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Research note

Title: Characterization of the first report of *Mycobacterium timonense* infecting an HIV patient in an Ecuadorian hospital

Running title: *Mycobacterium timonense* Ecuador

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ABSTRACT

Mycobacterium timonense is a Nontuberculous Mycobacteria (NTM) described in southern France in 2009, and to our knowledge, no reported again as a human pathogen in indexed literature. The aim of this work was to characterize the first clinical isolate of *M. timonense* in Ecuador. Time of growth, biochemical tests, thin layer, PCR-RFLP analysis of *hsp65* gene and

MALDI-TOF spectra analysis were no able to identify the species. The species identification was achieved throughout sequencing of *rrs, hsp65* and *rpoB* genes. The results highlight the necessity to set up a sequencing method to identification of emerging NTM in Ecuadorian clinical facilities.

Key words: Ecuador, Mycobacterium avium complex, *Mycobacterium timonense*, Nontuberculous Mycobacteria.

Mycobacterium genus comprises around 157 species, some of which are highly pathogenic to both humans and animals [1]. Nontuberculous Mycobacteria (NTM) are diverse and ubiquitous in the environment [2]. Human-Human transmission is rare, while the most of infections are acquired by aerolization, ingest or contact to injured tissue [3]. Mycobacterium avium complex (MAC) are the principal NTM associated with human infections in immunocompromised and elderly patients, this complex include *Mycobacterium avium*, *Mycobacterium intracellulare* and recently *Mycobacterium chimaerera, Mycobacterium colombiense, Mycobacterium arosiense, Mycobacterium vulneris, Mycobacterium marseillense, Mycobacterium bouchedurhonense* and *Mycobacterium timonense* [4]. The methods commonly used to NTM species identification in Ecuadorian hospitals, such as biochemical tests, thin layer, PCR-RFLP analysis (PRA) of *Hsp65* gene and MALDI-TOF are not able to identify the recently described species of MAC, like *Mycobacterium timonense* for which sequencing is necessary. To our knowledge, since the description of *M. timonesnse* in southern France in 2009, no cases of human infection associated

with this pathogen has been reported in indexed literature. We describe the first isolation and characterization of *M. timonense* from an HIV patient.

Colonies were isolated from synovial fluid sample and membrane biopsy from a 44-years-old man, with AIDS, classification C3 (Viral load: 207,000 copies/ml and CD4: 3) with highly active antiretroviral therapy (HAART). The patient had arthritis of right elbow so he was treated at the Traumatology Service of Vozandes Hospital in Quito, Ecuador. The samples were inoculated in Lowenstein-Jensen slants and MGIT 960 system, because the Ziehl-Neelsen stain (AFB) was positive in both samples. After ten days of incubation, the MGIT detected growth, while small colonies were observed in Lowenstein Jensen. A routine thin layer growth and biochemical tests were done manually.

The DNA of the isolate (denominated NTMZ&Z1) was obtained using High Pure PCR template preparation kit (Roche Diagnostic). DNA was subjected to PRA analysis [5]. Separate aliquots of PCR amplification of the 441 bp of the gene *hsp65* were digested with BstEII and HaeIII, and resolved in 2% agarose gel. Restriction patterns observed was compared to Chimara *et al*. 2008 [5].

MALDI-TOF analysis was carried out in a Microflex LT MALDI-TOF spectrometer (Bruker Daltonics Inc). The spectrum was examined in a MALDI-TOF Biotyper v. 3.1.

The PCR fragments of 1500 bp of the *rrs*, 441 bp of *hsp65* and 733pb of the *rpoB* genes were sequenced at Macrogen (Korea) facilities. Sequences were run against MAC sequences deposited in the GenBank. Sequences alignment and UPGMA tree (bootstrap with 1000 replicates) of the

concatenated sequences (2542 bp: 1428 bp of *rrs*, 707 bp *rpoB* and 407bp of *hsp65* genes) was built using Geneiuos v.7.0.6.

The isolate was identified as *M. chelonae*/*M. peregrinum* by routine biochemical tests. Thin layer showed a unique grow pattern (Figure 1A), MALDI-TOF analysis showed a high score (2.095), but was no able to differentiate between *M. chimera* and *M. intracellulare* (Figure 1B). PRA pattern was very similar to *M. chimaera* and *M. intracellulare*, according to the algorithm described by Chimara *et al*. 2008[5] and *M. marseillense* according Wallace *et al*. 2013[6] (Figure 1C). Finally, the sequences of *rrs* (KJ364652), *rpoB* (KJ364653) and *hsp65* (KJ364654) genes showed 100% of identity with *M. timonense* in Blast analysis and UPGMA tree generated using concatenated sequences (figure 1D, Table 1).

MAC are primarily pulmonary pathogens that affects individuals who are immune compromised (AIDS, immunosuppressive chemotherapy, hairy cell leukemia). In this clinical setting, MAC has been associated with osteomyelitis, tenosynovitis, synovitis and disseminated disease involving the lymph nodes, the Central Nervous System, the liver, the spleen and the bone marrow. MAC are the most common pathogens causing infection by NTM in patients with AIDS. *M. avium* is isolated in more than 95% of patients with AIDS who develop MAC infections [7, 8]. Still the epidemiology of distinct species of the MAC is not completely understood [9]. Recently, nine species have been described within the MAC, all of them with clinical implications. However, lack of genotyping techniques available has been one of the main reason why there is very few data available on species from this complex [9]. This could be illustrate by the results obtained using the most common methods applied in Ecuadorian clinical facilities which could not resolved between several species of *Mycobacterium* (Table 1, figure 1).

Single and concatenated sequences of *rrs*, *rpoB* and *hsp65* genes strongly suggest that the isolate found in the present study was *M. timonense.* Bootstrap values showed had high support in all nodes in UPGMA tree, confirming that these genes provide high resolution for identification of species of the MAC. Similar strategy has been used in order to identify emerging pathogen *Mycobacterium* species [10] (Figure 1). This lack of resolution in *Mycobacterium* identification by health-care facilities could be obscuring our knowledge of the real distribution and epidemiology of NTM strains at a global scale. This is relevant as understanding the distribution and epidemiology would be the first step to trying to assess differences in virulence between some of the newly described members of the MAC.

This is one of the first global reports since the species was described in 2009 [11]. We described an opportunistic infection caused by *Mycobacterium timonense* in AIDS patient, who was treated with ethambutol, clarithromycin and levofloxacin for 6 months. The outcome was favorable. This report has relevance from an epidemiological perspective since it suggests that MAC species in HIV patients are a potential source of infections and should be routinely screened and identified by sequencing. Countries in the region have found novel MAC species (*Mycobacterium colombiense*) causing infections in immunocompromised individuals [12]. As far as we know most hospitals do not routinely screen and identify NTM in our country, and the very few information that is generated in terms of epidemiology of this group of bacteria has not been published in indexed literature. Moreover, non-sequence based methods, which are commonly used in most Ecuadorian hospitals are no able to resolve between related NTM species. In this context, our findings suggest that many standard methods for identifying *Mycobacteria* could be misleading for accurate species identification of MAC and encourage us to recommend the use of sequence methods to identify these pathogens in local clinical facilities.

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TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Figure 1. Results of different identification methods used in strain NTMZ&Z1. a) thin layer growth pattern; b) MALDI-TOF spectra; c) PRA pattern; L1, 25bp ladder; L2, 1 kb ladder; BstEII digestion profile: 1, 231bp; 2, 116bp and 3, 94bp fragments; HaeIII digestion profile: 4, 145bp; 5, 127bp; 6, 59bp and 7, 40bp, 36bp and 34pb fragments. These fragments are the result of digestion of two separate aliquots of 441pb *hsp65* PCR fragment; d) 2542pb concatenated sequences; *rrs* (1420pb), *rpoB* (707pb) and *hsp65* (407pb) UPGMA tree. {

Table 1. Methods used in NTMZ&Z1 strain identification.

Method	Identification	Comment
Biochemical tests	*M. chelonae*/*M. peregrinum*	NTMZ&Z1 biochemical profile does not matched exactly with any profile [11][1].
Thin layer	ND	New growth pattern.
PRA	*M. chimera*/*M. intracellulare*	*Mycobacterium timonense* PRA pattern is not described in Chimara *et al*. 2008 PRA algorithm.
MALDI-TOF	*M. chimera*/*M. intracellulare*	MALDI-TOF Biotyper version 3.1 does not include *M. timonense* mass spectra.
Assemble of *rrs*, *rpoB* and *hsp65* genes sequences (2542pb)	*M. timonense*	Identify *M. timonense* with bootstrap 100% and identity 100%.

[1]Biochemical tests: Growth speed, growth in NaCl 5%, growth at 45 °C, pigmentation, tween 80 hydrolysis, utilization of citrate, alkaline phosphatase, pirazinamidase and urease.