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Genetic Diversity and Drug Resistance of *Mycobacterium tuberculosis* Complex Isolates and Nontuberculous Mycobacteria Identification from Presumptive Tuberculosis Cases in Oaxaca, Mexico

[1,a,b]Nakamura-Lopez Yuko, [1,2]Valencia-Carmona Oscar Daniel, [2,6]Martinez-Cruz Perla Monica, [3]Palma-Nicolas Jose Prisco, [4,a,b]Gonzalez-y-Merchand Jorge Alberto, [4]Rivera-Gutierrez Sandra, [5,a,b]Muñiz-Salazar Raquel and [2]Martinez-Martinez Lucia Lourdes

[1]Laboratorio de Biología Molecular, Consejo Estatal para la Prevencion y Control del Sida. San Bartolo Coyotepec, Oaxaca, Mexico
[2]Laboratorio de Biologia Molecular, Centro de Investigación, Facultad de Medicina UNAM-UABJO, Universidad Autonoma "Benito Juarez" de Oaxaca, Oaxaca, Mexico
[3]Laboratorio de Micobacterias, Centro Regional de Control de Enfermedades Infecciosas, Facultad de Medicina, Universidad Autonoma de Nuevo Leon, Monterrey, Nuevo Leon, Mexico
[4]Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Ciudad de Mexico, Mexico
[5]Laboratorio de Epidemiología y Ecología Molecular, Escuela de Ciencias de la Salud, Universidad Autónoma de Baja California, Ensenada, Baja California, México
[6]Division de Estudios de Posgrado e Investigacion, Instituto Tecnologico de Oaxaca, Oaxaca, Mexico
[a]Red Multidisciplinaria de Investigación en Tuberculosis (RemiTB; www.remitb.org)
[b]Red Nacional de Investigación para la Prevención y Control de la Tuberculosis en Mexico

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Corresponding Author:
Nakamura-Lopez Yuko
Laboratorio de Biología Molecular, Consejo Estatal para la Prevencion y Control del Sida. San Bartolo Coyotepec, Oaxaca, Mexico
Email: nakamura.yuko@gmail.com

Abstract: Tuberculosis (TB) is a re-emerging health problem worldwide. In Mexico, information about genetic diversity and drug resistance of *Mycobacterium Tuberculosis complex* (MTBc) is limited. Samples from 463 Presumptive TB cases were tested for TB by smear, culture and PCR, from which 19.2% were identify as MTBc and 16% as nontuberculous mycobacteria. MTBc isolates were characterized by Large Sequence Polymorphisms (LSPs), spoligotyping and MIRU-VNTR 24 loci typing methods. Clade designations showed 10 sub-lineages: Haarlem (35.7%), EAI (26.2%), LAM (9.5%), Ghana (4.8%), X (2.4%), New-1 (2.4%), H37Rv-like (7.1%), *M. bovis* (2.4%), S (7.1%), Uganda I (2.4%). The finding of EAI as one of the principal genotypes may be associated with high migration rates. Drug resistance was found in 35.71% of the isolates: 14.2% were multidrug-resistant (MDR-TB), 14.2% mono-resistant and 7.14% poly-resistant. This study provides the first description of genetic diversity and drug resistance profile of MTBc in Oaxaca, Mexico.

Keywords: *Mycobacterium tuberculosis Complex*, Genotyping, Tuberculosis

Introduction

Tuberculosis (TB) is considered since 1993 by World Health Organization as an emergent public health worldwide disease. In 2014, 9.6 million people developed TB and 1.5 million died by this disease (WHO, 2015). Mexico ranks third in TB cases in the Americas (OPS/OMS, 2014) and the state of Oaxaca has incidence and mortality rates above the national average (PUDI, 2014). According to ethnic and geographical criteria, Oaxaca is divided in eight geo-cultural regions with a high level of indigenous population and, in addition, immigration and emigration rates (nationally and internationally) are the highest among many other states of the south of Mexico. In Oaxaca, about 3.9 million people demand healthcare attention (PUDI, 2014) and 500-700 new TB cases were reported each year in the last decade.

Genotyping of *Mycobacterium tuberculosis complex* (MTBc) is a powerful tool that helps, at individual and population level to the disease management (Coll *et al*., 2014), however there is no information about the TB genotypic characteristics from Oaxaca. The inclusion of a few number of clinical isolates from this region in previous studies suggest that genotypic composition of circulating strains from the Southwest States (Guerrero, Oaxaca and Chiapas) differ from the rest of the country (Nava-Aguilera *et al*., 2011), but not conclusive data was previously available about Oaxaca. At the same time, the prevalence of drug resistance or MDR strains in Oaxaca was under represented in recent works (Macias Parra *et al*., 2011; Martinez-Guarneros *et al*., 2013). The genotyping methodologies more widely used due to its traceability, robustness and uniformity in the interpretation of results are spoligotyping, MIRU-VNTR and LSP (Allix *et al*., 2008; Reed *et al*., 2009; Supply *et al*., 2006).

In this work, we used the methodologies mentioned above to determine the first insight into the genetic diversity, drug resistance and comorbidities of MTBc isolates circulating in a southwest state of Mexico.

Materials and Methods

Sample Collection

Pulmonary and extra-pulmonary samples (sputum, lymph node biopsies, cerebrospinal fluids and bronchoalveolar lavages), were obtained from people with suspect of tuberculosis during the period 2014-2015 throughout Oaxaca State regions (Papaloapan, Cañada, Costa, Istmo, Mixteca, Sierra Norte, Sierra Sur and Valles Centrales (Fig. 1)).

Mycobacterial Identification and Drug Susceptibility Testing

Sputum samples were collected and decontaminated following the modified Petroff's method (Petroff, 1915). Mycobacteria isolation was carried out in Löwenstein-Jensen (LJ) medium. Characterization of AFB (+) isolates was achieved by phenotypic tests, macroscopic morphology of colonies, growth speed, niacin (+) and nitrite production (Campos and Flores, 1996; Huard *et al*., 2003). All clinical samples were manipulated under biosafety conditions using a Biological Safety Cabinet IIA in a BSL-2 facility.

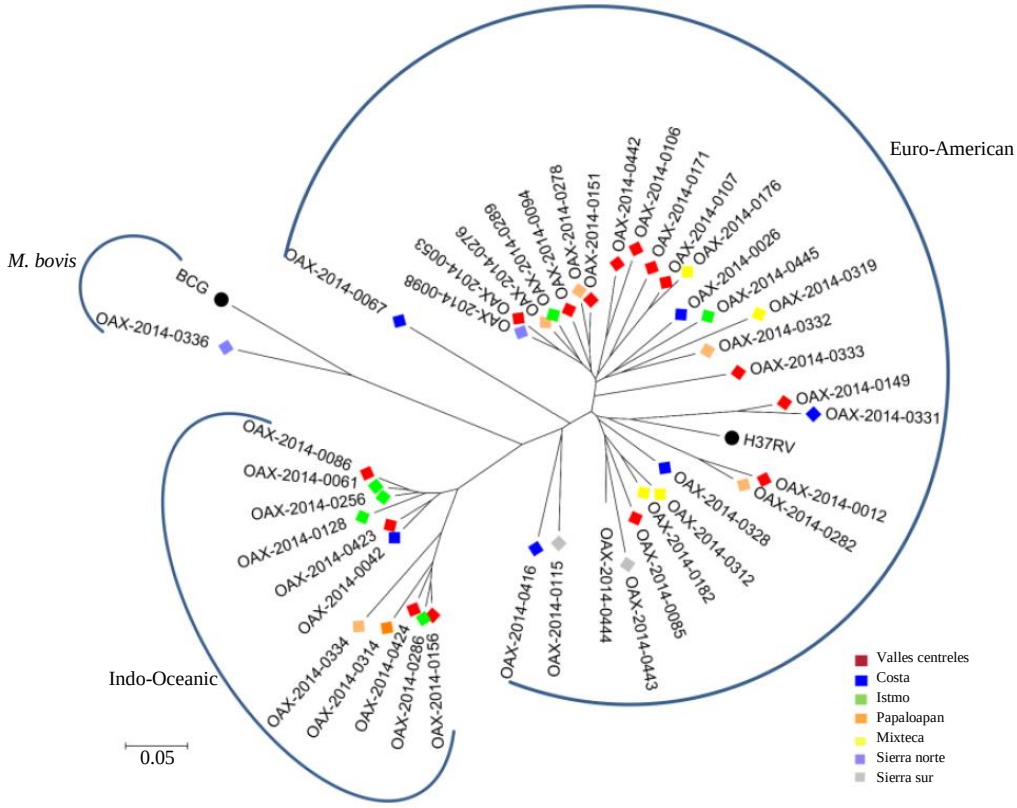

Fig. 1: Radial phylogenetic tree based on LPS, MIRU-VNTR and spoligotyping. The principal genetic lineage of each cluster is indicated and the geographical region where the isolates belonged to: Valles Centrales (red square), Costa (blue square), Istmo (green square), Papaloapan (orange square), Mixteca (yellow square), Sierra Norte (purple square) and Sierra Sur (gray square). Reference strains included *M. tuberculosis* H37Rv and *M. bovis* BCG (black circles)

Mycobacterial identification was done by Gene Xpert MTB/RIF (Cepehid, USA) according to manufacturer's specifications and by PCR using specific primers as described previously (Huard *et al*., 2003). Drug resistance was determined by the indirect proportion method on LJ medium at critical concentrations for Isoniazide (INH; 0.2 μg/mL), Rifampin (RIF; 40 μg/mL), Streptomycin (STR; 4.0 μg/mL) and Ethambutol (EMB; 2.0 μg/mL) (Workneh *et al*., 2016). Pyrazinamidase activity was used to determine susceptibility to Pyrazimide (PZA).

Genotyping

DNA of each clinical isolate was extracted using a commercial kit (DNAzol, Thermo Fisher Scientific, USA) and was quantified by UV spectrophotometry (UV Spectrophotometer Q3000, Quawell, USA). MTBc clinical isolates were genotyped by MIRU-VNTR 24loci (Allix *et al*., 2008), spoligotyping (Supply *et al*., 2006) and LSP including the regions IS1561, RD4, RD9, RD12 (Huard *et al*., 2003), RD105, RD239, RD750 (Reed *et al*., 2009), RD724 and RD726 (Rindi *et al*., 2014).

PCR products were evaluated by electrophoresis on agarose gels stained with ethidium bromide and by Bioanalyzer DNA chip (Agilent, Santa Clara, CA). *M. tuberculosis* H37Rv and *M. bovis* BCG were used as reference strains.

Bioinformatic Analysis

Spoligotype patterns as binary format, 24-loci MIRU patterns and presence/absence of RDs were analyze using the MIRU-VNTR *plus database* (http://www.miru-vntrplus.org) to determine the genotypes and the matrix of distances. MEGA6 software (MEGA software) was used to build the phylogenetic tree using the neighbor-joining algorithm.

Results

Mycobacterial Isolation and Drug Susceptibility Testing

From January 2014 to March 2015, 463 individuals with presumptive *Mycobacterium tuberculosis* infection of the eight regions of Oaxaca were tested. Some 21% samples were positive by PCR/XPERT and 16% were positive to Nontuberculous Mycobacteria (NTM). The samples from "Cañada" region were negative for MTBc nevertheless positive to NTM.

We recovered 42 clinical isolates of MTBc, all from 2014. Two isolates (4.7%) were recovered from extrapulmonar location (lymph node and cerebrospinal) and the rest were from respiratory samples. According to gender, 67% TB patients were men and 33% were women, all of them were diagnosed/treated as new TB cases. The most frequent comorbidities found were type 2 Diabetes Mellitus (DM) (33.3%), malnutrition (21.4%) and HIV co-infection (11.9%).

From 42 AFB(+) cultures, one niacin (-) and nitrite (-) isolate was identified as *Mycobacterium bovis* with intrinsic Pyrazinamide (PZA) resistance, while 15 (35.7%) isolates showed resistance to at least one drug. As shown in Table 1, one isolate (2.38%) was RIF mono-resistant and three (7.1%) were STR mono-resistant. MDR-TB strains recovered in this study account for 14.3% (6) of all isolates.

Genotyping Profiles of MTBc Strains

Using MIRU-VNTR 24 loci, spoligotyping and LSP, 42 MTBc isolates were analyzed. Considering data of the three markers, a distance matrix was calculated and a phylogenetic tree was constructed using the neighbor-joining algorithm. The isolates were classified into three major lineages: Indo-Oceanic, Euro-American and *M. bovis* (Fig. 1).

As shown in Table 1, the Euro-American was the principal genetic lineage represented with thirty isolates, including 13 Haarlem (30.9%), 4 LAM (9.5%), 5 H37Rv-like (11.9%) sub-lineages, accounting for 52% of all isolated strains. The second principal genetic lineage identified was the Indo-Oceanic with eleven (26.1%) *M. tuberculosis* isolates of EAI sub-lineage, which was also found highly associated to first-line drug resistance with five (45%) isolates being resistant to at least one drug (Table 1).

The frequency of other minor genotypes and their association with drug resistance is indicated in Table 1. It is noteworthy the high burden of EAI sub-lineage in Oaxaca and the high genetic diversity of the strains belonging to the Euro-American lineage.

The sub-lineages identified were located in seven of the eight geographical regions that conform the state of Oaxaca (Fig. 2). The "Valles Centrales" and "Costa" regions showed the highest diversity of lineages, with six and five sub-lineages respectively. In the "Valles Centrales" Euro-American Haarlem sub-lineage was the first most frequent (33.3%), followed by Indo-Oceanic EAI sub-lineage (26.6%) accounting 60% of the isolates; only one EAI isolate showed a monodrug resistance pattern to STR. In the "Costa" region, 66.6% of the isolates showed anti-tuberculosis drug resistance, the S sub-linage was the most frequent (33.3%), one of this strains was resistant to all the anti-tuberculosis drugs tested and another was MDR-TB.

Table 1: Demographic, genotyping data and drug resistance pattern of 42 MTBc isolates

Isolate	OAX-2014-0086	OAX-2014-0085	OAX-2014-0067	OAX-2014-0061	OAX-2014-0053	OAX-2014-0042	OAX-2014-0026	OAX-2014-0012
Region	VC	VC	C	I	VC	C	C	VC
Sex/Age	M/68	F/28	F/22	M/59	M/84	M/36	M/66	F/12
Co-morbidities	DM	None	Mn	HIV	HTA/Pn	None	Mn/DM	An/Pn
L	EAI	Haarlem	S	EAI	Haarlem	EAI	Haarlem	LAM
MIRU24								
MIRU-2	2	2	2	2	2	1	2	1
MIRU-4	5	2	2	5	2	5	2	2
MIRU-10	4	3	3	4	3	4	5	4
MIRU-16	3	4	3	2	3	3	2	2
MIRU-20	2	2	2	2	2	2	1	2
MIRU-23	6	5	5	6	5	5	5	5
MIRU-24	2	1	1	2	1	2	1	1
MIRU-26	2	3	7	2	7	2	6	5
MIRU-27	3	3	3	3	3	3	3	1
MIRU-31	3	3	4	3	1	2	3	3
MIRU-39	2	2	3	2	2	2	2	2
MIRU-40	2	2	3	2	3	2	3	2
ETR-A	4	2	4	4	3	3	3	1
ETR-B	5	2	2	5	2	3	2	2
ETR-C	4	4	4	4	3	4	3	4
QUB-11b	10	3	9	9	3	9	6	2
QUB-26	10	8	2	5	10	11	10	8
QUB-4156	1	2	2	1	3	1	3	3
Mtub04	1	2	5	1	4	1	2	3
Mtub21	10	2	5	10	2	10	3	3
Mtub29	3	4	3	3	0	3	3	0
Mtub30	2	2	4	2	4	2	4	1
Mtub34	3	3	1	3	2	3	3	3
Mtub39	2	3	4	3	3	2	3	2
LSP								
IS1561	X	X	X	X	X	X	X	X
RD4	X	X	X	X	X	X	X	X
RD9	X	X	X	X	X	X	X	X
RD12	X	X	X	X	X	X	X	X
RD105	X	X	-	X	X	X	X	X
RD239	X	X	X	-	X	-	X	X
RD750	X	X	X	X	X	X	X	X
RD724	X	X	X	X	X	X	X	X
Spoligotyping								
Octal code	657777777773771	177777777760771	777777777773771	677777777773771	777777777760771	777777777773771	775777777760771	777737607760771
Drug Resistance								
INH	S	S	S	S	S	S	S	S
RIF	S	S	R	S	S	R	R	S
EMB	S	S	R	S	S	S	S	S
PZA	S	S	S	S	S	S	S	S
STR	S	S	R	S	S	S	R	S

Isolate Region Sex/Age Co-morbidities L	OAX-2014-0094 VC F/70 Mn/DM/CA/H1/H1 Haarlem	OAX-2014-0098 SN M/31 DM/Sm Ghana	OAX-2014-0106 VC M/18 Mn/DM Haarlem	OAX-2014-0107 VC F/32 DM Haarlem	OAX-2014-0115 SS M/69 None S	OAX-2014-0128 I M/75 DM EAI	OAX-2014-0149 VC F/29 None LAM	OAX-2014-0151 VC M/88 DM X
MIRU24								
MIRU-2	2	2	0	2	2	2	2	2
MIRU-4	2	2	2	2	1	5	2	2
MIRU-10	5	5	1	3	3	4	4	3
MIRU-16	3	3	3	3	2	3	2	3
MIRU-20	2	2	2	1	2	2	2	2
MIRU-23	5	5	5	5	3	6	6	5
MIRU-24	1	1	1	1	0	2	1	1
MIRU-26	5	7	4	5	2	2	4	5
MIRU-27	3	3	3	3	3	3	3	3
MIRU-31	3	3	0	3	3	2	3	3
MIRU-39	2	2	2	2	2	1	2	2
MIRU-40	3	3	2	1	2	2	1	5
ETR-A	3	3	3	3	2	4	2	3
ETR-B	2	2	2	1	2	8	1	2
ETR-C	3	3	3	3	4	4	4	3
QUB-11b	5	3	5	3	2	6	4	5
QUB-26	12	10	12	9	7	10	11	8
QUB-4156	2	2	3	3	2	1	2	0
Mtub04	2	4	2	4	2	1	4	2
Mtub21	2	4	4	3	2	7	3	4
Mtub29	4	4	4	4	4	3	4	4
Mtub30	4	4	4	2	2	2	1	4
Mtub34	3	2	3	3	3	3	5	2
Mtub39	3	3	3	3	4	2	2	5
LSP								
IS1561	X	X	X	X	X	X	-	X
RD4	X	X	X	X	X	X	X	X
RD9	X	X	X	X	X	X	X	X
RD12	X	X	X	X	X	X	X	X
RD105	X	X	X	X	X	-	X	X
RD239	X	X	X	X	X	X	X	X
RD750	X	X	X	X	X	X	X	X
RD724	X	X	X	X	X	X	X	X
Spoligotyping								
Octal code	777777777720771	737777777760771	777777777760411	777777777773771	777777777773771	777777777773771	777777777760771	777777777760771
Drug Resistance								
INH	S	S	S	S	S	R	S	S
RIF	S	S	S	S	R	R	S	S
EMB	S	S	S	S	S	S	S	S
PZA	S	S	R	S	R	S	S	S
STR	S	S	S	S	S	S	S	S

Isolate	OAX-2014-0278	OAX-2014-0276	OAX-2014-0256	OAX-2014-0182	OAX-2014-0176	OAX-2014-0171	OAX-2014-0156
Region	P	P	I	MIX	MIX	VC	VC
Sex/Age	F/20	M/30	M/53	M/37	M/61	F/59	M/38
Co-morbidities	Sm	HIV/Cs	DM	None	Og/Eb	Mn/DM	HIV/Mn/Sm
L	Haarlem	Haarlem	EAI	NEW-1	Haarlem	Haarlem	EAI
MIRU24							
MIRU-2	2	2	2	2	2	2	2
MIRU-4	2	2	5	2	2	1	8
MIRU-10	0	3	4	3	4	2	4
MIRU-16	3	3	3	3	3	3	3
MIRU-20	0	2	2	2	1	2	2
MIRU-23	5	5	6	5	5	5	6
MIRU-24	1	1	2	1	1	1	2
MIRU-26	5	5	2	5	5	5	2
MIRU-27	3	3	3	3	3	3	3
MIRU-31	3	3	3	3	3	3	3
MIRU-39	2	2	2	2	1	2	2
MIRU-40	3	3	2	3	4	3	2
ETR-A	3	3	4	2	3	3	4
ETR-B	2	2	6	2	1	2	6
ETR-C	4	3	4	4	3	3	4
QUB-11b	5	2	7	4	3	5	9
QUB-26	9	6	10	7	11	10	11
QUB-4156	0	3	1	2	3	1	1
Mtub04	2	5	1	1	4	4	1
Mtub21	4	2	10	2	3	4	13
Mtub29	4	4	3	4	4	4	3
Mtub30	4	4	2	2	2	2	2
Mtub34	2	2	3	3	3	2	3
Mtub39	3	3	2	3	3	4	2
LSP							
IS1561	X	X	X	X	X	X	X
RD4	X	X	X	X	X	X	X
RD9	X	X	X	X	X	X	X
RD12	X	X	X	X	X	X	X
RD105	X	X	X	X	X	X	X
RD239	X	X	-	X	X	X	-
RD750	X	X	X	X	X	X	X
RD724	X	X	X	X	X	X	X
Spoligotyping							
Octal code	777777607760731	777777777760771	777777777773771	777777777773771	777777777773771	777777777773771	677777477413771
Drug Resistance							
INH	S	R	R	S	S	S	S
RIF	S	R	S	S	S	S	S
EMB	S	R	S	S	S	S	S
PZA	S	S	S	S	S	S	S
STR	S	R	R	S	S	S	R

Isolate	OAX-2014-0332	OAX-2014-0331	OAX-2014-0328	OAX-2014-0319	OAX-2014-0314	OAX-2014-0312	OAX-2014-0289	OAX-2014-0286
Region	P	C	C	MIX	P	MIX	I	I
Sex/Age	M/24	M/74	M/64	F/36	M/42	M/42	F/58	M/44
Co-morbidities	None	Mn/Sm	Mn/Sm	None	None	None	DM/HTA/Sm	HTA/Al
L	Ghana	LAM	S	Haarlem	EAI	Haarlem	Haarlem	EAI
MIRU24								
MIRU-2	2	2	2	3	2	2	2	2
MIRU-4	2	2	2	2	5	2	2	5
MIRU-10	3	4	3	2	4	3	5	4
MIRU-16	3	2	3	3	3	3	3	2
MIRU-20	1	2	2	1	2	2	2	2
MIRU-23	6	6	5	4	6	5	5	6
MIRU-24	1	1	1	1	2	1	1	2
MIRU-26	4	4	3	5	2	5	3	2
MIRU-27	3	3	4	3	2	4	3	3
MIRU-31	1	3	3	1	2	0	3	3
MIRU-39	2	2	2	2	3	2	2	2
MIRU-40	3	1	4	3	2	2	3	2
ETR-A	3	3	3	3	4	2	3	4
ETR-B	2	1	2	2	6	2	2	6
ETR-C	3	4	4	2	4	4	3	4
QUB-11b	5	4	5	6	9	4	2	9
QUB-26	2	2	4	7	6	7	5	11
QUB-4156	3	2	2	4	1	2	3	1
Mtub04	3	5	3	3	1	1	4	1
Mtub21	4	1	2	3	6	2	2	10
Mtub29	4	4	4	2	4	4	4	3
Mtub30	4	1	1	4	2	2	4	2
Mtub34	4	5	3	3	3	3	3	3
Mtub39	4	2	4	3	2	3	3	2
LSP								
IS1561	X	-	X	X	X	X	X	X
RD4	X	X	X	X	X	X	X	X
RD9	X	X	X	X	X	X	X	X
RD12	X	X	X	X	X	X	X	X
RD105	X	X	X	X	-	X	X	X
RD239	X	X	X	X	X	X	X	-
RD750	X	X	X	X	X	X	X	X
RD724	X	X	X	X	X	X	X	X
Spoligotyping								
Octal code	777777777720771	777777607760771	777777777760771	777777777020771	677777477413771	777777777760771	777777777760771	677777477413771
Drug Resistance								
INH	S	S	S	S	S	S	S	S
RIF	S	S	S	S	R	S	S	S
EMB	S	S	S	S	S	S	S	S
PZA	S	S	S	S	S	S	S	S
STR	S	R	S	S	S	S	S	S

Isolate	OAX-2014-0333	OAX-2014-0442	OAX-2014-0424	OAX-2014-0423	OAX-2014-0416	OAX-2014-0336	OAX-2014-0334	OAX-2014-0443
Region	VC	VC	VC	VC	C	SN	P	SS
Sex/Age	M/83	M/76	M/21	F/55	M/27	F/61	M/62	VC
Co-morbidities	None	None	Mn/Sm/As	Mn/Sm/HIV/HTA	HIV/Sm/Al/Cs	DM	DM	F?
L	Uganda I	Haarlem	EAI	EAI	H37Rv-like	Bovis	EAI	None
MIRU24								H37Rv-like
MIRU-2	2	1	2	2	2	2	2	1
MIRU-4	2	2	5	6	2	3	2	2
MIRU-10	3	4	4	4	3	2	3	3
MIRU-16	3	3	3	3	2	2	3	3
MIRU-20	2	2	2	2	2	2	2	2
MIRU-23	5	5	6	6	6	4	6	6
MIRU-24	1	1	2	2	1	2	2	1
MIRU-26	4	4	2	2	3	5	2	5
MIRU-27	1	3	3	3	2	3	3	0
MIRU-31	3	2	4	2	1	3	3	3
MIRU-39	2	2	2	2	2	2	4	2
MIRU-40	3	2	2	2	1	2	2	1
ETR-A	3	3	3	3	3	8	4	2
ETR-B	3	2	6	6	2	3	6	2
ETR-C	3	3	4	4	4	5	4	4
QUB-11b	2	5	9	9	2	3	7	4
QUB-26	4	9	7	6	4	4	7	6
QUB-4156	4	3	1	1	1	1	0	2
Mtub04	1	2	1	1	2	2	3	2
Mtub21	4	4	10	10	2	3	11	2
Mtub29	2	4	3	3	3	3	3	4
Mtub30	4	1	2	2	2	4	2	2
Mtub34	3	3	3	3	4	2	4	3
Mtub39	2	3	2	2	8	2	2	5
LSP								
IS1561	X	X	X	X	X	X	X	X
RD4	X	X	X	X	X	-	X	X
RD9	X	X	X	X	X	-	X	X
RD12	X	X	X	X	X	-	X	X
RD105	X	X	X	X	X	X	X	X
RD239	X	X	-	-	X	X	-	X
RD750	X	X	X	X	X	X	X	X
RD724	-	X	X	X	X	X	X	X
Spoligotyping								
Octal code	777777777760771	777777777773771	677777477413771	777777777773771	777777777773771	677777777777601	677777477413701	777777777760031
Drug Resistance								
INH	S	S	S	S	S	S	S	R
RIF	S	S	S	S	S	S	S	R
EMB	S	S	S	S	S	S	S	S
PZA	S	S	S	S	S	R	S	S
STR	S	S	S	S	S	S	S	S

Isolate	Region	Sex/Age	Co-morbidities	L	MIRU24																					LSP								Spoligotyping	Drug Resistance				
					MIRU-2	MIRU-4	MIRU-10	MIRU-16	MIRU-20	MIRU-23	MIRU-24	MIRU-26	MIRU-27	MIRU-31	MIRU-39	MIRU-40	ETR-A	ETR-B	ETR-C	QUB-11b	QUB-26	QUB-4156	Mtub04	Mtub21	Mtub29	Mtub30	Mtub34	Mtub39	IS1561	RD4	RD9	RD12	RD105	RD239	RD750	RD724	Octal code	INH	RIF	EMB	PZA	STR
OAX-2014-0444	?	M/?	None	H37Rv-like	2	2	3	3	2	6	1	5	3	3	2	4	3	2	4	3	8	2	2	2	4	2	3	5	X	X	X	X	X	X	X	X	777777557760771	S	S	S	S	R
OAX-2014-0445	I	M/?	None	Haarlem	2	2	4	5	2	3	1	2	3	3	1	3	3	2	3	5	4	3	2	3	2	3	3	3	X	X	X	X	X	X	X	X	777777777720771	S	S	S	S	S

Region: C (Costa); I (Istmo); MIX (Mixteca); P (Papaloapan); SN (Sierra Norte); SS (Sierra Sur); VC (Valles Centrales)
Sex/Age: M (Male); F (Female); ? (Unknown)
Co-morbidities: An (Anemia); Pn (Pneumonia); Mn (Malnutrition); DM (type 2 Diabetes mellitus); HTA (Arterial hypertension); HTP (Portal hypertension); HIV; Ci (Cirrhosis); HT (Hypothyroidism); Sm (Smoking); Og (Oligoclonal gamapathy); Ep (Epilepsy); Cs (Cannabis smoker); Ow (Overweight); Al (Alcoholism); As (Asthma); None
L: Lineage
LSP: X (Present region); - (Absent region)
Drug resistance: INH (isoniazid); RIF (rifampicin); EMB (ethambutol); PZA (pyrazinamide); STR (streptomycin); S (sensitive); R(resistant)

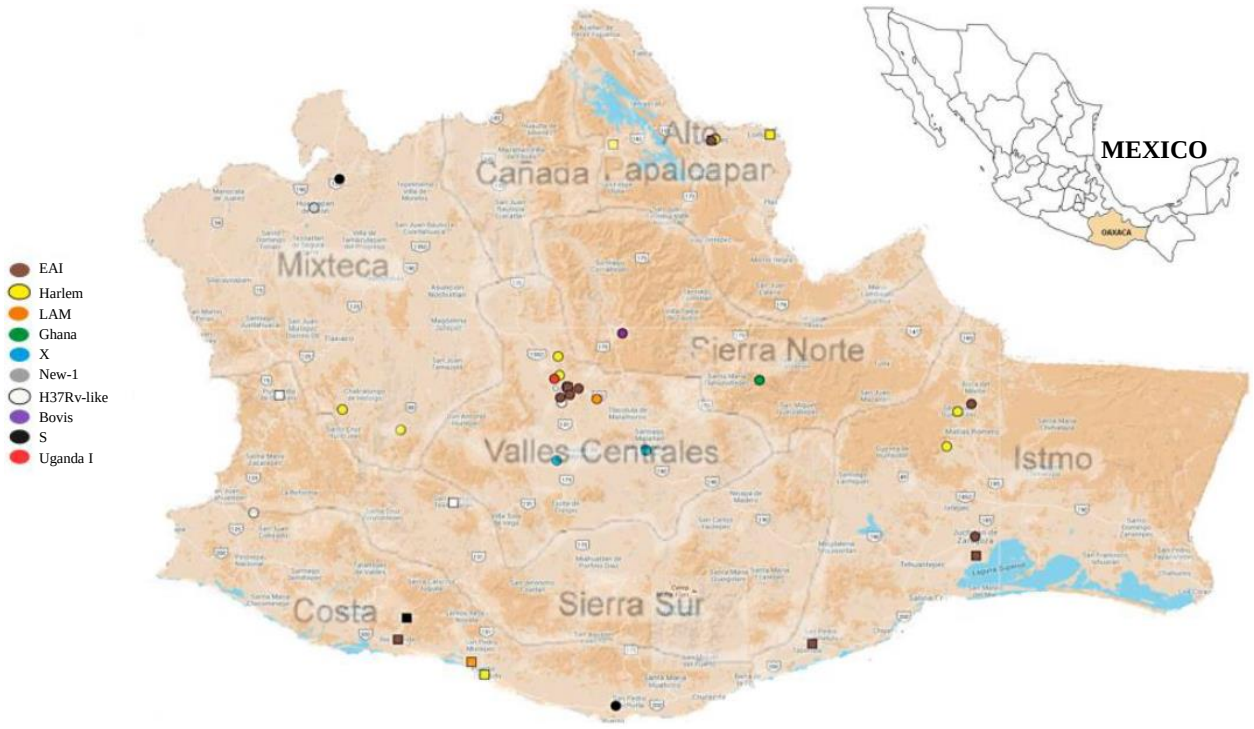

Fig. 2: Geographical localization of each sub-lineage and drug-resistance pattern within Oaxaca State, Mexico. Haarlem (yellow), EAI (brown), LAM (orange), Ghana (green), X (blue), New-1 (gray), H37Rv-like (white), *M. bovis* (purple), S (black) and Uganda I (red). Pan-susceptible isolates (circles) and isolates resistant to at least one first line drug (squares)

In the "Mixteca" and the "Papaloapan" regions, the Haarlem sub-lineage was predominant. However, in the "Mixteca" region the isolates were susceptible to all first line anti-tuberculosis drugs, while in "Papaloapan"

region, 66.6% were drug-resistant. In the "Istmo de Tehuantepec" region, the EAI sub-lineage was the most frequent (66.6%), showing a high incidence of first-line drug resistance (50%). In the "Sierra Sur" region there were two isolates belonging to H37Rv-like sub-linage, one of them showed anti-tuberculosis drug resistance to RIF/PZA and the second to RIF/INH, considered as MDR-TB. Finally, in "Sierra Norte" region *M. bovis* was isolated from a 61 year-old woman.

Discussion

The genotypic diversity of clinical isolates in the state of Oaxaca differs from other national reports (Macias Parra *et al*., 2011; Martinez-Guarneros *et al*., 2013; Zenteno-Cuevas *et al*., 2013), specifically those from the northern and central region of the country (Lopez-Alvarez *et al*., 2010; Molina-Torres *et al*., 2010), where the genotypes T, LAM and X are the most frequently reported. The T lineage was not detected in the oaxacan population, besides LAM and X which occurred in low frequency, 12% and 10% respectively. Unexpectedly we detected a high occurrence of EAI sub-lineage (26.1%), belonging to the Indo-Oceanic lineage, which has been reported at low frequencies throughout the country (Macias Parra *et al*., 2011; Molina-Torres *et al*., 2010), except for Acapulco City, in the state of Guerrero (Nava-Aguilera *et al*., 2011), where it was reported as the main genotype (44.6%). It has been proposed that high occurrence of Indo-Oceanic strains could be due to their arrival directly from Philippines between XVI and XIX centuries when both countries were Spanish colonies and kept frequent maritime communication through the Pacific Ocean, where Oaxaca and Guerrero are located (Nava-Aguilera *et al*., 2011).

Similar to previous studies that report type 2 Diabetes Melltitus (DM) as one of the most frequent comorbidities in TB patients (Workneh *et al*., 2016), in this study it was present in 30.9% of the cases. Besides, TB-HIV coinfection has been reported between 28.8% and 64% (Wejse *et al*., 2015; Middelkoop *et al*., 2015), here we found this binomial in just 11.9% of the patients. Haarlem sub-lineage predominates among DM patients (46%). Similar results were found in another Mexican population study, where 53% of patients infected by Haarlem strains were diabetic (Pérez-Navarro, 2014). The EAI sub-lineage was the most frequent among the HIV co-infected patients (60%), in concordance with another report in which this sub-linage was present in 46.2% of TB-HIV coinfected patients (Kibiki *et al*., 2007).

In our study we observed a high percentage of primary resistance to at least one first line drug (35.71%): MDR-TB (14.2%), mono-resistant (14.2%) and poly-resistant (7.14%); being 1.5 times higher than in a previous drug resistance report from Oaxaca (Granich *et al*., 2000).

H37Rv-like sub-lineage was more associated with drug resistance (60%) than the other lineages with two mono-resistant isolates to STR and RIF and one MDR-TB isolate. To our knowledge there is only one report about drug resistance in this sub-lineage (Niemann *et al*., 2010), where no MDR-TB isolates were found.

The Haarlem sub-lineage has been linked with drug-resistance and clonal expansion (Ramazanzadeh *et al*., 2015). We found that in "Papaloapan" region two of the three Haarlem isolates characterized were resistant to all drugs tested, in contrast with the isolates belonging to "Mixteca" region that were pan-susceptible. "Papaloapan" region is characterized by trade routes of agricultural products, is part of one of the migration routes from Central America to the USA and is better communicated with Veracruz than with Oaxaca. Reports about TB in Veracruz indicate that, together with Baja California, is the state with the highest prevalence of MDR-TB in the country (Juarez-Eusebio *et al*., 2017). So drug-resistant strains found in "Papaloapan" region may be associated with its geographical proximity to Veracruz. Furthermore, in the "Mixteca" region people prefer to use traditional medicine to treat diseases, in consequence they aren't exposed to drugs, which may not favour the development of drug-resistant strains.

There are some reports in which MDR-TB showed significant association with the EAI sub-lineage (Chen *et al*., 2017; Phyu *et al*., 2009). We observed that 45.5% of these strains were resistant to at least one first line drug: 9.1% monoresistant to RIF, 9.1% monoresistant to STR, 18.2% MDR-TB and 9.1% polyresistant to INH/STR.

It has been demonstrated that Mexican diabetic patients present 4.7-fold and 3.5-fold higher risk to develop drug-resistance and MDR-TB, respectively (Pérez-Navarro *et al*., 2015). We found that among DM patients, 35.71% showed resistance to at least one drug and 7.14% was MDR-TB. In fact, in our study the most frequent comorbidities associated with drug-resistance were malnutrition (40%) and HIV (40%). Regarding to HIV, there are reports of resistance to at least one drug that ranged from 10.6% in Tanzania (Kibiki *et al*., 2007), to 33.3% in Mexico City (Lopez-Alvarez *et al*., 2010), the latter more consistent with our results.

It is noteworthy that 16% of the analyzed isolates corresponded to NTM. This is an important percentage although lower than those previously reported for Mexico City (30.8%) (Hernández-Solís *et al*., 2017) and Northern India (27.4%) (Maurya *et al*., 2015). As in the above mentioned reports, we found *M. avium*, *M. fortuitum* and *M. intracellulare* as the most frequent infecting species. Now a days, AFB smear is still the preferred diagnosis method for TB, however it does not

provide accurate information about the infecting specie, consequently, patients receive TB drug treatment which is not effective in NTM infections. It is extremely important that public health official policies include molecular techniques as part of the initial diagnosis in TB suspicious cases, in order to differentiate between MTBc and NTM. This methods may also provide drug resistance information and give faster results than mycobacterial isolation by culture.

This is a first approach to the description of the lineages circulating in Oaxaca and its association with drug resistance and co-morbidities. It is necessary to increase the number of isolates to draw wider conclusions. Although by molecular methods the detection of members belonging to MTBc was significant, it was not possible to recover all AFB (+) strains in culture; this probably due to the grueling journey to reach some communities, climate conditions and time of delivery to the laboratory. All of this circumstances may have affected the viability of the bacillus. Improving sample transport conditions should be considered in future studies.

Conclusion

According to this study, ten different MTBc sub-lineages were identified in Oaxaca, being Haarlem and EAI the most prevalent and related to patients with DM, HIV and malnutrition.

Drug resistance, including MDR-TB cases, was observed in isolates belonging to these two lineages, while most isolates form the rest of the identified sub-lineages were mainly pan-sensitive to all first line drugs.

The high diversity of sub-lineages found in "Valles Centrales" could be associated to the location of the Capital City in this region which implies migration, tourism and regional trading.

The identification of highly pathogenic MTBc genotypes and NTM in Oaxaca reveals the significance of implementing strategic surveillance molecular systems in order to identify possible outbreaks which may impact public health management in the state of Oaxaca.

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Author's contribution

Nakamura-Lopez, Yuko: Designed and supervised the study and contributed to data analysis.

Valencia-Carmona, Oscar Daniel: Conducted MIRU-VNTR and LPS experiments

Martinez-Cruz, Perla Monica: Data analysis and manuscript writing.

Palma-Nicolas, Jose Prisco: Conducted drug resistance and spoligotyping analysis.

Gonzalez-y-Merchand, Jorge Alberto: Consultant for genotyping (MIRU-VNTR).

Rivera-Gutierrez, Sandra: Technical support in genotyping.

Muñiz-Salazar, Raquel: Molecular confirmation of drug resistance.

Martinez-Martinez, Lucia Lourdes: Provided laboratory facilities and contributed to data analysis.

Ethics

All individuals included in this study, signed a written informed consent and answered a questionnaire to obtain socio-demographic and clinical data. The Ethical Committee on Investigation of the Consejo Estatal para la Prevencion y Control del Sida, Oaxaca-Mexico, approved the protocol. As TB is a notifiable disease in Mexico, all confirmed TB cases were reported to the local public health authorities.

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