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Mycobacterium tuberculosis complex and detection of rifampicin and isoniazid resistance, with phenotypic study and massive genome sequencing

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Abstract

Objective: The objective of this study is to compare the results obtained from the detection of MTBC (*Mycobacterium Tuberculosis* Complex) and drug resistances between two commercial molecular methods, phenotypic Drug Susceptibility Testing (DST) and whole-genome sequencing.

Materials and methods: Clinical samples positive for MTBC were selected. Identification and study of mutations associated with rifampicin and isoniazid resistance were carried out using 2 commercial molecular techniques and phenotypic DST, using critical concentration in liquid medium. In parallel, the analysis of *Mycobacterium tuberculosis* complex genome was performed by sequencing complete genomes.

Results: The results obtained by the commercial molecular techniques and phenotypic DST included 4 TB-MDR samples. 31 positive MTBC samples that did not present mutations associated with rifampicin and/or isoniazid resistance in the molecular tests. The susceptibility/resistance profiles obtained by whole-genome sequencing were consistent with molecular and phenotypic results. The four sequenced MDR samples matched those obtained in the laboratory.

Conclusion: Our data showed a high correlation between commercial molecular techniques and massive genome sequencing for the identification and detection of TB-MDR strains, allowing for early and rapid diagnosis. Nevertheless, the feedback of data between the information obtained through genomic sequencing and clinical hospital microbiology is important.

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC). It mainly affects the lungs, but can also affect any other organ (extrapulmonary TB). It is transmitted from person to person through the air by coughing, sneezing or spitting by infectious patients [1]. According to the latest WHO report (2022) [2], approximately 10.6 million people become ill with TB each year. Despite being a preventable and curable disease, 1.6 million people die from tuberculosis each year, making it the second most deadly infectious disease after COVID-19. It is estimated that one quarter of the world's population is infected with MTBC. Only 5-10% will develop active TB, with the highest risk being in the first and second year after infection. The rest will have Latent Tuberculosis Infection (LTBI), where the immune system is able to control bacterial replication, so the individual will remain free from tissue damage and symptoms, and will not transmit the disease [3]. In terms of TB diagnosis, the WHO (2022) [2] emphasizes the fundamental aspects of bacilloscopy, rapid molecular diagnostic techniques and culture. It also points out that drug-resistant TB can be addressed by complete genome sequencing techniques.

The emergence of antimicrobial-resistant tuberculosis increases the morbidity and mortality of the infection worldwide. The new WHO definitions leave TB-MDR/RR unchanged, with TB-RR being resistant to at least Rifampicin (R) and MDR being resistant to at least isoniazid (H) and rifampicin. The concept of pre-XDR TB appears, which is TB-RR/MDR plus added resistance to any fluoroquinolone. Lastly, there is XDR tuberculosis: TB-RR/MDR, with resistance to any fluoroquinolone and at least one of the other drugs included in WHO Group A, meaning, at this time, resistance to linezolid or bedaquiline or both. It has been seen that TB-MDR/XDR is mainly the result of interpersonal transmission, rather than acquisition related to treatment [4]. This is where the importance of early diagnosis of MTBC lies, as well as the study of genotypic mutations associated with antimicrobial resistance. In this regard, the use of commercial molecular techniques stands out as diagnostic tools to assist in quick and efficient clinical intervention. On the other hand, Whole-Genome Sequencing (WGS) can also be considered as a promising alternative to phenotypic and molecular sensitivity testing methods, potentially aiding decision-making in clinical practice.

The objective of this study is to analyze the results obtained using two commercial molecular techniques in the detection of MTBC and mutations associated with rifampicin and isoniazid resistance, comparing them with those obtained by phenotypic DST and whole-genome sequencing.

Materials and Methods

This is an observational, retrospective study, in which a search was carried out in the laboratory's computer system, Gestlab®, selecting 35 MTBC isolates from clinical samples, belonging to 21 patients, that had been processed for mycobacterial culture according to our laboratory's standard protocol. Detection of the TB DNA and mutations associated with rifampicin resistance (rpoβ gene) were carried out using the commercial molecular technique Xpert ULTRA and XDR on decontaminated and frozen (-80°C) samples. The first assay simultaneously detects the MTBC and resistance to RIF by amplifying a specific region of the rpoB gene. Meanwhile, the Xpert XDR detects the TB genome and identifies resistance associated with isoniazid

(*inhA promoter, katG, fabG1, oxyR-ahpC intergenic region*), ethionamide (*inhA promoter*), fluoroquinolones (*gyrA, gyrB*), and aminoglycosides: amikacin, kanamycin, and capreomycin (*rrs, eis promoter*).

Moreover, using MCA (LiquidArray technology based on asymmetric PCR amplification and detection of the amplified product by Lights-ON and Lights-OFF hybridization probes), identification of the MTBC and detection of genes associated with resistance to rifampicin (*rpoB* gene) and isoniazid (*katG* and *InhA* genes) were carried out following the manufacturer's instructions.

Phenotypic Susceptibility Testing (DST) was conducted using the BACTEC™ MGIT™ 960 fluorometric system (Becton-Dickinson Diagnostics, Sparks, MD, USA) which uses the Middlebrook 7H9 liquid medium supplemented with PANTA. This semi-automated system is influenced by variations in oxygen levels, with decreasing levels exciting the fluorimetric substance. The system reader takes serial readings every hour and describes a logarithmic growth curve. The antibiogram is performed using a critical concentration method, interpreted by the system software as sensitive or resistant based on the sum of mycobacterial growth, following manufacturer guidelines. The drugs studied were: isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide.

Simultaneously, the 35 samples were sent to the Foundation for the Promotion of Health and Biomedical Research (FISABIO) and Biomedicine Institute of Valencia to perform whole-genome sequencing. DNA extraction, library preparation, sequencing in the Illumina MiSeq platform and bioinformatics analysis were carried out as described by *Cancino-Muñoz* et al. [5]. By using the Single Nucleotide Polymorphisms (SNPs) data obtained for each sample, resistance prediction was conducted following the methodology described by *Moreno et al*. [6]. For this, the resistance-associated mutations included in the World Health Organization (WHO)-endorsed catalogue [7,8] were used. To predict resistances, we equally considered variants described as associated with resistance and associated interim. This catalogue includes mutations to first-line drugs, with high sensitivity and specificity; and few mutations to second-line drugs administered during treatment. Particularly, by WGS we evaluated mutations associated with resistance to INH, RIF, EMB, PZA and FQ which are the best predicted in our setting [9]. In addition, by querying phylogenetic SNPs [10], we could determine the species and lineage of the strains.

Results

MTBC study by Xpert ULTRA and XDR

The results obtained by both commercial molecular techniques included 4 MDR-TB samples, belonging to 3 different patients. The remaining 31 samples did not show genotypic mutations associated with rifampicin or isoniazid, nor to other second-line antituberculosis drugs, in accordance with the phenotypic DST, except in two samples from a patient, where genotypic resistance to fluoroquinolones (*gyrA/gyrB* gene) was detected. In one patient (P21), Xpert XDR test showed indeterminate results for amikacin, kanamycin, and capreomycin.

Study of MTBC using MCA

Using this technique, MTBC was identified in all samples except for one. All 4 MDR-TB samples were detected coinciding with the rest of the methodologies.

No genotypic mutations to first- or second-line drugs were identified in the remaining 30 samples.

Phenotypic DST using critical concentrations

The sensitivity of all samples was studied using Middlebrook 7H9 liquid medium supplemented with PANTA for the five first-line drugs. Consistent with molecular techniques, 4 MDR samples were observed, two of them (belonging to the same patient) presented added resistance to ethambutol, pyrazinamide, and streptomycin. And the other two, resistance to streptomycin in addition. Finally, one strain showed resistance only to streptomycin. The remaining 30 samples were sensitive to all tested drugs.

Whole-genome sequencing

The median sequencing depth obtained was >40 (45-285). All samples were identified as M. tuberculosis, in agreement with lab results. Strains belonging to Lineage (L) 4.10, were the most frequent, representing 54% of all strains. The other lineages found in the study were L2.2.5, L2.25, L4.1.2, L4.2.1, L4.3.2, L4.3.3, L4.3.4, L4.4.1, L4.6.2, L6 and L3. Regarding genetic mutations associated with antimicrobial resistance, the results were as follows:

Four MDR samples, two ethionamide-resistant samples and two samples resistant to fluoroquinolones were identified

TBC-MDR

Sequencing of the 4 MDR strains matched the results obtained by commercial techniques and DST for isoniazid and rifampicin. The rifampicin resistance-associated mutation was Ser450Leu in rpoB, and for isoniazid were Ser315Thr in KatG. 3/4 MDR strains belonging to 2 patients (P1, P3) also presented resistance to streptomycin (*rpsL*: Lys43Arg), detected by both WGS and phenotypic DST; and ethambutol resistance (*embB*: Met306Val) detected by both methods in P3 (S3,S4) and only by WGS in P1. Streptomycin and pyrazinamide resistances were only detected by phenotypic DST in the case of P2 and P3, respectively (Table 1).

Table 1: We show results of phenotypic DST and drug resistance prediction by WGS. There were 4 MDR-TB samples belonging to 3 patients (grey), and one patient with 2 samples that presented monoresistance to ethionamide. Data is presented as phenotypic DST/WGS result and associated mutation in brackets.

	Isoniazid	Rifampicin	Ethambutol	Streptomycin	Pyrazinamide	Ethionamide
P1(S1)	Resistant/	Resistant/	*Susceptible/	Resistant/	Susceptible/ Susceptible	NC/
	Resistant	Resistant	Resistant	Resistant		Susceptible
	(*katG*: Ser315Thr)	(*rpoB*: Ser450Leu)	(*embB*: Met306Val)	(*rpsL*: Lys43Arg)		
P2(S2)	Resistant/	Resistant/	Susceptible/	*Resistant/	Susceptible/	NC/
	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
	(*katG*: Ser315Thr)	(*rpoB*: Ser450Leu)				

P3(S3,S4)	Resistant/	Resistant/	Resistant/	Resistant/	*Resistant/	NC/
	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible
	(*katG*: Ser315Thr)	(*rpoB*: Ser450Leu)	(*embB*: Met306Val)	(rpsL: Lys43Arg)		
P4(S5,S6)	[1] Susceptible/	Susceptible/	Susceptible/	*Resistant/ Susceptible	Susceptible/	NC/
	Uncertain	Susceptible	Susceptible		Susceptible	Resistant
	(*inhA*: Ser94Ala)					(*inhA*: Ser94Ala)

S: Sample; P: Patient; *discrepancy between phenotypic DST and WGS profile; 1 Mutation of uncertain significance according to WHO catalogue, but with evidence of conferring resistance to isoniazid; NC: phenotypic DST not conducted.

DR Ethionamide

A mutation in the *inhA* gene (Ser94Ala) associated with ethionamide resistance was detected in two samples from the same patient (P4: S5 and S6, Table 1). Its significance for isoniazid resistance is uncertain according to the WHO catalogue. However, the effect of this mutation was studied by allelic exchange experiments and its association with INH resistance was demonstrated [11]. No resistance was identified in either the MCA or phenotypic DST for this sample. It should be noted that this is a mutation of complex interpretation, as it may be present in strains that do not exhibit a resistant phenotype in standardized phenotypic tests (see WHO evidence).

On the other hand, previous analysis indicate that isolates containing this mutation would have a Minimum Inhibitory Concentration for isoniazid (MIC) of 0.063-0.125 ug/ml [9] compared to 0.031 ug/ml of the H37Rv reference strain (MICs obtained by the broth microdilution method using Middlebrook 7H9 supplemented with OADC [5]. These results would indicate that this mutation may provide low or intermediate levels of isoniazid resistance. This would also explain why it is so difficult to definitively associate it with resistance, and why phenotypic DST shows contradictory results.

In patient P21, sequencing did not identify mutations in genes associated with amikacin, kanamycin, or capreomycin resistance.

Sequencing of the remaining 30 did not detect mutations associated with isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide genes, in agreement with phenotypic and molecular results.

DR Fluoroquinolones

Patient P15 presented in its two samples resistance to fluoroquinolones by WGS (*gyrA*: Asp94Gly), according to the Xpert XDR results.

Discussion

In this study, the diagnostic accuracy of 2 commercial molecular methods was analyzed, comparing them with culture (the “gold standard”) and phenotypic DST for the identification of MTBC and detection of associated antimicrobial resistances, as well as whole-genome sequencing. Recently, the WHO reaffirmed the use of molecular techniques as part of the TB diagnostic process, in order to accelerate efforts to diagnose the disease and drug resistance, and to achieve the proposed goal of eliminating the global tuberculosis

epidemic by 2050.

In this study, it was observed, following in line with other studies [12], that the commercial molecular technique with the highest Sensitivity (S) for the identification of MTBC was Xpert ULTRA and XDR, with a S of 100%, compared to MCA, which showed a sensitivity of 97.14%. Regarding the detection of genotypic mutations, all techniques revealed 4 TB-MDR samples belonging to 3 patients, with mutations present in the *rpoB* gene (rifampicin) and in the *katG* gene (isoniazid), coinciding with the phenotypic DST.

Regarding WGS, it showed promising results by providing accurate information in all cases of TB-MDR, compared to current routine methods. It detected the 4 samples that showed resistance to isoniazid and rifampicin, consistent with DST.

Our results are supported by studies indicating the potential of WGS to identify various genetic polymorphisms, including Single Nucleotide Polymorphisms (SNPs) and small insertions and deletions (indels) potentially important for reliable prediction of drug susceptibility in clinically important timeframes and at a comparable cost range [13-15].

As for the performance characteristics of other first-line and reserved drugs, they varied considerably for streptomycin, ethambutol, and pyrazinamide. Of the 4 TB-MDR strains, three presented a mutation in the *embB* gene (Met306Val), which is associated with resistance to ethambutol according to WHO catalogue, and it was detected in two samples, belonging to one patient, by phenotypic DST and remained undetected for the other case. In addition, these two samples were resistant to pyrazinamide in phenotypic DST but no resistance mutation was identified by WGS. Accordingly, WGS sensitivity for pyrazinamide is low worldwide (less than 80%, [8]) and particularly in our setting (57.1% [9]). The last MDR-TB case presented resistance to streptomycin in DST, but no associated mutations for this drug were detected; it must be noticed that sensitivity of WGS for streptomycin is low because of the taxonomic filtering step implemented in the bioinformatics analysis [16]. Different studies show significant variations in sensitivity and specificity for streptomycin, ethambutol, and pyrazinamide, possibly due to the lack of data on the molecular mechanisms and genes involved in the resistance of these drugs [12]. The study by *TM Walker et al*. [8] shows that currently the overall sensitivity for the prediction of phenotypic resistance by WGS is over 90% for isoniazid and rifampicin, as well as values above 70% for most second-line antibiotics. On the other hand, another study analyzing the sensitivity of WGS for the prediction of resistance in our setting shows similar results, although more modest, due to the greater weight of rare mutations in the dataset, sensitivities were: 85.4% for isoniazid, 73.3% for rifampicin, 50% for ethambutol and 57.1% for pyrazinamide; all specificities were higher than 99.6% [9]. These results are quite good but not sufficient to draw robust conclusions about the role of WGS in clinical practice as a sole diagnostic tool, although it does have a promising future as an adjunctive tool.

Nucleic Acid Amplification Techniques (NAAT), such as MCA and Xpert, have had a great impact on the TB diagnostic process, because they are low-complexity, adequately biosecure, cost-effective methods that provide reliable diagnosis in less than two hours by identifying both the microorganism and the genotypic mutations associated with first-line TB treatment resistance. However, according to bibliographic sources,

the long-term impact on outcomes such as mortality remains unclear, with ambiguous data reported [17]. When comparing these techniques with traditional methodology for diagnosis, no reduction in TB morbidity and mortality is observed. Some of the reasons attempting to justify this situation are empirical antibiotic therapy, lack of adherence to treatment, and adverse treatment effects. Not all healthcare systems correctly carry out the care cascade, so as long as these weaknesses or deficiencies continue to exist, we will not see real reductions in the morbidity and mortality of this disease [18].

Current tests for identifying the microorganism and most antituberculosis drugs involve culturing the bacteria; however, everything points to direct sequencing of MTBC from non-cultured samples being the future of tuberculosis management.

Conclusion

Our data shows a high correlation between commercial molecular techniques and whole-genome sequencing, both for the identification of MTBC and for the accurate detection of rifampicin and isoniazid resistance. As the WGS completes the genotypic study, it is important to have a dynamic exchange between the information generated by genomic sequencing and clinical microbiology, so that it can have applicability at the care level, becoming a diagnostic aid tool aimed at the patient's treatment.

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