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Differentiation Of M. Tuberculosis Complex By PCR In Clinical Samples

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Abstract

Tuberculosis, caused by microorganisms of the so-called Mycobacterium tuberculosis complex (MTBC), continues to be a public health problem worldwide, as MTBC diverged into different lineages, gene expression of virulence y associated metabolites also did, this provides clear evidence that MTBC lineages likely reflect adaptation to different human populations.

The difficulties to manipulate mycobacteria, their slow growth and pathogenicity have made them a difficult model to genetically characterize. However, the development of tools for genetic manipulation has allowed advances in research, facilitating the understanding of genome organization, gene expression and phenotypic determination, which explain the mechanisms of pathogenicity, latency and drug resistance.

This paper reports the differentiation of the CMTB by Polymerase Chain Reaction (PCR) in samples from patients with clinical diagnosis of tuberculosis, from different genomic regions of the microorganisms of the complex in samples from patients with clinical diagnosis of tuberculosis. For the development of the present study, sputum samples were collected from patients with a microbiological diagnosis of tuberculosis, obtained from two hospitals in Arequipa.

The extraction and purification of DNA from clinical samples was carried out and then amplified by PCR. and identified PCR, for which a group of different DNA amplifiers were used. Initiators which amplifies the DNAn depending on the type of MTBC, allowing the results identified the Mycobacterium tuberculosis strains with 73%, followed by Mycobacterium of unidentified Mycobacterium with 20% and 2% of Mycobacterium of Mycobacterium canetti, strains that circulate in the region.

Descriptors: Mycobacterium tuberculosis complex, PCR.

Abstract

La tuberculosis, causada por microorganismos del denominado Complejo Mycobacterium tuberculosis (MTBC), sigue siendo un problema en salud pública a nivel mundial, a medida que el MTBC divergió en diferentes linajes, la expresión de genes de virulencia y metabolitos asociados también lo hizo, esto proporciona una evidencia clara de que los linajes de MTBC probablemente reflejan la adaptación a diferentes poblaciones humanas.

Las dificultades para manipular las micobacterias, su crecimiento lento y su patogenicidad han hecho de ellas un modelo difícil de caracterizar genéticamente, sin embrago el desarrollo de herramientas para la manipulación genética han permitido avances en la investigación, facilitando el entendimiento de la organización del genoma, la expresión de sus genes y la determinación fenotípica, que explican los mecanismos de patogenicidad, estado de latencia y resistencia a fármacos.

En este artículo reportamos la diferenciación del CMTB mediante Reacción en Cadena de la Polimerasa (PCR) de diferentes regiones genómicas de los microorganismos del complejo, en muestras de pacientes con diagnóstico clínico de tuberculosis. Para el desarrollo del presente estudio se recolectaron muestras de esputo de pacientes con diagnóstico microbiológico de tuberculosis, obtenidas de dos hospitales de Arequipa.

Se realizo la extracción y purificación de ADN de las muestras clínicas, para luego ser amplificadas e identificadas por PCR, para lo que se utilizaron un grupo de diferentes iniciadores, los que amplifican dependiendo del tipo de MTBC, permitiendo identificar las cepas Mycobacterium tuberculosis con un 73%, seguida de Mycobacterium no identificado con el 20% y el 2% de Mycobacterium canetti, cepas que circulan en nuestra región.

Descriptores: Complejo Mycobacterium tuberculosis, PCR.

1. Introduction

Tuberculosis (TB) is responsible for more than 1.8 million deaths per year (Ates, L. S. et al 2020), and affects 10.4 million new people, making it one of the deadliest infectious diseases in the world (Fonseca et al., 2020), and historically making it the largest cause of infectious death, having claimed more deaths than smallpox, malaria, plague, influenza and AIDS combined (Gonzalo-Asensio et al., 2018). Added to these figures is a pool of two billion people with latent infection (Gonzalo-Asensio et al., 2020). (Comas & Gagneux, 2009, Fonseca et al., 2020) because, Mycobacterium tuberculosis (Mtb), the causative agent of the disease has the ability to survive within macrophages, in a granuloma for weeks, months and even decades in an asymptomatic or latent state (Galagan, 2014).

Peru has 14% of TB cases in the Americas, with metropolitan Lima and Callao being the places with the highest percentage of infections, accounting for 64% of cases in the entire country. On the other hand, in the department of Arequipa, in 2015, 544 new cases of medium-grade TB were registered (PAHO/WHO. 2020). It is currently known that tuberculosis not only affects developing countries, but also considerably affects developed countries as is the case of Belgium (Vluggen et al., 2017). The high incidence of TB globally makes addressing TB a matter of urgency, as reflected in the current strategy put forward by WHO to end TB, aimed at a 90% reduction in TB incidence, reaching to less than 100 cases per million people by 2035 (Gröschel et al., 2018).

It is important to mention that, in Peru, within the few studies that have been carried out in this regard, it is mentioned that the lineages circulating in the national territory, mainly in the capital, correspond to Haarlem, T, Beijing, Mediterranean-Latin American (LAM), U, X and S; a considerable proportion of orphan lineages (Orphans) has also been described. Among the lineages mentioned, the most prevalent were Haarlem and LAM (Barletta et al., 2013; Cáceres et al., 2014).

In humans, the TB is caused mainly by members of the Mycobacterium tuberculosis complex (MTBC) (Coscolla & Gagneux Mycobacterium tuberculosis complex (MTBC) (Coscolla & Gagneux, 2014). Genotypic characterization of MTBC is important to better understand endemic and epidemic strains (Sanoussi et al., 2017).

MTBC comprises eight defined phylogenetic lineages. M. tuberculosis includes L1, L2, L3, L4 and L7 lineages. These human-adapted lineages are responsible for most of the world's human tuberculosis cases, whereas the M. africanum lineages (L5, L6) are restricted mainly to West African humans. The L8 comprises animal-adapted strains (Gonzalo-Asensio et al., 2018, Sanoussi et al., 2017), M. bovis, M. caprae, M. pinnipedii, M. microti, M. orygis, M. suricattae, M. mungi (Zimpel et al., 2017), therefore, these lineages are considered to have a differential impact on pathogenesis, disease outcome and vaccine efficacy.

It is known that modern lineages, which include strains with TbD1 deletion, are evolutionarily younger than strains without this deletion without this deletion. Consequently, strains with TbD1 deleted have been termed evolutionary modern, while M. africanum (Maf) and several other MTBC lineages were considered ancient. On the other hand, Euro-American Beijing and Haarlem strains, present more virulent phenotypes compared to ancient ones, such as East African Indian (Phelan, et al., 2016, Faksri et al., 2011). Similarly, other studies reflect the responsiveness of patients to different MTBC lineages, influencing the synthesis of cytokines important in post-treatment infection processes, as is the case of patients infected with Mtb y Maf in which they showed a more favorable recovery for those infected with Mtb than with Maf after antituberculosis treatment (Tientcheu et al., 2016). On the other hand, strains that infect animals have been found to cause zoonotic diseases, as is the case of M. bovis (Mbv), which causes TB bovine, a primitive infectious disease, which represents the most important zoonotic disease, usually affecting the lungs (pulmonary TB), but can also affect other sites (extrapulmonary TB) (Zahran et al., 2014).

In addition to this, these lineages were found to exhibit phylogeographic structure, meaning that specific lineages are associated with particular regions, and infect to a greater extent individuals originating from these regions (Yeboah-Manu, D. et al., 2016). Added to this, phylogenetically distinct Mtb lineages differ in their phenotypes and pathogenicity, thus, understanding phylogeographic population structures is essential for the design, interpretation and generalization of clinical trials (Gehre et al., 2016).

In general, ancient MTB lineages are less virulent than modern ones, lending some weight to the hypothesis that the emergence of newly evolved lineages leads to more virulent disease, a phenomenon termed interrupted coevolution. In the case of TB disease, virulence is highly correlated with the presence and severity of active TB symptoms, as they are necessary for Mycobacteria to spread (McHenry et al., 2020). All of the above, is reinforced in the context that, failure to identify MTBC will lead to delays in initiating appropriate treatment with dire consequences for the patient and their communities increasing the continued risk of TB transmission (Ofori-Anyinam et al., 2016).

Thus, the need for a reliable, early and sensitive diagnosis of MTBC for disease control is increasing, however, the sensitivity of sputum microscopy is low, and the culture method used for the diagnosis of tuberculosis, although sensitive, becomes slow, even taking several weeks to confirm a diagnosis, due to the slow growth of the microorganism (Hwang et al., 2015, Sun et al., 2009). In addition, several commercial serological tests have shown an unreliable diagnosis of TB, reporting inconsistent, imprecise and highly variable values of sensitivity and specificity, so, in consideration of this situation, the WHO in 2011 issued a policy recommending not to use these tests for the diagnosis of pulmonary and extrapulmonary TB (Tucci et al., 2014).

The modest decrease in the global incidence and mortality of TB in recent years should not go unmentioned, which must be accelerated if the objectives of ending TB by 2035 are to be met, and this can only be achieved if the implementation of strategies for prevention, detection, diagnosis and treatment are optimized. Currently, in most developing countries,

microbiological diagnosis is still based on microscopy, Ziehl Neelsen staining and culture, together with antibiogram to determine antibiotic sensitivity. The Polymerase Chain Reaction (PCR), method for DNA amplification is considered a rapid and accurate technique for the identification of mycobacterial species, and apparently proves to be more sensitive than that obtained by the culture technique (Zahran et al., 2014), playing an important role, both for the diagnosis of tuberculosis, and to know and study the lineages present in MTBC, especially in the field of places, where the implementation and use of this technique is slow, and the presence of tuberculosis is high. The WHO recommends the Xpert MTB/RIF test (Cepheid, Sunnyvale, California, USA) for the initial diagnosis of TB and MDR-TB. This test is performed using a closed, fully automated system (which prevents contamination), requires only minimal expertise, can be used in peripheral settings, and allows the result to be obtained within hours (WHO, 2015).

Insertion Sequences (IS) are useful genetic tools that have been used as taxonomic markers for diagnostic purposes (Thierry et al., 1990), of which, different sequences have been evaluated for the study of the M. tuberculosis complex, within them is included the IS6110 sequence, which belongs to the IS3 category family and is most frequently used for MTB detection because it is highly conserved (Kabir et al., 2018; McEvoy et al., 2007), in addition to occurring in different copy numbers and its positions in the genome are highly variable among different isolates (Arora et al., 2020; Turcios et al 2009). It is then that the high degree of polymorphism of IS6110, both numerically and positionally among different Mtb strains, has made it a useful marker for strain genotyping (McEvoy, C. R. R. E. et al 2007), thanks to these characteristics, these sequences have been exploited for molecular epidemiological purposes (Thorne et al., 2011).

However, some Mtb strains have been reported to lack this element (Farzam et al., 2015). Thus, the use of additional markers for complex differentiation helps to better identify MTBC members (Warren et al., 2006). On the other hand, new massive sequencing techniques (NGS) allow the establishment of regions that typify them. Based on the above considerations, the objective of this study was to differentiate the MTBC members by PCR technique in samples from two hospitals in the Arequipa region, with positive diagnosis for tuberculosis (culture or smear microscopy), in order to establish the predominant lineages.

Methodology

2.1. Samples

Sputum samples were requested from the Regional Honorio Delgado Espinoza Hospital and Hospital III Yanahuara ESSALUD. For tuberculosis diagnosis, each hospital used its own standardized methods of routine and confirmation, staining and culture. They were transferred in an airtight sealed medium maintaining the cold chain, with codes of the hospitals of origin, to the Human Morphology Research Laboratory of the Faculty of Medicine of the National University of San Agustin de Arequipa - UNSA.

The study lasted 24 months, and a total of 30 sputum samples were collected, 18 from the Honorio Delgado Espinosa Regional Hospital and 12 from the Yanahuara Hospital.

2.2. Decontamination and preservation of samples

All sputum samples were inactivated by the N -acetyl-l -cysteına-sodium hydroxide (NaLC) method, re-suspended sediment was used for all molecular assays.

The samples were stored in airtight containers and the cold chain was maintained until they were processed in the laboratory of the Faculty of Medicine of UNSA, maintaining the cold chain of each hospital. processing in the laboratory of the Faculty of Medicine of the UNSA, maintaining the cold chain of each of the hospitals.

2.3. Mycobacterial DNA extraction and purification

For the extraction and purification of mycobacterial DNA, inactive sputum samples were used, the DNA of the samples was extracted and purified with the clinical sample kit MACHEREY-NAGEL Moura, André et al. (2016) was performed according to the manufacturer's instructions.

For testing the extraction of bacterial DNA from sputum samples, the following is first performed

an amplification of the 16S ribosomal gene with the MyTaq Mix Bio Line amplification kit (Meridian BIOCIENCE™), amplification kit on a BioRad t100 thermal cycler.

Once DNA extraction by the rRNA16S gene was confirmed, PCR with specific TB2 primers for amplification of the mycobacterial genetic element IS6110 was performed to confirm the presence of Mtb. The reaction was carried out in 15 ul of Mix taq Polymerase, 1ul of Forward and 1ul of Reverse at a final concentration of 1ul of Reverse at a final concentration of 1uM each, 20 pMoles of DNA from each sample and ultrapure water to a final volume of 30 ul. TB2 amplification conditions are given in Table 1 (Ogusku et al., 2004)

Table 1. **Primers for the amplification of the mycobacterial genetic element IS6110 and PCR parameters for the Identification of Mycobacterium tuberculosis.**

Primer	Size	ncia del Primer	Tamaño	Size		Cicles
TB2	5´TGG TCC GGT TCA TAC TCG GGC TGG 3'	5´-CCT GCG AGC GTA GGC GTC GG-3´	123 pb	94°C – 1 min 66°C – 1 min 72°C – 0.4 min	35	
16SrRNA	5´-ACG GTG GGT ACT AGG TGT GGG TTTC-3´	5´-TCT GCG ATT ACT AGC GAC TCC GAC TTCA-3´	543 pb	94°C – 1 min 60°C – 1 min 72°C – 0.4 min	35	

2.4. **Identification of Mycobacterium tuberculosis complex (MTBC) by identification of regions of difference.**

For the identification of MTBC, the DNA samples extracted were used, classified by the laboratory and amplified with the initiators for the differentiation of the MTBC members with M. tuberculosis, M. africanum, M canétii, M. microti, M. pinnipedii, M. caprae, M bovis, M. bovis BCG, (Warren et al., 2006).

The identification of the different members MTBC was performed by electrophoresis through electrophoresis in 3% agarose gels.

Table 2. Primers and sizes of the amplification products of the different members of MTBC

			M. canettii	*M. tuberculosis*	*M. africanum*	*M. microti*	*M. pinnipedii*	*M. caprae*	*M. bovis*	*M. bovisBCG*
A (RD1)	MtbC-1A	5´-AAGCGGTTGCCGCCGACCGACCGACC-3´	146 pb	146 pb	146 pb	146 pb	146 pb	146 pb	146 pb	196 pb
	MtbC-2A	5´-CTGGCTATATTCCTGGGCCCGG-3´								
	MtbC-3A	5´-GAGGCGATCTGGCGGTTTGGG-3´								
B (RD4)	MtbC-1B	5´-ATGTGCGAGCTGAGCGATG-3´	172 pb	172 pb	172 pb	172 pb	172 pb	172 pb	268 pb	268 pb
	MtbC-2B	5´-TGTCACTATGCTGACCCATGCG-3´								
	MtbC-3B	5´-AAAGGAGCACCATCGTCCAC-3´								
C (RD9)	MtbC-1C	5´-CAAGTTGCCGTTTCGAGCC-3´	235 pb	235 pb	108 pb	108 pb	108 pb	108 pb	108 pb	108 pb
	MtbC-2C	5´-CAATGTTTGTTGCGCTGC-3´								
	MtbC-3C	5´-GCTACCCTCGACCAAGTGTT-3´								
D (RD12)	MtbC-1D	5´-GGGAGCCCAGCATTTACCTC-3´	AUSENTE	369 pb	369 pb	369 pb	369 pb	306 pb	306 pb	306 pb
	MtbC-2D	5´-GTGTTGCGGGAATTACTCGG-3´								
	MtbC-3D	5´-AGCAGGAGCGGTTGGATATTC-3´								
E (RD1mk)	MtbC-1E	5´-CGGTTCGTCGCTGTTCAAAC-3´			195 pb	127 pb	195 pb			
	MtbC-2E	5´-CGCGTATCGGAGACGTATTTG-3´								
	MtbC-3E	5´-CAATCAGCCAAGACGAGGTTG-3´								
F (RD2sea)	MtbC-1F	5´-TCAGCGGTCTCATAGCATTGC-3´			AUSENTE	293 pb	168 pb			
	MtbC-2F	5´-CGGGTTGGGAATGTCAGAAAC-3´								
	MtbC-3F	5´-GCGGCAAGGTACGTCAGAAC-3´								

3. Results and Discussion of Results

3.1. Comparison of clinical tuberculosis detection methods and PCR

For this study, the results of compared the results of tuberculosis molecular markers that amplify different regions of the MTBC genome were compared for this study.

The biological samples obtained were evaluated with three diagnostic techniques (i) smear microscopy with Ziehl-Neelse staining; ii) by microbiological culture in Kudohn (ZN); ii) by microbiological culture on Kudoh Ogawa; iii) by PCR enzymatic amplification

Table 3 Results, according to the hospital of origin, of the smear microscopy, culture and PCR molecular technique methods.

CODIGO ASIGNADO	PROCEDENCIA	TIPO DE MUESTRA	PACIENTE NUEVO		TRATAMIENTO		PRUEBAS		PCR							ESPECIE
			SI	NO	SI	NO	BK	CULTIVO	TB2	16sRNA	P-A	P-B	P-C	P-D	P-E	P-F	
001	H.General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
002	H.General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
003	H.General	Esputo	X			X	++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
004	H.General	Esputo	X			X	++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
005	H.General	Esputo	X			X	+++	+++	-	+	146	172	235	369	-	-	*M.tuberculosis*
016	H.General	Esputo	X			X	+	-	+	+	146	172	235	369	-	-	*M.tuberculosis*
017	H.General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
020	H. General	Esputo	X			X	+	+	+	+	146	172	235	369	-	-	*M.tuberculosis*
021	H. General	Esputo	X			X	+	+	+	+	146	172	235	369	-	-	*M.tuberculosis*
022	H. General	Esputo	X			X	+++	+++	+	+	-	172	235	-	195	168	*M. NI*
023	H. General	Esputo	X			X	+	+++	+	+	146	172	235	369	195	293	*M. NI*
026	H. General	Esputo	X			X	+++	++	+	+	146	172	235	369	-	-	*M.tuberculosis*
030	H. General	Esputo	X			X	+++	++	+	+	146	172	235	369	-	-	*M.tuberculosis*
027	H. General	Esputo	X			X	++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
031	H. General	Esputo	X			X	++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
028	H. General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
029	H. General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
032	H. General	Esputo	X			X	+++	+++	+	+	146	172	235	369	-	-	*M.tuberculosis*
006	Essalud	Esputo		X	X		+	-	+	+	146	172	235	369	-	-	*M.tuberculosis*
007	Essalud	Esputo		X	X		++	-	+	+	146	172	235	369	-	-	*M.tuberculosis*
008	Essalud	Lavado bronquial		X	X		+	++	+	+	146	172	235	-	-	-	*M.canettii*
010	Essalud	Esputo		X	X		+	++	+	+	146	172	235	-	-	-	*M.canettii*
009	Essalud	Esputo	X			X	++	++	+	+	146	172	235	369	-	-	*M.tuberculosis*
011	Essalud	Esputo	X			X	++	++	+	+	-	-	-	-	195	-	*M.NI*
012	Essalud	Esputo		X	X		+	++	-	+	146	172	235	369	-	-	*M.tuberculosis*
013	Essalud	Esputo	X			X	+++	+	+	+	146	172	235	369	-	-	*M.tuberculosis*
014	Essalud	Esputo	X			X	+	+	-	+	-	172	235	369	195	168/293	*M. NI*
015	Essalud	Biopsia epididimo	X			X	+	+	+	+	-	172	235	369	195	168/293	*M. NI*
018	Essalud	Esputo		X	X		+	-	+	+	-	172	235	369	195	293	*M.NI*
019	Essalud	Esputo		X	X		+	-	+	+	146	172	235	369	-	-	*M.tuberculosis*

***M. NI Mycobacterium not identified**

As can be seen from Table 2, three samples were negative with TB2 primers, which amplify a 123 bp fragment of the IS6110 insertion sequence. One of the samples (012) was from a patient who had been initiated on treatment. The remaining two samples were from patients without treatment initiation (Table 1).

3.2. Determination of Mycobacterium tuberculosis complex.

It was evaluated with six molecular markers that helped to identify the MTBC, for which Table 1 was used as a reference, thus determining the species of the MTBC complex.

A representative image of the amplification method of the MTBC clinical samples and the assignment of molecular weights is presented in Figure 1. As can be seen in Table 2, all processed samples gave positive results with the primers designed for the amplifications of difference regions. According to the amplified products, the prevalence of Mycobacterium tuberculosis was determined to be 73%, followed by Mycobacterium from unidentified Mycobacterium with 20% and 2% of Mycobacterium of Mycobacterium canetti.

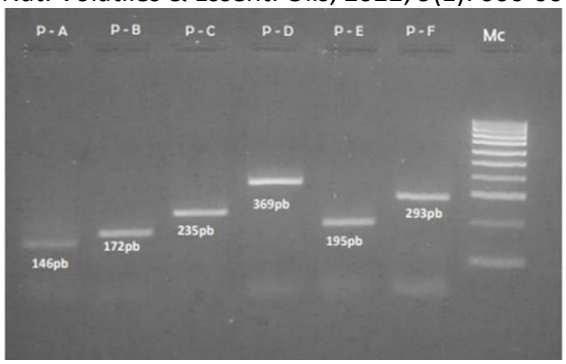

Figure 1. Image of Primer A- RD1 (146bp), Primer B- RD4 (172bp), Primer C- RD9 (235bp), Primer D - RD12 (369bp), Primer E- RD1mic (195bp), Primer F - RD2sed (293bp) and their respective 100bp marker.

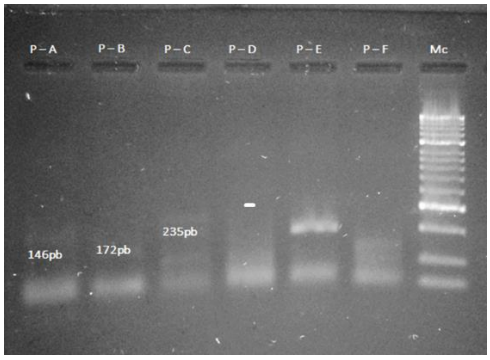

Figure 2. Image of Primer A- RD1 (146bp), Primer B- RD4 (172bp), Primer C- RD9 (235bp), Primer D - RD12 (369bp) Absent and their respective 100 bp marker.

4. Conclusions

The causative agent of TB tuberculosis, Mycobacterium tuberculosisis an obligate pathogen that evolved to persist exclusively in human populations. Mtb is a significant determinant of the association between Mtb and humans. On the other hand, the evolutionary success and permanence of the pathogenicity of Mtb would be determined by human demographic expansions, in addition to human and bacterial genetic polymorphisms.

Therefore, the differential distribution of MTBC lineages and sublineages could also reflect intrinsic characteristics of the strains. For example, some lineages could be considered "generalists", i.e., able to persist in different human populations, and others more "specialists", i.e., able to persist only in one or a few particular host populations (Kirzinger MW, Stavrinides J.).

The results show that the analysis with the primer (TB2) for the identification of the IS6110 sequence is very useful for the diagnosis of Mtb by PCR methodology in clinical samples. Of the samples analyzed, 80% were detected with the TB2 primer, these results indicate the importance of performing the diagnosis with more primers of the IS6110 sequence. Similar results were obtained in the studies of Ogusku et al., who obtained a positivity greater than 92.1%

There are reports in the literature of Mtb strains lacking this sequence. In the study, three samples did not amplify with the TB2 primer pair, however, these samples did amplify with primers designed to differentiate MTBC members, two of them were identified as Mtb while one (014) could not be identified. Based on this result, these Mtb strains circulating in Arequipa lack the IS6110 sequence.

The study reports the identification of the complex MTBC complex using six specific markers that will help in the study of the molecular epidemiology of Mycobacterium. It identified Mycobacterium tuberculosis, Mycobacterium tuberculosis,

Mycobacterium tuberculosis, Mycobacterium spp.m tuberculosis, Mycobacterium canetii and MTBC without defining their species, the polymorphisms found with different molecular markers show a strong mutual association because the M. tuberculosis complex has a strongly clonal structure (Sreevatsan et al., 1997).

Identification by PCR technique proved to be a robust, convenient and highly discriminatory technique, which is reproducible and suitable for typing isolates of the MTBC complex. PCR based on the differential region method appears to be stable enough to allow the design and conduct of meaningful epidemiological studies (Richard C. et al 2003), a technique that would be suitable for high-throughput automation using PCR workstations and DNA sequencing platforms running allele calling software (Skuce et al., 2002)

Understanding the molecular basis of pathogen variation is not only important for discriminating and tracking clinically relevant strains, but also provides information on pathogenesis, host adaptation and the origin of new pathogenic forms (Reid et al., 2001).

In this study, we have been able to rapidly identify individual strains of MTBC in laboratory. In the processed samples, the study reported for M. canetti complex (2%) and M. tuberculosis (73%). This PCR-based approach, which is simple to perform, can be incorporated into routine by many clinical mycobacteriology laboratories. While assays such as hybridization technique and DNA sequencing may not be within the scope of a clinical diagnostic laboratory, many laboratories use amplification procedures.

Determining the prevalence of individual MTBC members within a population is of paramount importance, as most of these isolates are clinically significant and patient management depends on correct and timely identification, suggesting that the use of this method is likely to improve primary care and public health services. (Parsons et al., 2002)

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References

1. Arora, J., Suresh, N., Porwal, C., Pandey, P., Pande, J. N., & Singh, U. B. (2020). Genotyping Mycobacterium tuberculosis isolates with few copies of IS6110: Value of additional genetic markers. Infection, Genetics and Evolution,81 , 104230. https://doi.org/10.1016/j.meegid.2020.104230.
2. Ates, L. S., Sayes, F., Frigui, W., Ummels, R., Damen, M. P. M., Bottai, D., Behr, M. A., van Heijst, J. W. J., Bitter, W., & Majlessi, L. (2018). RD5-mediated lack of PE_PGRS and PPE-MPTR export in BCG vaccine strains results in strong reduction of antigenic repertoire but little impact on protection. PLoS Pathog ,14 (6), e1007139. https://doi.org/10.1371/journal.ppat.1007139.
3. Barletta F, Otero L, Collantes J, Asto B, de Jong BC, Seas C, Rigouts L.Genetic variability of Mycobacterium tuberculosis complex in patients with no known risk factors for MDR-TB in the northeastern part of Lima, Peru. BMC Infect Dis. Aug 28, 2013; 13: 397. doi: 10.1186 / 1471-2334-13-397. PMID: 23984854; PMCID: PMC3765759. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3765759/.
4. Cáceres O, Rastogi N, Bartra C, Couvin D, Galarza M, et al. (2014) Characterization of the Genetic Diversity of Extensively-Drug Resistant Mycobacterium tuberculosis Clinical Isolates from Pulmonary Tuberculosis Patients in Peru. PLOS ONE 9(12): e112789. https://doi.org/10.1371/journal.pone.0112789.
5. Comas, I., & Gagneux, S. (2009). The Past and Future of Tuberculosis Research. PLoS Pathog,5 (10), e1000600. https://doi.org/10.1371/journal.ppat.1000600. https://doi.org/10.1371/journal.ppat.1000600.
6. Coscolla, M., & Gagneux, S. (2014). Consequences of genomic diversity in Mycobacterium tuberculosis. Seminars in Immunology,26 (6), 431-444. https://doi.org/10.1016/j.smim.2014.09.012
7. Faksri, K., Drobniewski, F., Nikolayevskyy, V., Brown, T., Prammananan, T., Palittapongarnpim, P., Prayoonwiwat, N., & Chaiprasert, A. (2011). Genetic diversity of the Mycobacterium tuberculosis Beijing family based on IS6110, SNP, LSP and

VNTR profiles from Thailand. Infection, Genetics and Evolution,11 (5), 1142-1149. https://doi.org/10.1016/j.meegid.2011.04.007.

8. Farzam, B., Imani Fooladi, A. A., Izadi, M., Hossaini, H. M., & Feizabadi, M. M. (2015). Comparison of cyp141 and IS6110 for detection of Mycobacterium tuberculosis from clinical specimens by PCR. Journal of Infection and Public Health,8 (1), 32-36. https://doi.org/10.1016/j.jiph.2014.08.005. https://doi.org/10.1016/j.jiph.2014.08.005
9. Fonseca, K. L., Rodrigues, P. N. S., Olsson, I. A. S., & Saraiva, M. (2017). Experimental study of tuberculosis: From animal models to complex cell systems and organoids. PLoS Pathog ,13 (8), e1006421. https://doi.org/10.1371/journal.ppat.1006421.
10. Galagan, J. E. (2014). Genomic insights into tuberculosis. Nat Rev Genet,15 (5), 307-320. https://doi.org/10.1038/nrg3664.
11. Gehre, F., Kumar, S., Kendall, L., Ejo, M., Secka, O., Ofori-Anyinam, B., Abatih, E., Antonio, M., Berkvens, D., & de Jong, B. C. (2016). A Mycobacterial Perspective on Tuberculosis in West Africa: Significant Geographical Variation of M. africanum and Other M. tuberculosis Complex Lineages. PLoS Negl Trop Dis,10 (3), e0004408. https://doi.org/10.1371/journal.pntd.0004408.
12. Gonzalo-Asensio, J., Pérez, I., Aguiló, N., Uranga, S., Picó, A., Lampreave, C., Cebollada, A., Otal, I., Samper, S., & Martín, C. (2018). New insights into the transposition mechanisms of IS6110 and its dynamic distribution between Mycobacterium tuberculosis Complex lineages. PLoS Genet,14 (4), e1007282. https://doi.org/10.1371/journal.pgen.1007282.
13. Gröschel, M. I., Walker, T. M., van der Werf, T. S., Lange, C., Niemann, S., & Merker, M. (2018). Pathogen-based precision medicine for drug-resistant tuberculosis. PLoS Pathog,14 (10), e1007297. https://doi.org/10.1371/journal.ppat.1007297.
14. Group A Streptococcus : allelic variation, population genetics, and host-pathogen interactions S. D. Reid, N. P. Hoe, L. M. Smoot, J. M. Musser J Clin Invest, 107: 393-399, 2001).
15. Hatherill, M., Chaisson, R. E., & Denkinger, C. M. (2019). Addressing critical needs in the fight to end tuberculosis with innovative tools and strategies. PLoS Med,16 (4), e1002795. https://doi.org/10.1371/journal.pmed.1002795
16. Hwang, S.-H., Kim, D.-E., Sung, H., Park, B.-M., Cho, M.-J., Yoon, O.-J., & Lee, D.-H. (2015). Simple Detection of the IS6110 Sequence of Mycobacterium tuberculosis Complex in Sputum, Based on PCR with Graphene Oxide. PLoS ONE,10 (8), e0136954. https://doi.org/10.1371/journal.pone.0136954.
17. Kabir, S., Uddin, M. K. M., Chisti, M. J., Fannana, T., Haque, M. E., Uddin, M. R., Banu, S., & Ahmed, T. (2018). Role of PCR method using IS6110 primer in detecting Mycobacterium tuberculosis among the clinically diagnosed childhood tuberculosis patients at an urban hospital in Dhaka, Bangladesh. International Journal of Infectious Diseases,68 , 108-114. https://doi.org/10.1016/j.ijid.2018.01.015.
18. Kirzinger MW, Stavrinides J. Determinants of host specificity as a genetic continuum. Trends Microbiol. 2012; 20 : 88- [PubMed] [Google Scholar].
19. Linda M. Parsons, Roland Brosch, Stewart T. Cole, Ákos Somoskövi, Arthur Loder, Gisela Bretzel, Dick van Soolingen, Yvonne M. Hale, Max Salfinger Journal of Clinical Microbiology Jul 2002, 40 (7) 2339-2345; DOI: 10.1128/JCM.40.7.2339-2345.2002)
20. McEvoy, C. R. E., Falmer, A. A., van Pittius, N. C. G., Victor, T. C., van Helden, P. D., & Warren, R. M. (2007). The role of IS6110 in the evolution of Mycobacterium tuberculosis. Tuberculosis,87 (5), 393-404. https://doi.org/10.1016/j.tube.2007.05.010.
21. McHenry, M. L., Bartlett, J., Igo, R. P., Wampande, E. M., Benchek, P., Mayanja-Kizza, H., Fluegge, K., Hall, N. B., Gagneux, S., & Tishkoff, S. A. (2020). Interaction between host genes and Mycobacterium tuberculosis lineage can affect tuberculosis severity: Evidence for coevolution? PLoS Genet,16 (4), e1008728. https://doi.org/10.1371/journal.pgen.1008728
22. Ofori-Anyinam, B., Kanuteh, F., Agbla, S. C., Adetifa, I., Okoi, C., Dolganov, G., Schoolnik, G., Secka, O., Antonio, M., & de Jong, B. C. (2016). Impact of the Mycobaterium africanum West Africa 2 Lineage on TB Diagnostics in West Africa:

Decreased Sensitivity of Rapid Identification Tests in The Gambia. PLoS Negl Trop Dis,10 (7), e0004801. https://doi.org/10.1371/journal.pntd.0004801.

23.Ogusku Mauricio Morishi, & Salem, Julia Ignez. (2004). Analysis of different primers used in the PCR method: diagnosis of tuberculosis in the State of Amazonas, Brazil. Jornal Brasileiro de Pneumologia, 30(4), 343-349.

24.PAHO/WHO. (2020). PAHO/WHO Peru - Tuberculosis (PAHO/WHO, Ed.). https://www.paho.org/per/index.php?option=com_content&view=category&layout=blog&id=1111&Itemid=101

25.PCR-Based Method To Differentiate the Subspecies of the Mycobacterium tuberculosis Complex on the Basis of Genomic Deletions Richard C. Huard, Luiz Claudio de Oliveira Lazzarini, W. Ray Butler, Dick van Soolingen, John L. Ho Journal of Clinical Microbiology Apr 2003, 41 (4) 1637-1650; DOI: 10.1128/JCM.41.4.1637-1650.2003

26.Phelan, J. E., Coll, F., Bergval, I., Anthony, R. M., Warren, R., Sampson, S. L., Gey van Pittius, N. C., Glynn, J. R., Crampin, A. C., & Alves, A. (2016). Recombination in pe/ppe genes contributes to genetic variation in Mycobacterium tuberculosis lineages. BMC Genomics,17 (1). https://doi.org/10.1186/s12864-016-2467-y. https://doi.org/10.1186/s12864-016-2467-y

27.Rapid and Simple Approach for Identification of Mycobacterium tuberculosis Complex Isolates by PCR-Based Genomic Deletion Analysis

28.Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination S. Sreevatsan, X. Pan, J. M. Musser Proc Natl Acad Sci USA, 94: 9869-9874, 1997

29.Sanoussi, C. N., Affolabi, D., Rigouts, L., Anagonou, S., & de Jong, B. (2017). Genotypic characterization directly applied to sputum improves the detection of Mycobacterium africanum West African 1, under-represented in positive cultures. PLoS Negl Trop Dis,11 (9), e0005900. https://doi.org/10.1371/journal.pntd.0005900. https://doi.org/10.1371/journal.pntd.0005900

30.Skuce RA, McCorry TP, McCarroll JF, Roring SMM, Scott AN, Brittain D, Hughes SL, Hewinson RG, Neill SD. Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology (Reading). 2002 Feb;148(Pt 2):519-528. doi: 10.1099/00221287-148-2-519. PMID: 11832515.

31.Sun, J.-R., Lee, S.-Y., Perng, C.-L., & Lu, J.-J. (2009). Detecting Mycobacterium tuberculosis in Bactec MGIT 960 Cultures by Inhouse IS6110-based PCR Assay in Routine Clinical Practice. Journal of the Formosan Medical Association,108 (2), 119-125. https://doi.org/10.1016/s0929-6646(09)60042-5.

32.Thierry, D., Cave, M. D., Eisenach, K., Crawford, J. T., Bates, J. H., Gicquel, B., & Guesdon, J.-L. (1990). IS6110, an IS-like element of Mycobacterium tuberculosis complex. 18(1), 188. https://doi.org/DOI:10.1093/nar/18.1.188.

33.Thorne, N., Borrell, S., Evans, J., Magee, J., Garcia de Viedma, D., Bishop, C., Gonzalez-Martin, J., Gharbia, S., & Arnold, C. (2011). IS6110-based global phylogeny of Mycobacterium tuberculosis. Infection, Genetics and Evolution,11 (1), 132-138. https://doi.org/10.1016/j.meegid.2010.09.011.

34.Tientcheu, L. D., Haks, M. C., Agbla, S. C., Sutherland, J. S., Adetifa, I. M., Donkor, S., Quinten, E., Daramy, M., Antonio, M., & Kampmann, B. (2016). Host Immune Responses Differ between M. africanum- and M. tuberculosis-Infected Patients following Standard Anti-tuberculosis Treatment. PLoS Negl Trop Dis,10 (5), e0004701. https://doi.org/10.1371/journal.pntd.0004701.

35.Tucci, P., González-Sapienza, G., & Marin, M. (2014). Pathogen-derived biomarkers for active tuberculosis diagnosis. Front. Microbiol. ,5 . https://doi.org/10.3389/fmicb.2014.00549

36.Turcios, L., Casart, Y., Florez, I., de Waard, J., & Salazar, L. (2009). Characterization of IS6110 insertions in the dnaA-dnaN intergenic region of Mycobacterium tuberculosis clinical isolates. Clinical Microbiology and Infection,15 (2), 200-203. https://doi.org/10.1111/j.1469-0691.2008.02107.x. https://doi.org/10.1111/j.1469-0691.2008.02107.x

37.Vluggen, C., Soetaert, K., Groenen, G., Wanlin, M., Spitaels, M., Arrazola de Oñate, W., Fauville-Dufaux, M., Saegerman, C., & Mathys, V. (2017). Molecular epidemiology of Mycobacterium tuberculosis complex in Brussels, 2010-2013. PLoS ONE,12 (2), e0172554. https://doi.org/10.1371/journal.pone.0172554.

38.Warren, R. M., Gey van Pittius, N. C., Barnard, M., Hesseling, A., Engelke, E., de Kock, M., Gutierrez, M. C., Chege, G. K., Victor, T. C., & Hoal, E. G. (2006). Differentiation of Mycobacterium tuberculosis complex by PCR amplification of

genomic regions of difference. 10(7), 818–822. https://web.archive.org/web/20200621175734/https://pubmed.ncbi.nlm.nih.gov/16850559/

39.Yeboah-Manu, D., Asare, P., Asante-Poku, A., Otchere, I. D., Osei-Wusu, S., Danso, E., Forson, A., Koram, K. A., & Gagneux, S. (2016). Spatio-Temporal Distribution of Mycobacterium tuberculosis Complex Strains in Ghana. PLoS ONE,11 (8), e0161892. https://doi.org/10.1371/journal.pone.0161892.

40.Zahran, R. N., El Behiry, A., Marzouk, E., & Askar, T. (2014). Comparison of LCD array and IS6110-PCR with conventional techniques for detection of Mycobacterium bovis isolated from Egyptian cattle and Buffaloes. International Journal of Mycobacteriology,3 (3), 197-204. https://doi.org/10.1016/j.ijmyco.2014.06.002. https://doi.org/10.1016/j.ijmyco.2014.06.002

41.Zimpel, C. K., Brandão, P. E., de Souza Filho, A. F., de Souza, R. F., Ikuta, C. Y., Ferreira Neto, J. S., Camargo, N. C. S., Heinemann, M. B., & Guimarães, A. M. S. (2017). Complete Genome Sequencing of Mycobacterium bovis SP38 and Comparative Genomics of Mycobacterium bovis and M. tuberculosis Strains. Front. Microbiol. ,8 . https://doi.org/10.3389/fmicb.2017.02389

42.Moura, André et al. (2016) Comparison of nine DNA extraction methods for the diagnosis of bovine tuberculosis by real time PCR. Ciência Rural [online]. 2016, v. 46, n. 7 [Accessed 4 June 2021] , pp. 1223-1228. Available from: <https://doi.org/10.1590/0103-8478cr20151489>. ISSN 1678-4596. https://doi.org/10.1590/0103-8478cr20151489.

43.World Health Organization. Global tuberculosis report 2015. WHO/HTM/TB/2015.22. Geneva: World Health Organization; 2015.