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Application of whole-genome sequencing for distinguishing relapse from reinfection in tuberculosis patients from Lithuania

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ABSTRACT

Objectives: Tuberculosis (TB) can reoccur even after successful treatment due to endogenous reactivation or exogenous reinfection. Understanding the aetiology of TB recurrence might prevent further transmission and development of resistance. Therefore, this study aimed to assess the rate of true TB relapses *versus* reinfection among patients with TB recurrence in Lithuania using whole-genome sequencing (WGS).
Methods: This study included 62 *Mycobacterium tuberculosis* complex (MTBC) strains recovered from 29 pulmonary TB patients who had at least one reported TB recurrence or treatment failure episode between 2016 and 2023. To investigate potential sources of transmission in reinfected patients, 4 additional MTBC sequences were included in the analysis. The analysis of WGS results was performed using an in-house bioinformatic pipeline. A cut-off of 5 single nucleotide polymorphisms was used to differentiate between relapse and reinfection.
Results: Majority (60%) of all recurrent TB cases were caused by true relapse, while reinfections with a different strain accounted for 40%. Moreover, half of the treatment failures were also found to be reinfections.
Conclusions: The risk of reinfection is underestimated in Lithuania, highlighting the need for rapid changes in diagnostics and infection control strategies to contain the transmission of extensively drug-resistant TB (XDR-TB) strains in Lithuania.

Introduction

Although global tuberculosis (TB) treatment success rates have improved, reaching 86% for new patients on first-line treatment and 63% for those with rifampicin-resistant/multidrug-resistant TB (RR/MDR-TB) in 2020, a significant number of patients still do not achieve long-term recovery even after successful treatment [1]. Unfortunately, some patients may experience a recurrence of the disease caused either by endogenous reactivation of *Mycobacterium tuberculosis* complex (MTBC) strains responsible for the first episode, referred as "relapse" or exogenous reinfection with a distinct MTBC strain or even a mixed infection [2–4]. The source of the recurrence can be identified by comparing MTBC strains isolated from the initial and recurrent episodes. For many years, only limited-resolution techniques covering <1% of the MTBC genome were available for MTBC strains genotyping and relatedness analysis, such as spoligotyping or mycobacterial interspersed repetitive unit-variable number of tandem repeat typing [5]. The introduction of WGS has brought a significantly greater discriminative power compared to previous methods, allowing accurate characterization of MTBC strains [6]. However, neither technique has ever been implemented for routine genotyping and relatedness analysis in Lithuania.

Distinguishing the cause of TB recurrence is crucial for tracing transmission chains and guiding timely treatment. TB reinfection

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can be mistaken for a relapse or treatment failure due to a changed drug resistance pattern after initially successful treatment [3]. TB reinfection could likely occur in the household [7], within the community [8,9], or even in the hospital during treatment [10].

Despite significant progress in TB control, Lithuania still has the second-highest TB burden (26.3 TB cases per 100,000) after Romania (46.3 cases per 100,000) in the European Union and European Economic Area (EU/EEA) [11]. TB incidence in Lithuania remains substantially above the average incidence (8 TB cases per 100,000) in the EU/EEA countries [11]. Moreover, the percentage of recurrent TB should not be underestimated, as in 2022 it accounted for 13.3% (3.5 recurrent TB cases per 100,000) of the annual TB incidence in Lithuania (according to data provided by the Department of Programs and State Tuberculosis Information System in Lithuania).

Targeted interventions could reduce the incidence of the disease if the aetiology of TB recurrence were better understood [12]. However, this requires the implementation of sequencing-based methods. Therefore, the aim of this study was to assess the rate of true TB relapses *versus* reinfection among patients with TB recurrence in Lithuania using whole-genome sequencing (WGS).

Methods

Study sample

This retrospective study included 62 MTBC strains, recovered from 29 pulmonary TB patients who had at least one reported TB recurrence (20/29; 69%) (>6 months between visits) or treatment failure (9/29; 31%) episode, that were treated in Vilnius University Hospital Santaros Klinikos between 2016 and 2023. The ID of every strain consisted of the patient's number, the month, and the year of the isolate collection. These 29 patients do not represent all recurrent TB cases identified during this period, as some individuals who developed recurrent TB between 2016 and 2023 had experienced their previous episode before 2016, for which no corresponding isolates had been preserved. The systematic storage of MTBC isolates in our hospital was implemented only from 2016 onward; therefore, comparative analysis was not possible. In addition, 4 more MTBC sequences from our sequence database were later included in the analysis to investigate potential sources of transmission in reinfected patients. This database (n = 469) consisted predominantly of RR/MDR/pre-extensively drug resistant (pre-XDR)/extensively drug resistant (XDR) strains (96.2%), with only a small proportion of isoniazid (INH)-/poly-resistant/drug-susceptible strains (3.8%), which had a very low likelihood of being detected as part of possible transmission events.

DNA extraction

Frozen bacterial isolates were recovered on Löwenstein-Jensen slants and cultivated at 37°C until visible growth (about 3 weeks). Subsequently, colonies were transferred to tubes containing 150 ml of Tris-EDTA (TE) buffer and heat-killed in an 80°C water bath for 20 minutes. Genomic DNA was extracted using the column-based QIAamp DNA Mini Kit (Qiagen, Germany). The purified DNA was dissolved in TE buffer and subsequently quantified with the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA).

Whole-genome sequencing and bioinformatic data processing

WGS was done with Illumina NovaSeq 6000 sequencer (Illumina, USA) in 2 × 150 bp paired-end mode. The key bioinformatic analysis steps, as read mapping to the *Mycobacterium tuberculosis* H37Rv reference genome (NCBI RefSeq accession number NC_000962.3), variant calling, identification of low-frequency variants (with a reduced threshold of 1 for both forward and reverse reads), strain identification, and single nucleotide polymorphisms (SNP)-based comparative analysis, were carried out using MTBseq v1.0.4 [13]. Deletion identification analysis was conducted using Delly v1.1.6 [14].

To assess drug resistance-associated mutations, the second edition of the World Health Organization *Mycobacterium tuberculosis* mutation catalogue [15] was employed. For the detection of resistance-associated mutations in ribosomal genes such as *rrs*, a higher frequency threshold (≥40%) was applied to minimize the risk of false resistance due to minor contamination from other species with similar ribosomal sequences. A 10% variant frequency was used for other non-ribosomal with resistance-associated genes.

The raw reads were deposited under NCBI Bio-Project accession number PRJNA1267974.

Phylogenetic analysis

The multiple sequence alignment of alleles was constructed using the MTBseq bioinformatic pipeline, setting the distance at 5. To differentiate between relapse and reinfection, a cut-off of 5 SNPs was used [16]. Subsequently, Randomized Axelerated Maximum Likelihood v.8.2.12 was used to produce a robust bootstrap-supported phylogenetic tree. The resulting phylogenetic tree was visualized and annotated using the Interactive Tree of Life.

Results

Patient characteristics

The study population (n = 29; age 43 ± 11 years) predominantly consisted of males (26/29; 89.7%). Notably, a high proportion of patients were unemployed (23/29; 79.3%), smokers (24/29; 82.8%), and alcohol abusers (15/29; 51.7%) (Table 1). None of the patients reported being homeless.

Genotyping of MTBC strains

The MTBC isolates in this study belonged to Lineage 2 (30/62; 48.4%) and Lineage 4 (32/62; 51.6%) (Figure 1). The most prevalent genotype was Beijing (2.2.1) (30/62; 48.4%), followed by LAM (4.3.3) (15/62; 24.2%), mainly T (4.8 and 4.7) (10/62; 16.1% and 1/62; 1.6%, respectively), Ural (4.2.1) (5/62; 8.1%), and H37Rv-like (4.9) (1/62; 1.6%) (Supplementary Table S1).

Relapse vs reinfection vs treatment failure

The majority (12/20; 60%) of all reported relapses were caused by true relapse, while reinfections accounted for 40% (8/20). Of the 9 patients reported as treatment failures, 4 were reinfections, 1 was both a reinfection and a subsequent treatment failure, and the remaining 4 were true treatment failures (Figure 1). Out of the total of 13 reinfected patients, 7 were reinfected with the same MTBC genotype (mostly Beijing 2.2.1), while 6 were reinfected with a completely different genotype (Figure 1). The time between reinfection strain pairs varied from 7 to 82 months, while that between relapse strain pairs was 8-69 months. The SNP distance between reinfection strain pairs ranged from 9 to 2239 SNPs, while relapse strain pairs differed by 0-3 SNPs (Figure 2). However, relatedness analysis for patient 12 and patient 13 was challenging, as both pairs belonged to the Beijing 2.2.1 genotype and had only 9 SNPs difference. Nonetheless, other patients with relapse caused by the Beijing 2.2.1 genotype, even those with similar or longer time intervals between TB episodes (1-5 years), had an

Table 1
Patient characteristics of the initial episode stratified by the cause of TB recurrence.

Characteristics	The cause of tuberculosis recurrence determined by whole-genome sequencing			
	Relapse	Treatment failure	Reinfection[a]	Total
Gender				
Male	10	4	12	26
Female	2	0	1	3
HIV positive				
Yes	2	0	0	2
No	10	4	13	27
Diabetes mellitus				
Yes	3	1	0	4
No	9	3	13	25
Viral hepatitis C				
Yes	2	0	0	2
No	10	4	13	27
Drug addiction				
Yes	2	0	0	2
No	10	4	13	27
Alcohol use				
Abstinent	1	0	1	2
Occasionally	4	2	6	12
Alcohol abuse	5	1	5	13
Mental/behavioural disorders due to the use of alcohol	1	0	1	2
Smoking				
Yes	12	3	9	24
No	0	1	4	5
Employment				
Yes	3	1	2	6
No	9	3	11	23

[a] Patient 6 with reinfection and subsequent treatment failure was included in the reinfection category.

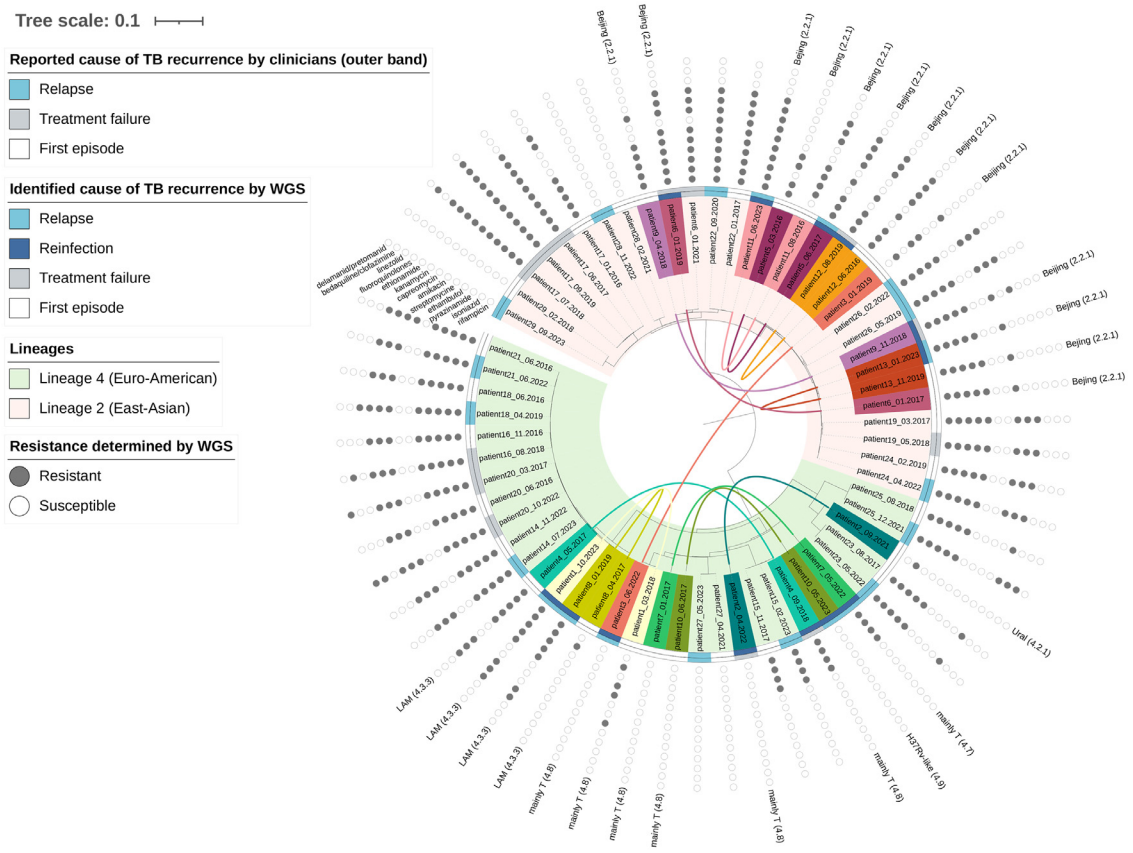

Figure 1. Phylogeny and drug resistance profile of 62 *Mycobacterium tuberculosis* strains from 29 recurrent/treatment failure patients. The ID of every strain consists of the patient's number, the month, and the year of the isolate collection. The inner band indicates the identified cause of tuberculosis (TB) recurrence/treatment failure (reinfection represents strain pairs differences >5 single nucleotide polymorphisms (SNPs), whereas relapse/treatment failure represents strain pairs differences ≤5 SNPs) by whole-genome sequencing (WGS), and the outer band indicates TB recurrence cause or treatment failure reported by clinicians (see legend). White bands represent the first available isolate of the patient that was available for sequencing. Strains of reinfected patients are highlighted with coloured rectangles and connected by curved lines, indicating paired strains. The identified *Mycobacterium tuberculosis* genotype is specified for strains in the reinfection case pair. Solid circles indicate drug resistance detected by whole-genome sequencing. Resistance to the delamanid/pretomanid and the bedaquiline/clofazimine is merged, as resistance to both drug pairs is caused by loss-of-function mutations associated with cross-resistance.

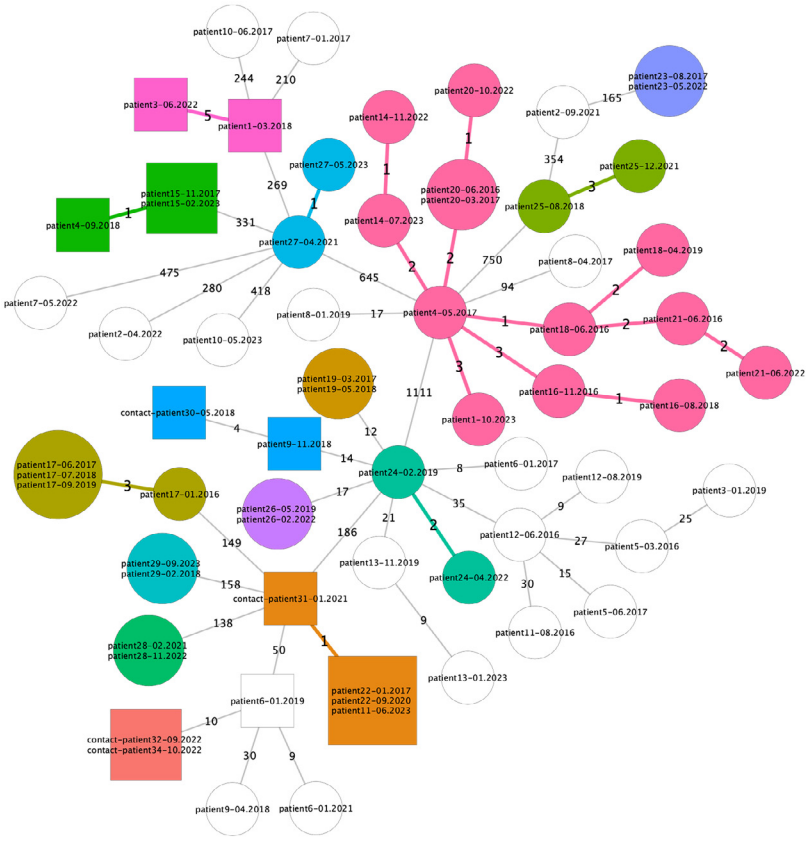

Figure 2. Minimum spanning tree of all *Mycobacterium tuberculosis* complex strains (n = 66) included in the study. The ID of every strain consists of the patient's number, the month, and the year of the isolate collection. Coloured nodes and branches indicate strains that are in ≤5 single nucleotide polymorphism distance. Rectangular shapes indicate strains that belong to five clusters of potential transmission in reinfected patients.

SNP distance of only 0-3. Subsequent analysis revealed that none of the mutations in the respective strains occurred in resistance-related genes, suggesting that these cases are more likely reinfections rather than relapses. In addition, treatment failure in patient 12 (patient12_06.2016) was confirmed in December 2018, and a new treatment regimen was initiated promptly in January 2019. As the patient was not discharged during the previous treatment period, reinfection within the hospital setting is more likely than a relapse.

Notably, an exception was made for the strain pair of patient6_01.2019 and patient6_01.2021. Although these isolates are at 9 SNP distance and do not meet the clustering threshold of 5 SNPs, they were still considered a relapse as patient6_01.2021 harboured the same mutations found in the earlier isolate (patient6_01.2019) at low frequencies (30-40%). The clustering algorithm only considers fixed mutations (≥75%) or wild-type positions, which leads to low-frequency variants being excluded from the distance calculation, even though they were present in a subpopulation.

Investigation of possible sources of reinfection

We investigated possible contacts for reinfected patients and performed relatedness analysis, including other available sequences in our database (469 MTBC genomes). Analysis revealed that 5 reinfected patients belonged to 5 clusters, of which, in 3 clusters, possible contacts included strains from this study sample (Supplementary Table S2). Furthermore, there were 4 strains from our MTBC genome database (4/469) that belonged to 3/5 clusters, and for clarity, were called "contact patients" (Supplementary Table S2).

Patient9_11.2018 was possibly in contact with contact_patient30_05.2018 (Supplementary Table S2; Figure 2), as both strains differed by only 4 SNPs. Patient3_06.2022 differed by 5 SNPs from patient1_03.2018 (first episode) (Supplementary Table S2; Figure 2). Patient11_06.2023 differed by 0 SNPs from both patient 22 isolates (patient22_01.2017; patient22_09.2020) and 1 SNP from contact_patient31_01.2021 (Supplementary Table S2; Figure 2). Patient4_09.2018 differed by 1 SNP from both patient 15 strains (patient15_11.2017; patient15_02.2023) (Supplementary Table S2; Figure 2). Patient6_01.2019 differed by 10 SNPs from strains in the additional database (contact_patient32_09.2022, contact_patient34_10.2022). However, both distant contacts developed TB later, in 2022 (Supplementary Table S2; Figure 2).

Primary drug resistance vs acquired drug resistance

The resistance pattern has not changed for ten relapse and two failure patients (including patient 6 who contracted treatment failure after the reinfection), while strains belonging to the rest two (patient 18 and 25) relapse and four failure (patient 6, 16, 17, and 20) cases gradually acquired more resistance-associated mutations. Strains isolated from patients 1, 5, 6, 7, 8, 9, and 11 showed an increase in drug resistance-associated mutations, as a result of reinfection. In contrast, strains isolated from reinfected patients 4 and 12 were less resistant, while those from patients 2 and 3 became fully susceptible after reinfection. Nevertheless, two patients (13 and 10) were reinfected with strains showing the same resistance profile of the initial strain. (Supplementary Table S1).

Resistance profiling

The majority of MTBC strains were pre-XDR (32/62; 51.6%), followed by susceptible (11/62; 17.7%), MDR (9/62; 14.5%), XDR (5/62;

8.1%), poly-resistant (3/62; 4.8%), and INH-monoresistant strains (2/62; 3.2%) (**Supplementary Table S1**). However, it is important to note that for some strains in the study sample, the new definitions for pre-XDR and XDR-TB, which were introduced in early 2021 [17], have been applied retrospectively.

The most prevalent rifampicin (RIF) resistance-conferring mutation was *rpoB* Ser450Leu, accounting for a total of 24 (24/62; 38.7%). Roughly half (33/62; 53.2%) of the INH-resistant variants harboured a single substitution in the codon 315 of the *katG* gene or were double mutants by *katG* Ser315Thr and mutations in the *inhA* gene (18/62; 29.0%). Most fluoroquinolone (FQL)-resistant mutants harboured *gyrA* substitutions in codons 94 (18/62; 29.0%), 90 (5/62; 8.1%), or 91 (2/62; 3.2%), while the *gyrB* Asn499Asp mutation was present in 11 isolates (11/62; 17.7%). Less frequent mutations conferring resistance to RIF, INH, and FQL in the remaining strains are listed in the Supplementary Table S1.

Loss-of-function (LoF) mutations in the *Rv0678* and *ddn* genes associated with bedaquiline/clofazimine (BDQ/CFZ) and delamanid/pretomanid (DLM/PA) cross-resistance were detected in four and two strains, respectively. Additionally, two strains harboured the *rplC* Cys154Arg mutation associated with resistance to linezolid (LZD) (Supplementary Table S1).

Discussion

To the best of our knowledge, this is the first study analyzing the aetiology of TB recurrence using WGS in Lithuania. In our study, reinfection was a more predominant cause of TB recurrence compared to other studies [2,18,19]. However, higher rates of reinfection were reported in countries with a high burden of TB compared with countries with a low TB incidence [20–23]. A portion of the isolates in our dataset was collected between 2016 and 2018, when Lithuania was still considered a high TB incidence country in the EU/EEA [11]. Nevertheless, we cannot rule out the possibility of misclassifying relapse as reinfection due to slightly distinct sequences, particularly in Beijing genotypes (e.g., patients 12 and 13). The Beijing genotype is known to mutate at a higher rate compared to other strains [24], and it is prevalent in Lithuania [25]. Conversely, in our study sample, the Beijing genotype strain pairs exhibited smaller SNP differences compared to other genotypes. Interpreting genetically similar or even identical genotypes as relapse can be challenging, as reinfection may occur with a genetically similar strain, leading to inconclusive results [26]. Other studies have also found possible reinfections with closely related strains [27], even comparing consecutive samples from single-episode patients [28]. It has been shown that reinfection typically occurs later than relapse [2,28], which aligns with our findings (reinfection: 7-82 months vs relapse: 8-69 months).

We intentionally included treatment failures, which are typically excluded from study samples [2,19,29]. Our findings indicate that including treatment failures can reveal the actual extent of TB reinfection (e.g., patients 2, 6, 9), as was also shown in neighbouring Latvia [28]. Furthermore, reinfection should have been suspected at least for some patients, especially when the strain from the initial episode shows a resistant phenotype and the strain from the second episode becomes less resistant (patients 4 and 12) or even completely susceptible (e.g., patients 2, 3).

On the other hand, a high rate of relapse and treatment failure suggests the need to improve patient management and diagnostics rather than transmission control. A relatively few studies have investigated sequencing-based methods for drug resistance prediction among MTBC strains from Lithuania [30,31]. While phenotypic resistance to BDQ and CFZ had been reported previously in strains from Lithuania [31], this is the first study to report genotypic resistance to BDQ, LZD, DLM, CFZ, and PA. Resistance to the latter drugs was acquired as patients received the corresponding treatments, except for PA. BDQ was not yet available for routine treatment in Lithuania in 2017; therefore, we assume that these patients participated in a clinical drug trial. However, this information is not available to us. Although phenotypic resistance in strains from Lithuania was reported previously, WGS failed to confirm it, likely due to the different bioinformatic pipeline used [31]. However, the authors noted that nonsynonymous nonsilent mutations were detected in phenotypically BDQ-resistant strains in the *Rv0678* gene. Our findings revealed that resistance to new and repurposed drugs existed already in 2017, before routine phenotypic drug susceptibility testing for these drugs became available in Lithuania. These results underscore the importance of having the capacity to test for resistance before a drug is implemented into treatment, a point increasingly emphasized by TB experts [32]. Unknown resistance could have contributed to unfavourable outcome due to a suboptimal treatment regimen, possibly consisting of fewer than four active drugs, as shown by other authors [4]. For instance, in 2017, patient 17 was transferred to another hospital and treated with a complex, frequently modified regimen that included BDQ, FQL, ethambutol, pyrazinamide, cycloserine, and capreomycin. In fact, there is no available evidence that phenotypic or genotypic drug susceptibility testing was performed at that time. According to retrospective WGS analysis, this patient received fewer than four active drugs, leading to the emergence of a clone harbouring cross-resistance to BDQ and CFZ. In 2022, a new regimen consisting of BDQ, FQL, DLM, LZD, and CFZ was initiated; however, WGS analysis indicates that LZD was the only active drug. These poorly designed treatment regimens contributed to the patient's death from TB in 2023.

The use of WGS could enable a rapid response to prevent transmission of XDR-TB strains, endangering the success of new treatment regimens [33].

Only two patients (3 and 4) reported possible TB contact among friends or family members, and this applied only to their first episode. The remaining reinfected patients were unaware of any potential exposure during either the first or second episodes. The investigation of potential clusters that strains from reinfected patients might belong to led us to speculate whether a few transmission events (e.g., patients 4, 9, 11) may have occurred in the hospital. Interestingly, patient 4 struggled to achieve culture conversion during his previous episode (patient4_05.2017), and this may have been influenced by reinfection from confirmed contact with patient 15, which later on caused TB recurrence. Patient 9 may have been reinfected from patient 30, as both patients had been treated in the same hospital unit at the same time. Considering the microscopy, culture results, and the timeline, we suspect that patient11_06.2023 may have been reinfected during the first hospitalization in 2017 from patient 22 but developed disease with this new strain later. However, this reinfection could also have occurred in the community. None of the reinfected patients reported household contacts; therefore, transmission for the rest of the patients most likely occurred in the community, or the actual contacts are missing. Studies have shown that transmission usually happens outside the household among individuals with shared interests or similar social backgrounds [34,35]. Our findings highlight the need for an extensive use of WGS when no direct contacts are identified, especially considering the distinct behavioural characteristics of our study group, such as unemployment and alcohol abuse. To establish an effective integrated surveillance system capable of rapidly responding to high-risk scenarios, multiple criteria should be taken into consideration [12,36–38].

There were several limitations to this study. First, for reinfected patients, we had no capacity to further investigate potential recent transmission within the household, as has been done in other studies [7]. Furthermore, including a control group of patients with only a single TB episode could enable the comparison of risk fac-

tors associated with TB recurrence. Lastly, the first available isolate used for sequencing did not always originate from the patient's first episode, as some patients in our dataset had previously contracted relapses or treatment failures.

Conclusions

Only 55% (16/29) of patients were correctly differentiated as relapse or treatment failure by clinicians. A high rate of misclassified reinfections as relapse or treatment failure due to limited diagnostics poses a challenge to TB control efforts. The nearly equal proportion of reinfection compared to relapse suggests a high risk of local transmission and highlights the need for effective public health interventions. Moreover, our findings revealed that XDR-TB strains were circulating in Lithuania before phenotypic drug susceptibility testing for new and repurposed drugs became available. Thus, the implementation of WGS may improve timely diagnosis and TB control in Lithuania.

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Ethics approval

This study was approved by the Lithuanian Regional Biomedical Research's Ethics Committee (decision no 2024/6-1569-1053). The study was exempted from the requirement for informed consent by the Lithuanian Regional Biomedical Research Ethics Committee. All methods were carried out in accordance with guidelines and regulations of the Vilnius University, and Vilnius University Hospital Santaros Klinikos.

Author contributions

LV conceptualized the study, drafted the manuscript, analysed and visualized clinical and bioinformatic data; AZ, FM performed bioinformatic analysis and data visualization; VED, BN collected and revised patient data; LV, VED obtained approval for the study; LV, AG extracted mycobacterial DNA; TK, DMC edited and provided critical review of the manuscript. All authors participated in reviewing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2025.108203.

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