Characterisation of *pks15/1* in clinical isolates of *Mycobacterium tuberculosis* from Mexico

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Tuberculosis (TB) is an infectocontagious respiratory disease caused by members of the Mycobacterium tuberculosis *complex. A 7 base pair (bp) deletion in the* locus *polyketide synthase* (pks)15/1 *is described as polymorphic among members of the* M. tuberculosis *complex, enabling the identification of Euro-American, Indo-Oceanic and Asian lineages. The aim of this study was to characterise this* locus *in TB isolates from Mexico. One hundred twenty clinical isolates were recovered from the states of Veracruz and Estado de Mexico. We determined the nucleotide sequence of a ± 400 bp fragment of the* locus pks15/1, *while genotypic characterisation was performed by spoligotyping. One hundred and fifty isolates contained the 7 bp deletion, while five had the wild type* locus. *Lineages X (22%), LAM (18%) and T (17%) were the most frequent; only three (2%) of the isolates were identified as Beijing and two (1%) EAI-Manila. The wild type* pks15/1 locus *was observed in all Asian lineage isolates tested. Our results confirm the utility of* locus pks15/1 *as a molecular marker for identifying Asian lineages of the* M. tuberculosis *complex. This marker could be of great value in the epidemiological surveillance of TB, especially in countries like Mexico, where the prevalence of such lineages is unknown.*

Key words: tuberculosis - W-Beijing - *pks15/1* - Mexico

Tuberculosis (TB) is a respiratory infectocontagious disease caused by members of the *Mycobacterium tuberculosis* complex. In spite of global efforts to lessen or eradicate disease burden, TB remains one of the most significant diseases affecting mankind. In 2011, the World Health Organization (WHO 2011) estimated that one third of the world´s population was infected, with an incidence of 8.8 million cases and a mortality of 1.4 million people.

The pathogenesis of *M. tuberculosis* involves mechanisms to reside and proliferate inside host phagocytic cells (Glickman & Jacobs 2001, Nguyen & Pieters 2005). Several major virulence factors of *M. tuberculosis* are cell wall components that play an important role in modulating the host immune response (Brennan 2003, Astaire-Dequeker et al. 2010). Phenolic glycolipid (PGL) is one such component (Reed et al. 2004, Caws et al. 2008) and, depending on the host genetic background, is associated with suppressing pro-inflammatory cytokine production in human macrophages (Sinsimer et al. 2008).

The polyketide synthase (*pks*)*15/1 locus* is involved in the biosynthesis of PGL (Constant et al. 2002) and is reported to be polymorphic among members of the *M. tuberculosis* complex (Gagneux & Small 2007). This genetic region has an intact open reading frame in the W-Beijing, Asian (non-W-Beijing) and Indo-Oceanic lineages, while Euro-American lineages contain a 7 base pair (bp) deletion (Constant et al. 2002, Reed et al. 2004, Gagneux & Small 2007).

The W-Beijing lineage is one of the most described lineages world-wide (Glynn et al. 2002) and is endemic in many parts of East Asia, where it has been the predominant strain lineage. The W-Beijing lineage is predominantly drug sensitive; however, epidemic spread of this strain outside of endemic regions is frequently associated with multidrug resistance (MDR) (Glynn et al. 2002, 2006, Caws et al. 2008). Furthermore, animal models have provided clear evidence that W-Beijing strains are highly virulent, resulting in higher bacillary load, increased dissemination and premature death (Aguilar et al. 2010).

Recently, the *pks15/1 locus* has been considered a potential marker for identifying W-Beijing, Asian (non-Beijing) and Indo-Oceanic lineages in countries with a low prevalence of these strains and a significant immigrant

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population presence (Alonso et al. 2008). However, characterisation of this marker is practically unknown in isolates from Latin America. Therefore, the aim of our study was to characterise the *pks15/1 locus* in clinical isolates of *M. tuberculosis* from different locations in Mexico.

MATERIALS AND METHODS

Isolation of mycobacteria, culture and drug susceptibility tests - Sputum samples from 120 patients with clinically confirmed TB were collected from 2007-2010 by the Mycobacteriology Departments of the Public Health Laboratories of Estado de Mexico and Veracruz. Sputum decontamination was performed using Petroff's modified method and primary isolation of mycobacteria was carried out using Löwenstein-Jensen medium. Susceptibility testing was performed following the fluorometric method (BACTEC, MGIT 960 Becton-Dickinson) for the first line drugs streptomycin, isoniazid, rifampin, ethambutol and pyrazinamide.

Variables such as age, gender, place of residence, type of treatment, co-occurrence of diabetes, cancer, malnutrition, anaemia, co-infection by human immunodeficiency virus (HIV), as well as addiction to tobacco, alcohol and other drugs were obtained from the patients' clinical files.

DNA purification and polymerase chain reaction (PCR)-amplification of the pks15/1 fragment - Extraction of DNA from the clinical isolates was conducted with one loop of cultured mycobacteria, according to Van Soolingen et al. (1991). DNA was re-suspended in nuclease-free water and concentration was determined by spectrophotometry using a Nanodrop 1000 (ThermoScientific, USA). The DNA solution was stored at -20ºC until use.

The 405 bp fragment of the *pks15/1* gene, including the 7 bp polymorphic fragment, was amplified by PCR using the primers PKR 5'-CTGCCCAGGAAACAC-GAC-3' and PKF 5'-GTGTCCTCCTTTGGGATCAG-3' (Martínez-Gamboa et al. 2008). The PCR reaction mixture consisted of: 10 mM Tris pH 8, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 10 nmoles of PKF and PKR primers, 1.25 U *Taq* polymerase (Promega, USA), 5% glycerol, 200 ng DNA template and nuclease-free water added to a final volume of 25 µL. Amplification was performed in a Veriti thermocycler (Applied Biosystems, USA) according to the following cycling parameters: 95ºC for 5 min, 30 cycles of 95ºC for 45 s, 60ºC for 45 s and 72ºC for 45 s, with a final extension at 72ºC for 8 min.

PCR products were electrophoretically separated in a 1.5% agarose gel and further purified using Amicon ultra centrifugal filters (Millipore, Ireland). Final DNA concentration of the PCR product was determined by electrophoresis using the Mass Ruler low range DNA Ladder (Fermentas, USA).

Sequencing of pks15/1 - Sequencing reactions were performed in forward directions using 6 µL of the Big Dye Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, USA), 3.2 pM of PF primer and 20 ng of purified PCR product in a final volume of 20 µL. Amplification cycling parameters were 25 cycles of 95ºC for 30 s, 50ºC for 15 s and 60ºC for 4 min.

The amplified products were purified using the ZR DNA Sequencing Clean-up Kit™ (Zymo Research, USA), re-suspended in Hi-Di formamide (Applied Biosystems, USA), heated to 95ºC for 5 min, cooled on ice and finally loaded onto a 96-well MicroAmp reaction plate (Applied Biosystems, USA).

Sequencing of DNA products was conducted by capillary electrophoresis in a Genetic Analyser 3500 (Applied Bio-systems, USA). Fluorescence spectra were analysed with the software Data Collection V1.01 (Applied Biosystems, USA). Sequence analysis was performed using the Sequencing Analysis V5.4 and SeqScape V2.7 programs (Applied Biosystems, USA). The *pks15/1* gene from *M. tuberculosis* H37Rv (GenBank accession 887291) was used as the reference DNA sequence.

Spoligotyping analysis of the clinical isolates - Spoligotyping was carried out with the clinical isolates following the previously described standard technique (Kamerbeek et al. 1997, Driscoll 2009). The DR region was amplified with oligonucleotides *DRa* (5'-GGTTTTGGGTCTGACGAC-3' biotinylated) and *DRb* (5'-CCGAGAGGGGACGGAAAC-3'). The biotinylated PCR products were hybridised to a membrane containing a set of 43 oligonucleotides corresponding to each spacer. DNA from *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551 and *Mycobacterium bovis* BCG was used for control reactions. Hybridised PCR products were incubated with streptavidin peroxidase conjugate. The membrane was then exposed to the chemiluminescence system, followed by exposure to X-ray film according to the manufacturer's instructions. The film was developed by standard photochemical procedures (Amersham International plc, Buckinghamshire, UK). The spoligotype international type (SIT) and family assignment were performed using the MIRU-VNTR plus (miru-vntrplus.org/MIRU/index.faces) and SITVIT web (pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) databases.

RESULTS

Epidemiological characteristics of population - Of the 120 patients included in the study, 75 (62.5%) were from Estado de Mexico and 45 (37.5%) were from Veracruz. The average age was 48 (± 16) years and there was no significant difference in age between the states ($p > 0.05$). There were 56 (47%) male patients, of which 41 (34%) were from Estado de Mexico and 15 (13%) were from Veracruz (Table I).

Regarding co-morbidities, 23 (19%) patients had a history of type 2 diabetes mellitus (DM-2), with Estado de Mexico accounting for 13 (11%) patients and Veracruz accounting for 10 (8%) patients (Table I). Only two individuals were identified with HIV and a history of alcohol and tobacco consumption.

Forty-two (35%) individuals were undergoing retreatment, of which Estado de Mexico accounted for 31 (26%) patients and Veracruz accounted for 11 (9%) patients (Table I). Resistance to at least one drug was noted in 50 (42%) patients and MDR was observed in 26 (22%) individuals. The most frequent drug resistances observed were to isoniazid and rifampicin, with 33 (28%) and 28

(23%) cases, respectively. The next most common drug resistances were to streptomycin with 19 (15%) cases, ethambutol with 15 (12%) cases and pyrazinamide with 12 (10%) cases.

Lineages identified - Table II shows the lineages identified in the study population. The most frequent lineage was X, with 26 (22%) isolates divided into two sub-lineages: X1 and X2. This was followed by 22 (18%) isolates distributed into six LAM sublineages. Twenty (17%) isolates were located in the sublineages T1 and T2. Seventeen isolates (14%) were found in the sub-lineages H1 and H3. The families S and U were observed in one isolate each and 28 isolates (23%) were located as orphans. Finally, five isolates were found in two Asian lineages, with three (2%) belonging to the W-Beijing lineage and two (1%) to the EAI-Manila lineage. Only one W-Beijing lineage isolate came from Veracruz; the remainder came from Estado de Mexico.

Occurrence of locus pks15 /1 and association with Asian lineages - The sequence of the *pks15/1 locus* was determined in all clinical isolates recovered. Only five (5%) had an intact *pks15/1 locus*: MC130, MC335, MC21, MC169 and VC131 (*pks15/1*$^{+}$*).* The remaining isolates, including the reference strain H37Rv, were found to contain the characteristic 7 bp deletion, GCCGCGG (*pks15/1*$^{-}$*).* The association between the wild type *locus pks15/1*$^{+}$ and the Asian lineages, EAI-Manila (MC130 and MC335) and W-Beijing (MC21, MC169 and VC131), was absolute (Table III).

DISCUSSION

There was a significant presence of TB drug-resistance (42%) and MDR (22%) in the recovered isolates, supporting previous observations indicating MDR as a growing problem in Mexico (Quitugua et al. 2002, Molina-Torres et al. 2010, Nava-Aguilera et al. 2011, Zenteno-Cuevas et al. 2012). Similarly, 19% (23) of the population had a medical history of DM-2, coinciding with reports illustrating a high association of this co-morbidity within Mexico (Ponce-de-Leon et al. 2004, Nava-Aguilera et al. 2011, Pérez-Navarro et al. 2011, Zenteno-Cuevas et al. 2012, Cuevas-Córdoba et al. 2013, Jiménez-Corona et al. 2013).

Mexico is one of the most significant contributors of TB cases in Latin America (PAHO/OMS 2011); however, reports describing the genotypic characteristics of these isolates are limited. In the patient population examined in this study, eight lineages were found, with the X, LAM, T and H lineages accounting for 68% of the isolates. All of these lineages have been previously described in Mexico; however, direct comparison with other lineage reports reveals an unusually high frequency of lineages X and LAM (Molina Torres et al. 2010, Nava-Aguilera et al. 2011, Zenteno-Cuevas et al. 2012, Martínez-Guarneros et al. 2013).

Information regarding the prevalence of isolates with Indo-Oceanic and Asian lineages in America is scarce and contradictory. Some reports from the United States of America (USA) and Peru show significant prevalence of these lineages in these countries (Quitugua et al. 2002, Iwamoto et al. 2012, Rodwell et al. 2012), while reports concerning countries in Central and South America suggest they are either scarce or completely absent (Ritacco et al. 2008, Rosales et al. 2010, Gomes et al. 2012). Reports from Mexico describe a significant variation of isolates with Indo-Oceanic and Asian lineages, according to geographic region. In northern states, the Indo-Oceanic and Asian lineages are scarce or absent, despite the strong human migration patterns that exist with proximity to the USA (Molina-Torres et al. 2010, Martínez-Guarneros et al. 2013). In central and southeastern Mexico, occurrences of less than 3% are observed for these lineages (Martínez-Gamboa et al. 2008, Zenteno-Cuevas et al. 2012). However, a significant occurrence of the EAI-CAS lineage (26%) has been reported in southwest Mexico (Nava-Aguilera et al. 2011). In this regard, less than 5% of the isolates analysed in our study were found to be of the Indo Oceanic and Asian lineages. This pattern is consistent with the lineage prevalence described for the states of central and southeastern Mexico. However, it is necessary to increase the number of genotyping studies in order to evaluate the frequency of these lineages in Mexico with greater detail.

Of the five isolates identified with an intact *pks15/1 locus*, three belong to the W-Beijing lineage (SIT 1) and two were of the EAI-Manila lineage (SIT 19). This relationship between Asian lineages and the presence of

TABLE I

Epidemiological description of the population

State of origin	Individuals (n)	Age (years) average (± SD)	Gender male (n)	Diabetes mellitus (n)	Re-treatment (n)	Drug-resistance (n)					
						S	H	R	E	Z	MDR
Estado de Mexico	75	47 (17)	41	13	31	11	19	14	5	6	12
Veracruz	45	49 (11)	15	10	11	8	14	14	10	6	14
Total	120	48 (16)	56	23	42	19	33	28	15	12	26

E: ethambutol; H: isoniazid; MDR: multidrug resistance; R: rifampin; S: streptomycin; SD: standard deviation; Z: pyrazinamide.

an intact *pks15/1 locus* has also been described in clinical isolates from Spain (Alonso et al. 2008), Myanmar (Stavrum et al. 2008), Vietnam (Caws et al. 2008) and Mexico (Martínez-Gamboa et al. 2008).

A detailed comparison between our findings and those described by Martínez-Gamboa (2008) reveals that carriers of the Asian TB lineages share the same SITS (1 and 19), despite the fact that that the diagnoses of TB in these populations are 10 years apart and that they had no epidemiological linkage or contact with any person or relative who was reported as having travelled to foreign locations. All of this information suggests that these lineages have been circulating within the population for a long time, which raises questions regarding why these lineages do not develop epidemic outbreaks and why they seem to be contained or limited. This is an intriguing issue, considering that a hypervirulent phenotype has been attributed to strains that have the

TABLE II

Lineages of *Mycobacterium* clinical isolates from Estado de Mexico and Veracruz

Lineage	Estado de Mexico	Veracruz	n (%)
X1	18	0	20
X2	1	3	1
X3	1	3	5
Total X	20	6	26 (22)
LAM1	0	3	1
LAM2	4	0	4
LAM3	1	2	3
LAM4	2	1	2
LAM7	1	0	1
LAM9	8	2	10
Total LAM	14	8	22 (18)
T1	13	4	17
T2	2	1	3
Total T	15	5	20 (14)
H1	2	0	2
H3	8	7	15
Total H	10	7	17 (14)
S	1	0	1 (1)
U	0	1	1 (1)
Beijing	2	1	3 (2)
EAI-Manila	2	0	2 (1)
Orphan	11	17	28 (23)
Total	75	45	120

TABLE III

Correlation between polyketide synthase (*pks*)*15/1* intact *locus* and Asiatic and Indo-Oceanic lineages in isolates from Estado de Mexico and Veracruz

ID	pks15/1 sequence	Lineage	SIT	Octal code	Spoligotype
H37Rv	AGCGAAAGCACCGGGG------GCGGCCGCCGTCGATGG	H37Rv	451	777777477760771	■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■■□□□□■■■■■■■
C130	AGCGAAAGCACCGGGG*GCCGCGG*GCGGCCGCCGTCGATGG	EAI-Manila	19	675777477403771	■■□■■■■□■■■■■■■■■■■□□■■■■■■■□□□□□□■■■■■■■
C335[a]	AGCGAAAGCACCGGGG*GCCGCGG*GCGGCCGCCGTCGATGG	EAI-Manila	19	677777477413771	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■
C21	AGCGAAAGCACCGGGG*GCCGCGG*GCGGCCGCCGTCGATGG	Beijing	1	000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
C169	AGCGAAAGCACCGGGG*GCCGCGG*GCGGCCGCCGTCGATGG	Beijing	1	000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
V122[b]	AGCGAAAGCACCGGGG*GCCGCGG*GCGGCCGCCGTCGATGG	Beijing	1	000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■

a: resistant to isoniazid; *b*: resistant to ethambutol; SIT: spoligotype international type.

pks 15/1^{+} gene, resulting in higher production of a PGL (Reed et al. 2004). While we acknowledge the limited number of isolates used in this study, our results enable us to propose that the *locus pks15/1* is a valuable molecular marker in terms of identifying W-Beijing and Asian lineages in *M. tuberculosis* patient isolates in Mexico. Use of the *locus* as a marker can have important implications for the molecular-epidemiological surveillance of TB in Mexico and countries where the prevalence of the Asian (including W-Beijing) and Indo-Oceanic lineages are still unknown.

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