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CASE REPORT

Pulmonary Tuberculosis with Polyclonal Infection – Diagnostic Challenges and the Importance of Sequencing to the Improvement of Case Management – Case Report

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Abstract: Many studies on the molecular epidemiology of tuberculosis (TB) have shown that 10–20% of patients may be infected with more than one strain of *Mycobacterium tuberculosis* (MTB), during a single episode of the disease. However, data on the frequency of this phenomenon are underestimated, and methodological difficulties mean that most cases are not detected. Below we present the first case of polyclonal/mixed infection documented in Poland. We described the case of a 33-year-old Ukrainian man, imprisoned in a Polish prison, who was diagnosed with pulmonary TB confirmed by culture. During the 6-month therapy, the patient was treated in three Polish hospitals, but no negative sputum test result was obtained. In the course of microbiological and molecular diagnostics, conducted from June 2021 to February 2022, divergent results were obtained from drug susceptibility testing and genotyping of MTB strains isolated from clinical materials from the patient. In the final stage of the tests, it was confirmed that the patient was infected with two strains of MTB - one of them was drug-sensitive and belonged to the T1 267 genotype, the other was pre-extensively drug resistant (pre-XDR) and belonged to the Beijing 265 genotype. The existence of clonally complex TB infections resulting in heteroresistance to basic antituberculosis drugs has important implications for patient care. Established molecular methods complemented by routine microbiological diagnostics allow rapid detection of these infections and appropriate adjustment of therapy.

Keywords: *Mycobacterium tuberculosis* mixed infection, polyclonal infection, heteroresistance, genotyping methods, next-generation sequencing

Introduction

A polyclonal/mixed infection is a situation when at least two phylogenetically different strains of the same pathogen are simultaneously present in one macroorganism. This may be the result of a single transmission event involving more than one distinct strain, or multiple transmission events (superinfection) during an episode of a single disease. Simultaneous transmission of multiple strains resulting in mixed infection may occur in susceptible individuals, with both strains able to evade the host defence system and resist attempts to kill them.[1] Failure to detect all strains in clinical material obtained from the patient with suspected TB may affect the effectiveness of treatment and clinical results. This is because unidentified strains may have different characteristics, such as drug resistance or virulence. This leads to a more complex picture of TB transmission and infection.

Case Study

In June 2021, a 33-year-old Ukrainian man, incarcerated in Polish prison in Lublin, was diagnosed with pulmonary TB culture confirmed. Until the antibiotic susceptibility testing results were obtained, the patient was treated empirically with a standard regimen for drug-sensitive TB. At the time, the patient was transferred to a prison hospital in the Pomeranian Voivodship, where he still revealed sputum-positivity confirmed by fluorescent stain and Ziehl-Neelsen microscopy (AFB+++) and

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cultured of *Mycobacterium tuberculosis* complex strains (MTBC). Antibiogram performed using phenotypic methods on liquid medium, both in the regional laboratory and hospital laboratory, showed resistance of the strains to streptomycin (SM), isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). In response to these results, the treatment regimen was modified according to the Polish national guidelines, which are aligned with WHO recommendations for MDR-TB at the time. The modified regimen included second-line anti-TB drugs such as fluoroquinolones (FQ), linezolid (LIN), amikacin (AMK), and cycloserine (CS). Despite the change in treatment, sputum-negativity was not achieved during the six-month therapy (September 2021 – February 2022).

In February 2022, after serving his sentence, the patient was transferred to a civil hospital, where MTBC bacilli with multidrug-resistance (MDR) profile were again cultured in the sputum during a follow-up examination. At the time, the patient discontinued treatment and left the hospital, probably returned to his country of origin.

Three MTB isolates obtained from patient at different sampling times (in June 2021 – before treatment, in September 2021 – during treatment recommended for drug-sensitive TB and in February 2022 – after 6 months of WHO-recommended treatment for MDR TB) were cultured on Löwenstein–Jensen (L-J) medium in three regional laboratories and sent for verification to the National Reference Laboratory of Mycobacteria in Warsaw, where their genotype and phenotype were analysed.

Molecular drug resistance to INH, RMP, EMB, FQ, AMK, capreomycin (CAP), and kanamycin (KAN) was analysed using a commercial tests GenoType (Hain Lifescience GmbH, Nehren, Germany); their spoligotypes were determined (Ocimum Biosolutions, Hyderabad, India). All strains were identified as drug-sensitive and belonging to the SIT 267 genotype. At the same time, the drug resistance phenotype of the strains was determined on liquid medium using the Bactec MGIT 960 system, and in each case pre-XDR resistance (MDR + FQ resistance) was obtained. Cultures carried out on liquid medium during antibiotic susceptibility determination were screened onto L-J solid medium. After growth was obtained, DNA was isolated from random 3 colonies and genotyped. Spoligotyping of drug-resistant random isolates (A, B, C) identified 3 different spoligotypes: SIT 267, SIT 265 and 777700003763771 (Figure 1A). The Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeats (MIRU-VNTR) analysis revealed three different genotypes of the strains, one of them (C) had double alleles detected in 5 PCR reactions (Figure 1B).

The next stage of molecular analysis involved sequencing using the Deeplex Myc-TB® system (Genoscreen, France).

It was confirmed that isolate A belonged to spoligotype SIT 267 and was a drug-susceptible strain. Isolate B belonged to the Beijing 265 molecular family and had mutations causing resistance to RIF, INH, PZA, EMB, FQ, LIN, bedaquiline (BDQ), clofazimine (CFZ), SM, ethionamide (ETH) and KAN (XDR resistance). Isolate C was a mixture of two genetic variants, one of which accounted for 91.67% and the other for 8.33% of the mixture (Figure 1C).

In the final stage of the analysis, the strains were subcultured on Middlebrook 7H10 agar to isolate pure cultures of the two phylogenetic variants previously identified through spoligotyping and sequencing: (1) a drug-susceptible strain with the SIT 267 spoligotype, and (2) a multidrug-resistant strain belonging to the Beijing family, identified as SIT 265 (Figure 2). Six colonies were randomly selected; their genotype and phenotype were again determined. Colonies 1–3 were drug-sensitive with the SIT 267 spoligotype, and colonies 4–6 were identified as pre-XDR strains with the SIT 265 spoligotype.

Discussion and Conclusions

The phenomenon of polyclonal/mixed infection in TB was first detected and described in 1975 using phage typing.[2] Currently, in line with the international standards for MTB genotyping,[3] methods such as MIRU-VNTR, IS6110-Restriction Fragment Length Polymorphism typing (IS6110-RFLP typing) and genome sequencing, are commonly used in molecular epidemiological investigations, in studies on TB transmission and are applied to identify mixed infections. The rate of detected mixed infections can vary (from 0.4% to 57.1%), which often depends on the genotyping method.[4–6]

The authors of the study used methods recommended in molecular epidemiological investigations in TB. In the case of spoligotyping, difficulties were encountered in interpreting the DNA pattern, which, on further analysis, turned out to be a hybrid of two spoligotypes. As a result of the overlap of Direct Repeat sequences from two different strains,

a unique genetic pattern was created which has not yet been registered in the international spoligotype database (SITVIT2). As described previously, mutual masking of two spoligotypes may lead to incorrect identification of infection with a single MTBC strain. This phenomenon significantly limits the usefulness of spoligotyping in detecting mixed infections and the independent use of this method in this case.

In our research, the MIRU-VNTR method was applied as a complement to spoligotyping. It is characterised by higher sensitivity and discrimination power in identifying mixed infections than other methods of phylogenetic analysis. It is especially useful for identifying polyclonal infections caused by strains with the same spoligotype.[7,8] In MIRU-VNTR analysis, mixed infection caused by two strains is defined as the presence of two alleles in at least two *loci*.[9] Our analysis revealed double alleles in 5 PCR reactions (MIRU16, VNTR42, VNTR47, QUB11b, QUB26), which clearly confirmed the presence of polyclonal infection. However, MIRU-VNTR analyses only part of the MTB genome, and this can limit the usefulness of this method.[10]

Additionally, for a more complete analysis, the strains isolated after passage through liquid medium were sequenced using the Deeplex Myc-TB system. A mixture of two phylogenetic variants was detected in isolate C, with the drug-sensitive variant predominating (91.67%). For this reason, there was no clear result of the drug resistance profile. Interpretation of drug resistance results obtained by sequencing requires comparison with phenotype analysis. For

A

Isolate A – Spoligotype SIT 267

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Isolate B – Spoligotype SIT 265

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Isolate C – Spoligotype 777700003763771

(a unique genetic pattern – a hybrid of spoligotype SIT 267 and SIT 265)

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B

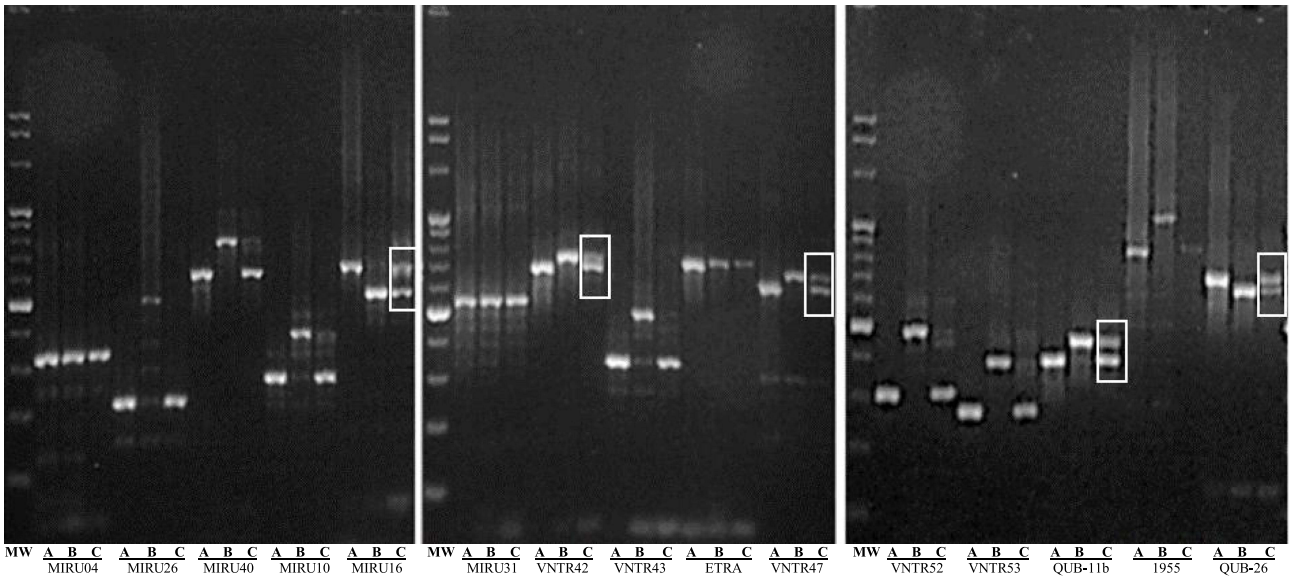


Figure 1a Continued.

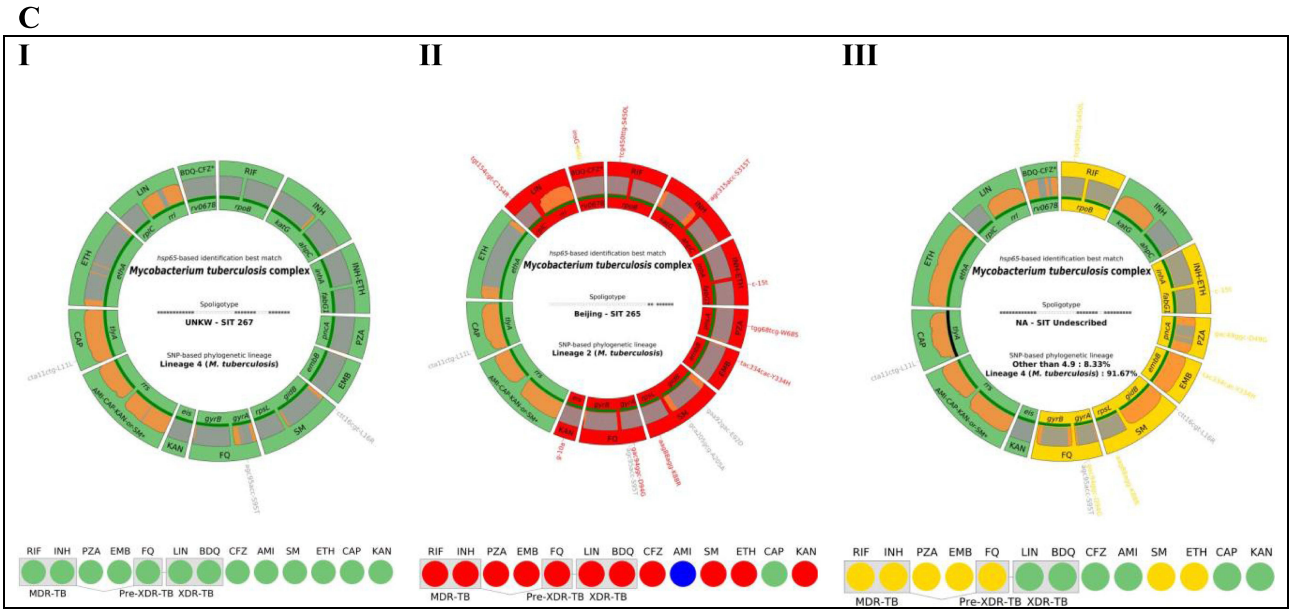


Figure 1b Molecular analysis of strains using applied genotyping methods. (**A**) Spoligotyping of the strains passed through liquid medium – the analysis of three different DNA isolates from cultures in liquid medium; (**B**) The results of Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeats (MIRU-VNTR) analysis of isolate A, B and C. The products obtained in 15 PCR reactions, visualised during gel electrophoresis. MW – molecular weight marker. Double PCR products identified in 5 reactions (MIRU16, VNTR42, VNTR47, QUB11b, QUB26) obtained for C isolate are marked; (**C**) Sequencing results obtained with Deeplex Myc-TB® system. The results of genome sequence analysis for isolate A, B and C. I (isolate (**A**) – spoligotype SIT 267, drug-susceptible strain; II (isolate (**B**) – spoligotype SIT 265, strain with XDR resistance; III (isolate (**C**) – spoligotype 777700003763771 (unregistered), a mixture of two phylogenetic variants (one from lineage 4–91.67%, the other belonging to a different phylogenetic lineage - 8.33%). The information on hsp65 best-match-based identification, spoligotype and phylogenetic SNP-based identification of MTBC lineage is shown in the centre of the circle. The information on drug susceptibility and drug resistance predictions for 13 anti-tuberculous drugs/drug classes is as follows. Target gene regions are grouped within sectors in a circular map according to the anti-tuberculous drug resistance (based on associations). Sectors in red and green indicate targets in which resistance-associated mutations, no mutations or only mutations not associated with resistance (shown in grey) are detected, resulting in predictions of resistant or susceptible phenotypes, respectively. Sections in yellow indicate targets where drug resistance-associated mutations have been detected, but due to the preponderance of drug-sensitive variants, the result is inclusive.

Abbreviations: RIF, Rifampicin; INH, Isoniazid; PZA, Pyrazinamide; EMB, Ethambutol; FQ, Fluoroquinolones; LIN, Linezolid; BDQ, Bedaquiline; CFZ, Clofazimine; AMK, Amikacin; SM, Streptomycin; ETH, Ethionamide; CAP, Capreomycin; KAN, Kanamycin.

example, in isolate B, numerous mutations in genes associated with drug resistance were observed, however, they did not produce a phenotypic effect in the Bactec MGIT 960 system. This discrepancy between genotypic and phenotypic results indicates the need for further research and analysis in the context of creating algorithms for determining drug resistance of mycobacteria. This will allow for more precise determination which genetic mutations actually translate into phenotypic resistance, and this is crucial for the effective treatment and control of TB.

Opinions on the usefulness of sequencing methods in detecting mixed infections vary. The method has been shown to be more valuable in detecting heteroresistance within a single strain than in the case of infections caused by more than one MTBC strain.[11] Despite its numerous advantages, there are factors that limit the use of whole-genome sequencing, such as high cost and difficulty in data interpretation.[12]

The use of Deeplex Myc-TB® system allowed for a detailed analysis of the genetic material of mycobacteria and for detection of various genetic variants present in the sample. More-over, both genetic variants were detected in different proportions (91.67% and 8.33%), and different drug resistance profiles (drug-susceptible and XDR) were identified, further emphasising the complexity of mixed infection.

So far, little data has been published on the usefulness of Deeplex Myc-TB for detecting polyclonal infections in TB. Single reports focus mainly on the identification of drug-resistant strains and heteroresistance.[13,14] In our opinion, the system can be used to detect a mixture of genetic variants with different spoligotypes. However, since in a mixture of strains with different drug sensitivities the system does not identify them separately, the determination of individual drug susceptibility profiles for each strain should still be based on other genetic and phenotypic methods, including isolation of single colonies. The investigation was justified due to inconsistent results of drug resistance tests and treatment failure, which resulted in long-term sputum-positivity despite therapy. After passing the strains through the liquid medium, the

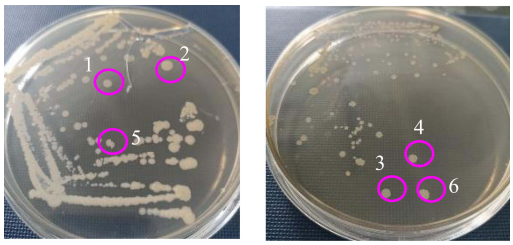
Isolation of single colonies on Middlebrook 7H10 agar	
DNA isolation from randomly selected colonies	
Spoligotyping	Colony 1, 2, 3 – Spoligotype SIT 267 ■■■■■■■■■■■■□□□□□■□□□□□□□■■■■■■■□□□□■■■■ ■■■ Colony 4, 5, 6 – Spoligotype SIT 265 □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■□■■■ ■■■
Drug resistance genotype of isolates obtained from single colonies	Colony 1, 2, 3 – drug sensitive Colony 4, 5, 6 – pre-XDR resistance, strains resistant to RMP, INH, FQ
Drug resistance phenotype of isolates obtained from single colonies	Colony 1, 2, 3 – drug sensitive Colony 4, 5, 6 – Pre-XDR resistance, strains resistant to SM, INH, RMP, PZA, RIF, FQ

Figure 2 Isolation of single colonies on Middlebrook 7H10 agar. Single colonies obtained on agar 7H10; the analysis of phenotype and genotype. Pink circles in the photos indicate colonies from which DNA was isolated; spoligotyping, genotype and drug resistance phenotype analysis were performed. Six colonies were randomly selected; their genotype and phenotype were again determined. Colonies 1–3 were drug-sensitive with the SIT 267 spoligotype, and colonies 4–6 were identified as pre-XDR strains with the SIT 265 spoligotype.

mixture became dominated by the drug-resistant Beijing 265 strain, while the drug-sensitive strain was no longer detectable and became a "hidden genotype". As a consequence, the pre-XDR resistance phenotype was identified and the treatment was modified according to the antibiogram for the drug-resistant strain.

However, it is important to note that treatment guided solely by the dominant genotype may not always yield the expected therapeutic results. For instance, even when the dominant strain is drug-resistant and treatment is adjusted accordingly, the presence of other, less abundant strains — possibly with different resistance profiles — may compromise treatment efficacy. This highlights a potential limitation: if these minority strains are also resistant to drugs included in

the modified regimen, they can undermine the overall therapeutic outcome.[15] It is believed that direct isolation of DNA from clinical material obtained from the patient may provide a more representative sample of the entire population of strains.[16] With this approach, it appears that sequencing using the Deeplex Myc-TB® system is a promising method for detecting mixed infections. Biobanking of clinical materials obtained from patients could be a potential solution to improve detection of mixed infections. Preservation of clinical samples would allow for retrospective analysis in the event of inconsistent drug resistance results and in the case of treatment failure. Such a strategy would enable a more accurate assessment of the genetic variability of a given bacterial population and a better understanding of drug resistance mechanisms. Biobanking of clinical materials could be a key element in improving the diagnosis and treatment of TB, allowing for a more precise and personalised approach to therapy.

However, the isolation of mycobacterial DNA directly from clinical material is a very problematic step that may significantly limit further sequencing steps.[17]

Additional prospective studies with larger sample sizes are planned to further evaluate challenges related to the implementation and characteristics of the Deeplex-MycTB system in settings with diverse epidemiology and infrastructure. The aim of the research will be to conduct more detailed analysis of the effectiveness and accuracy of the system in detecting mixtures of strains and to evaluate its suitability in various clinical contexts.

Our observations confirm the existence of clonally complex TB infections, which indicates an increasing trend in recent years. The awareness of this phenomenon has important implications for patient care because polyclonal infections may be associated with heteroresistance to basic anti-tuberculous drugs.

In our study, the spoligotype SIT 267 was predominant among the isolates, with additional detection of spoligotypes SIT 265 and a unique, previously unregistered pattern. According to international databases (SITVIT2), SIT 267 is relatively uncommon in global datasets but has been sporadically reported in Central and Eastern Europe. SIT 265 is more prevalent in East Asian settings and is often associated with the Beijing lineage. The presence of both spoligotypes in a single patient, and the emergence of a novel pattern resulting from hybridization, highlight the dynamic nature of MTBC transmission and evolution. These findings are in line with previous reports that suggest both common and rare genotypes can co-occur within the same host, contributing to complex patterns of infection and resistance. Further surveillance studies are warranted to determine whether these genotypes represent recent importations, local evolution, or underrecognized endemic clades.[10,13,15]

Molecular testing, on the other hand, involves analyzing the genetic material of individual organisms or colonies to identify specific genetic markers. While molecular testing can provide precise insights into genetic make-up, one limitation is that individual colonies might only be tested for certain assays and not others. For example, if only specific genetic markers are applied, you may miss out on important variations that could affect the phenotype. This limitation can affect the overall accuracy or comprehensiveness of the results when comparing them to phenotypic methods.

Abbreviations

MTB, *Mycobacterium tuberculosis*; Pre-XDR, Pre-extensively drug resistant; AFB, Acid Fast Bacilli; MTBC, *Mycobacterium tuberculosis* complex; SM, Streptomycin; INH, Isoniazid; RIF, Rifampicin; EMB, Ethambutol; PZA, Pyrazinamide; MDR, Multidrug-resistance; L-J, Löwenstein–Jensen; FQ, Fluoroquinolone; AMK, Amikacin; CAP, Capreomycin; KAN, Kanamycin; MIRU-VNTR, Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeats; LIN, Linezolid; BDQ, Bedaquiline; CFZ, Clofazimine; ETH, Ethionamide; IS6110-RFLP, IS6110-Restriction Fragment Length Polymorphism.

Informed Consent Statement

During the patient's hospitalization, written informed consent was obtained for publication of the findings and case report.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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