novel NTM species, we recommend Deeplex Myc-TB as the primary method for identifying NTM. WGS should be employed in cases where Deeplex Myc-TB fails to provide identification or validate its results in patients experiencing unsuccessful treatment outcomes.

The majority of rare and potentially novel NTM species in this study were fully susceptible to a range of commonly used first-line antituberculosis drugs, as demonstrated by MIC testing. In addition, all patients treated with antibiotics, most often a combination of ethambutol, rifampicin and clarithromycin, showed culture conversion after treatment. However, as we don't have follow-up data on the patients, we cannot exclude the occurrence of relapses. Resistance to clarithromycin was observed in only one isolate, one of the potential new species, likely due to the presence of an inducible methyltransferase gene (*erm*). Two isolates (*M. branderi* and *M. kumamotonense*) were identified as multidrug-resistant, and the patients infected with these isolates met the ATS/ERS/ESCMID/IDSA criteria. These findings and previous studies indicate that ciprofloxacin and linezolid might not be effective in treating *M. branderi* infections [28]. While the results suggest potential risks associated with using fluoroquinolones to treat *M. kumamotonense* resistant to these drugs, a successful treatment outcome involving ciprofloxacin was also observed in a previous study [29].

This study has several limitations. The sample size is relatively small, although it represents a bi-nation-wide collection of isolates collected over five years. However, it may reflect the rarity of the NTM species studied. Future

studies with larger sample sizes may provide insights into their prevalence, global dissemination and characteristics and would further help to provide treatment guidelines for novel and rare species.

Conclusions

This study emphasizes the clinical utility of targeted deep-sequencing, coupled with WGS, in identifying rare (variants) and potential novel NTM species. While most isolates were susceptible to standard antibiotics, the emergence of multidrug-resistant strains underscores the importance of ongoing surveillance and the development of new therapeutic strategies. Future research should focus on expanding our understanding of NTM epidemiology, developing more rapid and affordable diagnostic tools that also consider rare species, and exploring novel treatment options to address the growing burden of these infections.

Abbreviations

NTM	Non-tuberculous mycobacteria
WGS	Whole-genome sequencing
LPA	Line probe assay
M	Mycobacterium
AFB	Acid-fast bacillus
ITS	Internal transcribed spacer
tNGS	Targeted next-generation sequencing
ATS	American Thoracic Society
ERS	European Respiratory Society
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
IDSA	Infectious Diseases Society of America
CLSI	Clinical and Laboratory Standards Institute
ANI	Average nucleotide identity
TYGS	Type strain genome server
DDH	Digital DNA: DNA hybridization
GGDC	Genome-to-Genome Distance Calculator
GBDP	Genome BLAST Distance Phylogeny approach
RGM	Rapidly growing mycobacterium
erm	Methyltransferase
MIC	Minimum inhibitory concentration

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12941-025-00781-z.

Supplementary Material 1

Supplementary Material 2

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Author contributions

Conceptualization, M. D., M. Di., N. W; Methodology, M.D, M. H., S. M., P. K., M. Š., I. P., I. S., V.D.; Software, M. Di.; Investigation, M. D., N. W., M. H., S. M., E. M. R., P. K., I. P., J. H., M. Di.; Data Curation, M. D., V. D.; Writing– Original Draft M.D. and M. Di.; Visualization: M. Di.; Supervision: S. N., J.M., M. Di.; Funding Acquisition: J. Hn., S. N., J. M. and M. Di.

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Data availability

All sequence data generated in this project has been deposited under ENA project number PRJEB81778.

Declarations

Ethical approval

This study was approved by the Ethical Committee of the Jessenius Faculty of Medicine in Martin, Comenius University Bratislava (EK65/2021). All methods were carried out following relevant guidelines and regulations.

Consent for publication

Not applicable.

Content of the publication

During the preparation of this work the author(s) used chatgpt in order to enhance the clarity and readability of the text. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Competing interests

The authors declare no competing interests.

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