RPDI
Maio > Dezembro 2023 / Vol. 18 > N.os 2-3

CASO CLÍNICO / *CLINICAL CASE*

Deteção de mutações do gene *rpoB* fora da região determinante de resistência à rifampicina do *Mycobacterium tuberculosis*

Detection of rpoB *Gene Mutations Outside the Rifampicin Resistance-Determining Region of* Mycobacterium tuberculosis

/ **Carla Sofia da Costa Correia Amorim**
/ **Maria José Padre-Santo Janeiro**
/ **Marta Filomena Melo Ferreira Rijo**
/ **Tânia Isabel Dias Vermelhudo Batalha**
/ **Teresa Leonor Dimas Araújo**

Clinical Pathology Laboratory. Hospital de Dona Estefânia. Centro Hospitalar Universitário de Lisboa Central. Rua Jacinta Marto, 1169-045 Lisboa, Portugal

Correspondência:
Carla Sofia da Costa Correia Amorim
Laboratório de Patologia Clínica. Hospital de Dona Estefânia.
Centro Hospitalar Universitário de Lisboa Central.
Rua Jacinta Marto, 1169-045 Lisboa, Portugal.
Tel.: 917034131
E-mail: carlaamorim80@gmail.com

Patrocínios:
O presente estudo não foi patrocinado por qualquer entidade.

Artigo recebido em
02/10/2023

Artigo aceite para publicação em
15/01/2024

/ Resumo

O diagnóstico da tuberculose pulmonar deve ser alcançado o mais precocemente possível devido ao risco de contágio, mas embora os exames e as técnicas usadas na abordagem diagnóstica tenham vindo a sofrer grandes avanços nomeadamente na área laboratorial, continuam ainda a haver muitos desafios que nos vão sendo colocados quotidianamente, sobretudo no que respeita às estirpes do complexo *Micobacterium tuberculosis* com resistências aos fármacos antibacilares. Os testes moleculares são rápidos e muito importantes no diagnóstico e início do tratamento da tuberculose, contudo, os exames fenotípicos são indispensáveis. O isolamento cultural permite confirmar a viabilidade dos bacilos presentes na amostra e a realização posterior do teste de sensibilidade aos antibióticos (TSA) fenotípico, o qual, apesar de ser um teste lento e requerer infraestrutura especializada e pessoal altamente qualificado, no caso clínico que apresentamos permitiu identificar uma discordância de resultado em relação ao do teste molecular, confirmada posteriormente por sequenciação genómica, no que respeita à resistência da rifampicina.
Através do TSA também foi possível detetar que se estava perante uma estirpe multirresistente e alertar para a necessidade de realização do antibiograma de segunda linha e sequenciação genómica.

Palavras-chave: TSA fenotípico, *Mycobacterium tuberculosis*, genótipo Beijing

/ Abstract

Pulmonary tuberculosis shlould be diagnosed as early as possible due to the risk of contagion. Although the tests and techniques used in the diagnostic approach have undergone great advances, namely in the laboratory, there are still many challenges being faced on a daily basis, especially regarding strains of the Mycobacterium tuberculosis *complex that are resistant to antituberculosis drugs. Molecular tests are rapid and very important in the diagnosis and start of treatment of tuberculosis, but phenotypic tests are still mandatory. Culture isolation allows confirmation of the viability of the bacilli present in the sample and identification for subsequent phenotypic Drug-Susceptibility Testing, which, despite being a slow test, requires specialized infrastructure and highly qualified personnel. In the clinical case presented here, a discordance was identified between the molecular test and the phenotypic Drug-Susceptibility Testing, regarding rifampicin resistance, confirmed by genomic sequencing. Drug-Susceptibility Testing allowed the detection of a multidrug-resistant strain, alerting to the need to perform a second-line antibiogram and detection of the mutations involved through genomic sequencing.*

Keywords: *Phenotypic Drug Resistance;* Mycobacterium tuberculosis*;* Beijing genotype

/ Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (MTC), which in most cases develops in the lung, and its transmission is mainly through the air. About a quarter of the world's population has a TB infection [1]. Individuals infected with the bacteria may take some time to develop signs and symptoms of the disease and to transmit it (active infection). When TB passes into its active state, the most common symptoms include prolonged cough (accompanied or not by secretions), chest pain, weakness or fatigue, weight loss, fever and night sweats. Often, these symptoms will be mild for many months, thus leading to delays in seeking care and increasing the risk of spreading the infection to others, which may lead to uncontrolled transmission of the disease among the population [2].

According to World Health Organization (WHO), in 2021 the highest number of new cases were in the WHO regions of South-East Asia (45%), Africa (23%) and the Western Pacific (18%), with smaller proportions in the Eastern Mediterranean (8.1%), the Americas (2.9%) and Europe (2.2%). However, only eight countries accounted for more than two thirds of the global total: India (28%), Indonesia (9.2%), China (7.4%), Philippines (7.0%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%) and the Democratic Republic of the Congo (2.9%) [3].

TB is a communicable disease that is a major cause of morbidity and one of the leading causes of death worldwide [3]. Despite, over 95% of cases and deaths occurring in developing countries, in European Union (EU) countries TB remains a public health issue even though, most are considered low-incidence countries (with a notification rate below 10 per 100,000). In the EU, TB predominantly affects populations such as migrants, prisoners and individuals co-infected with HIV [3,4].

In Portugal, there has been a sustained reduction in the notification of new TB cases since 2000. However, in order to control the disease and accelerate the annual reduction of new cases, it is important to reduce the time of active TB diagnosis, ensure the interruption of transmission chains in the community and identify susceptible individuals so that preventive actions can be taken [5].

In the context of TB surveillance and monitoring in Lisbon and Tagus Valley region (2016/2020), it was found that immigration is the main social determinant related to TB with 47.3% of new TB cases in the region being associated with the immigrant population [5]. In Lisbon city, where the Centro Hospitalar Universitário Lisboa Central (CHULC) is located, the notification rate was 30.4% with a total of 771 cases in this period, corresponding to the highest value in the whole country [5].

According to European Centre for Disease Prevention and Control (ECDC), in it's 2022 TB Surveillance and Monitoring in Europe report, in the EU, from all TB cases in which Drug-Susceptibility Testing (DST) was performed, 3.8% were found to be to Multidrug-

Resistant Tuberculosis (MDR-TB), i.e. *Mycobacterium tuberculosis* complex (MTBC) with resistance to rifampicin (RIF) and isoniazid (INH), the two most importante first- line therapeutic drugs against TB. Of these, in cases in which sensitivity to fluoroquinolones (FQ) was tested, 27.6% were classified as pre-extensively resistant (Pre-XDR) tuberculosis, i.e. a strain of MDR-TB that is also resistant to any FQ [6,7].

The Beijing family strains have a high prevalence among MDR-TB cases and are associated with rapid dissemination, higher virulence, transmissibility and mutation characteristics. The prevalence of the Beijing strain is 44.7% in Asia, 27.9% in Europe, 12.5% in Africa and 8.9% in the Americas [5,8].

DNA sequencing of MDR-TB strains, has been carried out to detect codons associated with resistance to anti-TB drugs, mainly to RIF and INH. Studies have shown that 95% of the isolates with resistance to RIF present a mutation in the rifampicin resistance-determining region (RDRR) - mutational "hotspots" - of the *rpoB* gene, a region of 81 base pairs (bp) that codes for the RNA polymerase B subunit, and the most frequent mutations affect codon 531 with substitution of the amino acid (aa) serine (Ser) by leucine (Leu), but also in codons 516 and 526. Of the INH-resistant strains, 30-70% have mutations in the *katG* and *inhA* genes. The *inhA* gene and its regulator encodes the inhA protein involved in fatty acid synthesis while *katG* gene encodes an enzyme involved in mycolic acid synthesis, catalase peroxidase. The most common mutation in this gene is the substitution (Ser315Thr), and its highest prevalence has been observed in regions with high TB incidence, often where Beijing strains predominate [9,10]. However, the percentages of mutations associated with these genes vary according to geographical areas, with consequent implications for the implementation of molecular methods for the detection of MDR-TB and for TB diagnosis in general.

/ Clinical Case / Methods

Male, 25 years, born in Nepal, living in Portugal since November 2019, unemployed. In May 2021, he was referred to the emergency department of Centro Hospitalar do Oeste with a prolonged fever for 20 days, cough and hemoptysis. A CT scan showed condensation in the upper left lobe with cavitation and a positive sputum smear for acid-fast bacilli (AFB). From the smear-positive result, it was possible to make the presumptive diagnosis of tuberculosis and start antibiotic therapy with INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). The pacient was transferred to CHULC where a sputum sample was cultured for mycobateria after decontamination procedure, digestion and concentration of sample by N-Acetyl L-cysteine-NaOH method, Mycoprep BBL® MycoPrep™[11,12].

The molecular detection of multidrug resistance (MDMR) was performed with the GenoType MTBDRplus VER 2.0 (Hain Lifescience GmbH, Germany), which is a molecular genetics test for identification of MTBC and its resistance to RIF and/or INH from the study of the most significant mutations in the r*poB*, *katG* and *inhA* genes [13]. The mutations studied in this test are located between strands 505 and 533 of the *rpoB* gene, in codon 315 of the *katG* gene and in positions -15, -16 and -8 of the aa of the *inhA* gene, from decontaminated clinical samples based on DNA-STRIP technology [13]. A mutation conferring a probable resistance to INH was found in the katG gene but no mutation was detected on the rpoB gene.

The sample was also inoculated in two culture media, a solid Lowenstein-Jensen medium (BBL Lowenstein-Jensen Medium) [14] and a liquid medium, Middlebrook 7H9 in a BBL™ MGIT™ tube (Mycobacteria Growth Indicator Tube) which was incubated in the automated Bactec™ MGIT™ 960 system (Becton Dickinson, MD) [15].

After growth in liquid medium, susceptibility testing was performed in the BACTEC™ MGIT™ 960 system (Becton Dickinson Microbiology Systems®) for the following antibiotics: streptomycin (STR) (1.0mg/L), isoniazid (0.10mg/L), rifampicin (1.0mg/L), ethambutol (5.0 mg/L) and pyrazinamide (100.0mg/L) [16,17]. The strain showed resistance to all antibiotics tested in their respective concentrations, all first-line drugs in the treatment of TB.

Given the discordant results between susceptibility testing and MDMR, a new culture-based GenoType MTBDRplus VER 2.0 (Hain Lifescience GmbH, Germany) test was performed, confirming the initial result of INH resistance. Since we were in the presence of a MDR-TB strain, the culture was sent to the National Institute of Health Dr. Ricardo Jorge (INSA), National Micobacterium Reference Laboratory to perform the second-line susceptibility testisng and a genomic sequencing.

/ Results / Discussion

The latest TB surveillance and monitoring report in Portugal (2020) of the Direção Geral de Saúde (DGS) states that therapeutic success in TB treatment is fundamental to avoid relapses and the development of resistant forms, associated with greater morbidity and mortality. As such, treatment must be standardized, effective and include drugs with bactericidal action and the lowest possible toxicity. Therefore, INH and RIF are the most important drugs in treatment [5].

The GenoType MTBDRplus VER 2.0 Molecular Detection test (Hain Lifescience GmbH, Germany) carried out allowed the isolated strain to be identified as an MTBC and didn't reveal mutations in the RDRR of the *rpoB* gene, nor the *inhA* promoter region, a single mutation was found in the *KatG* coding region. Thus, the result of the MDMR test indicates that the strain under study, in the areas of bacterial DNA tested, is probably sensitive to RIF and resistant to INH [13].

In parallel, phenotypic susceptibility testing was performed on BACTEC™ MGIT™ 960 (Becton Dickinson Microbiology Systems®) showed the strain to be resistant to all drugs tested [16,17].

The MTBC strain was sent to the National Respiratory Infections Reference Laboratory - Micobacterium (INSA, Porto) to perform the second-line antibiotic susceptibility testing. We also sent the MTBC strain to National Reference Laboratory for Mycobacteria (INSA Lisboa), for genomic sequencing to obtain the identification (lineage prediction) and detection of possible mutations [18].

The determination of susceptibility to second-line antibiotics for MTC revealed that the strain was resistant to levofloxacin (1,0mg/L), moxifloxacin (0.25mg/L) and sensitive to moxifloxacin (1.0mg/L), linezolid (1.0mg/L), clofazimine (1.0mg/L), amikacin (1.0mg/L), ethionamide (5.0mg/L) and cycloserine (40mg/L). A second-line MDMR test was also performed, which detected mutation in the *gyrA* gene, but not in the *gyrB* gene,conferring resistance to fluoroquinolones. No mutation was detected for aminoglycoside resistence in the *rrs* gene nor in the *eis* gene. Accordingly, the pacient was treated with second-line antibiotics: linezolide 600mg; cycloserine 500mg; bedaquiline 200mg; clofazimine 100mg; moxiflxacin 400mg.

The sequencing result was obtained using the TBProfiler database, which allows analysis of complete *M. tuberculosis* genome sequencing data [19] and demonstrated that it was a strain of MTBC from the 2.2.1.2 East-Asian (Beijing) strain of the Spoligotype Rd Family: Beijing RD142; RD105; RD207; RD181; RD142. It's genotypic profile of drug resistance mutations is consistent with an MDR strain where the following mutations were found: rifampicin (rpoB p Phe424Leu and rpoB p Leu430Pro); isoniazid (katG p Ser315Thr); ethambutol (embB p Met306Val); pyrazinamide (pncA p Asp49Ala); streptomycin (rpsL p Lys43Arg); fluoroquinolones (gyrA p Asp94Gly) and ethionamide (ethA c 1047del),the latter being a silent mutation with no expressed resistance.

Rapid and reliable detection of RIF and INH resistance is critical for initial empiric treatment of resistant and multidrug-resistant tuberculosis. However not all resistant strains can be detected and not all mutations translate to a resistant phenotype so a susceptibility test from cultured sample is still a fundamental step in the study of M. tuberculosis.

Genetic sequencing of MTBC detected mutations that may confer resistance to RIF (rpoB p Phe424Leu and rpoB p Leu430Pro), in line with the phenotypic susceptibility test resistance profile. It is relevant to point out that no mutations were found between codon 505 and 533 of the *rpoB* gene analysed on the original genotyping test used [8,20].

This MTC strain was identified as belonging to Beijing family and had two RIF resistance mutations observed in the *rpoB* gene, in codons 424 and 430, i.e. in locations outside the mutational "hotspots" used by the molecular detection test performeds.

/ Conclusion

Phenotypic susceptibility testing, despite of being a slow test requiring specialised infrastructure and highly qualified personnel, continues to be the standard reference for most anti-TB drugs. In this case, it was also decisive for the correct diagnosis of the patient, because it allowed the laboratory to classify the strain as a MDR-TB and institute appropriate therapy.

Based on MDMR, whose results are faster than phenotypic susceptibility testing, it was possible to identify the strain as MTBC and to identify a mutation in the *katG* gene, which indicated a probable resistance to INH. As regards the *rpoB* gene, no mutations were evident in the regions of the genes studied, which indicated that the strain was probably susceptible to RIF.

Genomic sequencing of the isolated MTBC strain was important to corroborate the first-line susceptibility test result, the second-line susceptibility test (levofloxacin resistance at the concentration tested), the second-line MDMR, and it allowed the reclassification of the strain as pre-extensively drug resistant (pre-XDR) i.e. a MDR strain that also is resistant to any FQ [6].The complementarity of the phenotypic and genotypic approach is important to obtain a diagnosis of high sensitivity and specificity in the detection of drug resistance, an accurate MDR/XDR classification of MBTC and in obtaining relevant data for resistance surveillance.

Culture based Phenotypic susceptibility testing is essential to confirm the susceptibility of new cases of TB, as it was demonstrated in this clinical case, as without it, resistance to rifampicin would not have been detected. This type of mutations, are very rare and it was the first time that one was detected in our laboratory as mutations outsider the hotspot they do not normally translate into resistance and they are not detected in routine testing.

The increase in resistance to first-line drugs, especially resistance to INH and RIF, is related to immigration from high prevalence areas, delay in diagnosis and therapeutic failure. With regard to immigration, although the majority of TB cases continue to occur in the native population, according to the most recent data from the DGS, the proportion of TB cases in immigrants continues to increase. This clinical case is in line with the DGS data [5] and the publications of recent years that refer that the Beijing family strains seem to have a greater relationship with anti-TB resistance, a significant relationship with INH and RIF resistance and an increasing worldwide distribution [8].

Acknowledgements

We would like to thank Dr. Hermínia Choon (Head of the Mycobacteria Laboratory at CHULC, Lisbon, Portugal) and Dr. Helena Peres (Laboratory of Microbiology and Mycobacteria at CHULC, Lisbon, Portugal) for reviewing the article.

We also thank Dr. Rita Macedo (Head of the National Reference Laboratory for Mycobacteria. Department of Infectious Diseases, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisboa, Portugal) for the sequencing, and Dr. Anabela Silva (Responsible for the Respiratory Infections Reference Laboratory - Mycobacteria, Instituto Nacional de Saúde Doutor Ricardo Jorge, Porto, Portugal) for the Determination of Susceptibility to antibiotics for M. tuberculosis: 2nd Line Antibiogram of the strain under study, whose results were primordial for the completion of the article.

/ References

1. WHO. Health Topics. Tuberculosis. Overview. Available at: https://www.who.int/health-topics/tuberculosis#tab=tab_1

2. WHO. Health Topics. Tuberculosis. Symptoms. Available at: https://www.who.int/health-topics/tuberculosis#tab=tab_2

3. WHO. Global Tuberculosis Report. 2022. Available at: https://www.who.int/teams/global-tuberculosis-programme/tb-reports

4. WHO Europe, ECDC. Tuberculosis Surveillance and Monitoring in Europe. 2022.

Available at: https://www.ecdc.europa.eu/sites/default/files/documents/Tuberculosis-surveillance-monitoring-europe-2022_0.pdf

5. Direção-Geral da Saúde, Portugal. Relatório de Vigilância e Monitorização da Tuberculose em Portugal. 2021. Available at: https://www.sppneumologia.pt/uploads/subcanais2_conteudos_ficheiros/relat%C3%A3%C2%B3rio-tuberculose_dgs2021.pdf

6. WHO. WHO Operational Handbook on Tuberculosis: Module 3: Diagnosis - Rapid diagnostics for tuberculosis detection. 2021 update. Available at: https://apps.who.int/iris/rest/bitstreams/1354706/retrieve

7. Gupta A, Sinha P, Nema V, Gupta P, Chakraborty P, Kulkarni S, et al. Detection of Beijing strains of MDR M. tuberculosis and their association with drug resistance mutations in kata, rpoB and embB genes. BMC Infectious Diseases. 2020; 20:752.

DOI: https://doi.org/10.1186/s12879-020-05479-5

8. Keikha M. There is significant relationship between Beijing genotype family strains and resistance to the first-line anti-tuberclosis drugs in the Iranian population. J Clin Tuberc Other Mycobact Dis. 2020; 21: 100161. DOI: https://doi.org/10.1016/j.jctube.2020.100161

9. Zenteno-Cuevas R, Cuevas-Córdoba B, Parissi-Crivelli A. rpoB, katG and inhA mutations in multi-drug resistant strains of Mycobacterium tuberculosis clinical isolates from southeast Mexico. 2019; 37(5): 307-13. DOI: https://doi.org/10.1016/j.eimc.2018.09.002

10. Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey B, Maharjan B, et al. Molecular Characterization of Multidrug-Resistant Mycobacterium tuberculosis Isolated in Nepal. Antimicrod Agents Chemother. 2012; 56 (6): 2831-6.DOI: https://doi.org/10.1128/AAC.06418-11

11. Chapter 4 - Diagnosis of Tuberculosis Disease. Available at: https://www.cdc.gov/tb/education/corecurr/pdf/chapter4.pdf

12. Procedures - BBL ® MycoPrep™: Specimen Digestion/Decontamination Kit. Available at: https://legacy.bd.com/ds/technicalCenter/clsi/clsi-MYCOPREP.pdf

13. Genotype MTBDRplus VER 2.0 instruction for use. Hain Lifescience GmbH. 2005. Available at:

https://www.hain-lifescience.de/downloadcenter/MTBDRplusV2_0615_304A-06-02%20(5).pdf

14. BBL ® Lowenstein-Jensen Medium. Becton, Dickinson and Company. Available at: https://www.bd.com/resource.aspx?IDX=22837

15. BACTEC™ MGIT™ 960 System. Becton, Dickinson and Company. Available at: https://legacy.bd.com/ds/technicalCenter/clsi/clsi-mgt960-7ml.pdf

16. BBL ® MGIT AST SIRE System. Becton, Dickinson and Company. Available at: https://www.bd.com/resource.aspx?IDX=21366

17. BACTEC™ MGIT™ 960 PZA Kit. Becton, Dickinson and Company. Available at: https://www.bd.com/resource.aspx?IDX=18343

[18] Direção-Geral da Saúde, Portugal. Manual de Tuberculose e Micobactérias não tuberculosas. Recomendações. 2020. Available at: https://www.sip-spp.pt/media/rsrc5013/manual-tuberculose-e-micobacte-rias-na-o-tuberculosas.pdf

[19] TB Profiler; https://tbdr.lshtm.ac.uk

[20] Shea J, Halse T, Kohlerschmidt D, Lapierre P, Modestil H, Kearns C, et al. Low-Level Rifampin Resistance and rpoB Mutations in Mycobacterium tuberculosis: an Analysis of Whole-Genome Sequencing and Drug Susceptibility Test Data in New York. J Clin Microbiol. 2021; 59 (4): e01885-20. DOI: https://doi.org/10.1128/JCM.01885-20

[21] Schön T, Miotto P, Köser C, Viveiros M, Böttger E, Cambau E. Mycobacterium tuberculosis drug-resistance testing: challenges, recent developments and perspectives. Clin Microbiol Infec. 2017; 23 (3): 154-60. DOI: https://doi.org/10.1016/j.cmi.20