17

Study of Mycobacterium Tuberculosis by Molecular Methods in Northeast Mexico

H. W. Araujo-Torres[1,3], J. A. Narváez-Zapata[2], M. G. Castillo-Álvarez[1], MS. Puga-Hernández[4], J. Flores-Gracia[5] and M. A. Reyes-López[1,*]

[1]*Conservation Medicine Lab., Centro de Biotecnología Genómica del Instituto Politécnico Nacional, Cd. Reynosa, Tamps,*

[2]*Industrial Biotechnology Lab., Centro de Biotecnología Genómica del Instituto Politécnico Nacional, Cd. Reynosa, Tamps,*

[3]*Centro de Investigación en Ciencia Aplicada y Tecnología Avanzada del Instituto Politécnico Nacional, Altamira, Tamps,*

[4]*Laboratorio Estatal de Salud Pública de Tamaulipas, Cd. Victoria, Tamps,*

[5]*Instituto Tecnológico de Ciudad Victoria, Cd. Victoria, Tamps*

México

1. Introduction

One third of the world population is afected by TB and one million people did die this year 2011 in undeveloped countries (Venkatesh et al., 2011). In Tamaulipas, a Northern State of Mexico and a border state between USA and Mexico, frequency is 26.9 new TB cases per 100,000 people, twice of national rate of 12.85 cases per 100,000 people (Ferrer et al., 2010). Only on the border of Tamaulipas about 320 cases are diagnosed each year. Many of these cases correspond to people from other states of Mexico, probably by geographic position and by migration problematic of this study zone (Fitchett et al., 2011). Only 92% of the treated population are cured mainly because much of these people are poor and whose nutritional status directly affects the possibility of quick recovery (SSA, 2009).

The long presence of this disease has increased the need to know specifically which *Mycobacterium tuberculosis* strains are circulating in the region. Additionally, it is necessary to know the antibiotic/susceptibility profile of these strains since many of them acquire resistance against the traditional antibiotics along time.

In general, the diagnostic of this disease is traditionally conducted by using gold standard techniques focused to identify the presence of *M. tuberculosis* in clinical specimen of humans or cattle. These techniques included the strain of microorganism in Ziehl-Neelsen and culture in Lowenstein-Jensen medium (Cadmus et al., 2011), both regarded as reference techniques in the diagnosis of TB. Differentiation among mycobacteria of the *M. tuberculosis* complex (MTC) and other than MTC (NMTC) is accomplished by applying biochemical tests: niacin production, catalase activity, thermostable at 68 ° C and reduction of nitrate.

* Corresponding Author

Actually, detection for *M. tuberculosis* has been shorted due mainly to application of molecular methods directly to clinical samples. Usually, the detection of this bacterium takes 2 to 4 weeks (Marhöfer et al., 2011). Some molecular techniques are already on the market, being the most commonly used *AMPLICOR M. tuberculosis PCR test* (Roche), *M. tuberculosis* Direct test (MTDT) (GenProbe) and LCX *M. tuberculosis* assay (Abbott). In addition, PCR amplification of ribosomal sequences (Ribotyping) or amplification of repetitive intragenic consensus sequences (i.e. ERIC-PCR, spoligotyping, MIRU-VNTR), among others, are the usual methods used to specifically discriminate among different *M. tuberculosis* strains (Rodwell et al., 2010; Pang et al., 2011).

The use of molecular approach is also applied in the analysis of the antibiotic resistance of these isolates. In this sense, molecular detection of specific mutations in genes involved with drug resistance has successfully been applied in the identification of these (i.e. detection of mutations on *rpo*B, *kat*G, *mab*A, etc.) (Sala & Hartkoorn, 2011).

As an example of the conjunction of the background described above, this chapter briefly presents a work of potential tuberculosis patient samples, to which mycobacteria were isolated to determine whether any of them were resistant to some antibiotics and if it could be grouped by health districts of Tamaulipas and also grouped the isolates strains in MTC and NMTC, and identify them, potentially.

Therefore, the main aim of this study was determinated by using a molecular approach the specific *M. tuberculosis* strains presents in the region and identify specific mutations in these strains related with drug resistance. The information here generated helps to take epidemiological decisions aimed to control and to prevent this disease in the Northeast of Tamaulipas.

1.1 TB statistical

This main issue is due to produce a resistance against antibiotics used traditionally to control the disease. The first antibiotic against TB was created in the 40's decade. Consequently, the incidence of this disease declined in the following decades, especially in developed countries. However, in the last 20 years it has been observed an increase in the TB cases around of the world, particularly due to generation of new *M. tuberculosis* strains with resistance of the traditional antibiotics, or multidrug resistance (Yew et al., 2010). The death associated to TB may increase in undeveloped countries since some others diseases as the HIV may duplicate the death frequency in patients with both diseases (Havlir & Barnes 1999; Sonnenberg et al., 2005). Given this situation the TB was declared as global emergency by WHO (WHO 1993).

1.2 Situation of TB in northern Mexico

The Northeast state of Tamaulipas exhibits a high peak of occurrence of TB with regarding at Mexican rate and the frequency of this disease has remained stable during the last 10 years (Ferrer et al., 2010). Besides, this region is a natural corridor for exporting of cattle between Mexico and EE.UU. Therefore, both countries have commitment to keep safe their borders (Fitchett et al., 2011). Among the factors that may partially explain it, the high migrations rate reported in this region with people from other Mexican states (mainly

Veracruz, Coahuila, Mexico city, among others) and other Central America countries (i.e. Guatemala, Honduras, among others).

One of the reasons of this migration may be high number of manufacturing factories that offer a high number of jobs and that appeal and wait to travel. In addition, many of these people only remain of 4 to 5 years here and wait to travel to U.S.A. As it was previously mentioned, many of these people are poor and with low nutritional status and their lifestyle (i.e. drug or alcohol consumes) could prompted the TB disease (Wagner et al., 2011). Therefore, TB will become a big issue between Mexico and USA. From here, both countries have agreements on health and security cooperation. These agreements include the fast detection of this disease and the discrimination among *M. tuberculosis* strains (Fitchett et al, 2011).

1.3 Multidrug resistant in Mycobacterium tuberculosis

Recently, it has been reported an increase in the TB cases around of the world, particularly due to generation of new *M. tuberculosis* strains with resistance to traditional antibiotics, or multidrug resistance (Sougakoff, 2011). In 2007, the 14th edition of the Merck list shows 30 different anti-TB drugs, many analogues or prodrugs of antibiotics, as the first line of defense against this disease. In Mexico, the antibiotics most commonly used are the rifampicin (RIF), the isoniazid (INH), the pyrazinamide (PZA), the streptomycin (STR) and the ethambutol (EMB) (Borrell, Gagneux., 2011).

Worldwide, rifampicin is the drug mostly in the control of this bacterium (Connell et al., 2011). These antibiotics are not enough to halt the emergence and spread of multidrug resistant (MDR) strains causing a serious problem for the TB control and increasing public health problems (Zumi, et al. 2001). This have prompts the development of fast and reliable diagnostic process to detect, to discriminate, and to evaluate resistance of *M. tuberculosis* strains against main drugs.

1.4 Molecular approaches

Molecular biology has allowed detection of DNA or RNA sequence of different mycobacteria. An example of these approaches is using probes. Probes were prepared from nucleic acid sequences complementary to the DNA or RNA sequences from different species (including *M. tuberculosis, M. avium, M. kansasii, M. gordonae*.), which may be labeled with radioactive isotopes (hot probes) or chromogenic substances (cold probes). The gene probe is capable of binding or hybridizing with a homologous fragment of the study sample, which has been previously denatured by physical means. Hybridization of the probe to its complementary fragment is easily detected with addition of a marker. The main advantages of these techniques are fast and specific. Its disadvantages high cost and that many probes cannot identify species within the MTC.

Typing techniques based on amplification of nucleic acids by PCR provide a fast and reliable approach to obtain genetic information about bacteria or microorganism groups. Molecular typing methods for tuberculosis are based on that those infected by strains of *M. tuberculosis* have the same genotype (genetic fingerprinting) and are epidemiologically related, while those infected with different genotypes (unique patterns) are not.

Among the techniques of molecular biology that are currently used, as: ribotyping, the PCR amplification of repetitive extragenic palindromic sequences (REP-PCR) and the repetitive intragenic consensus sequences of *Enterobacteriaceae* (ERIC-PCR). These techniques can also be used in clinical studies to establish patterns of colonization and to identify sources of transmission of infectious microorganisms, which may contribute to a better understanding of the epidemiology and pathogenesis thereby helping to develop disease prevention strategies (Struelens, MESGEM, 1996).

1.5 Ribotyping

Ribotyping technique applied in the diagnostic of diseases has been used for differentiation of bacterial serotypes involved with the occurrence of outbreaks. Additionally, this technique has an extended use in the study of nosocomial fungus (Pavlic and Griffiths, 2009). Ribotyping is also used to study the ecology, the genotypic variation and the transmission of Streptococcus mutants from person to person (Alam et al., 1999). The patterns are simplified to ribotyping, making visible the DNA fragments containing parts or all of ribosomal genes, sometimes detected bacterial serotypes (Pavlic and Griffiths, 2009).

Because of the epidemiological and clinical importance of some bacterial strains such as *M. tuberculosis*, it is interesting the application of related techniques like typing by PCR, in breaking through in a better understanding of the ecology and epidemiology of these bacteria. Some studies show this approach to evaluate the discriminatory power of different methods for genotyping of MTC isolates, they compared the performance of i) IS6110 DNA fingerprint, ii) spoligotyping and iii) 24-loci MIRU-VNTR (mycobacterial interspersed repetitive units - variable number of tandem repeats) typing in a long term study on the epidemiology of tuberculosis (TB) in Schleswig-Holstein, the most-northern federal state of Germany (Roetzer et al, 2011), other group studied the clustered cases identified using a population-based universal molecular epidemiology strategy over a 5-year period. Clonal variants of the reference strain defining the cluster were found in 9 (12%) of the 74 clusters identified after the genotyping of 612 M. tuberculosis isolates by IS6110 restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive units-variable-number tandem repeat typing. Clusters with microevolution events were epidemiologically supported and involved 4 to 9 cases diagnosed over a 1- to 5-year period (Pérez-Lago et al, 2011), another study was to compare polymerase chain reaction (PCR)-based methods--spoligotyping and mycobacterial interspersed repetitive units (MIRU) typing--with the gold-standard IS6110 restriction fragment length polymorphism (RFLP) analysis in 101 isolates of Mycobacterium tuberculosis to determine the genetic diversity of M. tuberculosis clinical isolates from Delhi, North India (Varma-Basil et al 2011) and finally, a study where Forty three isoniazid (INH)-resistant *M. tuberculosis* isolates were characterized on the basis of the most common INH associated mutations, katG315 and mabA -15C→T, and phenotypic properties (i.e. MIC of INH, resistance associated pattern, and catalase activity). Typing for resistance mutations was performed by Multiplex Allele-Specific PCR and sequencing reaction (Soudani et al, 2011).

1.6 ERIC-PCR

Amplification of enterobacterial repetitive intergenic consensus by PCR (ERIC-PCR) has only been used sporadically to detect mycobacteria. ERIC sequences are repetitive elements

of 126 bp that appear to be restricted only to transcribed regions of chromosome. Its position in the genome appears to be different in different species. As any technique, ERIC-PCR is used as typing, to study the clonal relationship in various Gram-negative bacteria such as *Acinetobacter baumannii*. The DNA patterns obtained with the ERIC-PCR are usually less complex than those generated by other techniques such as REP-PCR. The technique is quick and easy to perform, and provides highly reproducible results (Gillings & Holley 1997.).

Additionally, the presence of ERIC sequences has been detected in genome of *M. tuberculosis* (Sechi et al, 1998). Studies showed that the level of differentiation obtained by ERIC-PCR is superior to that obtained by the RFLP-IS6110 genetic profile comparable to that obtained by (*GTG*) *5-PCR* fingerprinting (PCR-GTG). The use of the PCR-GTG, a repetitive marker in the *M. tuberculosis* chromosome with an IS6110 sequence has been successfully applied to a PCR-based fingerprinting method. This method is fast and sensitive and can be applied to the study of the epidemiology of infections caused by *M. tuberculosis* and therapeutic implications for health, particularly when the IS6110 RFLP-DNA profile does not provide any help.

2. Objective

The aim of this study was to conduct a molecular characterization of mycobacteria strains by typing and drug resistance gene mutations from samples of potential TB patients in Northeast Mexico.

3. Methods

Two strategies were conducted to study the samples isolated from patients with probable TB clinical diagnosis from the State Public Health Laboratory of Tamaulipas (LESPT) from The State of Tamaulipas, MX. The first one was the identification of strains as belonged or not to MTC. Second one was to detect mutations on the genes related to drug resistance to major antibiotics against *M. tuberculosis.*

3.1 Samples and cultures

Specimens included in this study were collected over a period of 16 months (October 2008 to January 2010) from acid-fast bacilli AFB-positive sputum obtained from the State Public Health Laboratory (LESPT). Basically, LESPT concentrates most of the TB cases from Tamaulipas. All the samples were taken under the informed consent of the patients. . In addition, a structured test was used to obtain standard demographic and epidemiologic data of the patients. Two sputum consecutive specimens were collected from each individual. These samples were mixed with 1% cetylpyridinium chloride and immediately transported to the LESPT where they were stored at 4o C (Kent and Kubica, 1985). All strains cultured were identified to species level by standard microbiological procedures in the LESPT.

3.2 DNA extraction

Samples were first lysed (tissue samples were mechanically disrupted) and proteins simultaneously denatured in the appropriate lysis buffer. QIAGEN Proteinase K was then

added and after a suitable incubation period, lysates were loaded onto the QIAGEN Genomic-tip. DNA binds to the column while other cell constituents passed through. Following a wash step to remove any remaining contaminants, pure, high-molecular-weight DNA was eluted and precipitated with isopropanol. Hands-on time for the complete procedure was just 45 minutes for samples.

Bacterial strains obtained from patients with TB were preliminary analyzed by an antibiogram test to verify if these strains exhibit some class of antibiotic resistant. Approximately, One hundred consecutive strains were selected to further molecular characterization. Bacteria selected were growth in solid Lowenstein-Jensen and 7H9 Middlebrook broths supplemented with 10% (vol/vol) of oleic acid-albumin-dextrose-catalase. After that, the samples were incubated for at least 8 weeks. DNA from bacterial samples was obtained from those grew strains by used the QIAGEN kit (QIAGEN) of according to manufacturing instructions

3.3 Molecular detection of *M. tuberculosis*

The following primers were used (Yeboah-Manu et al. 2001): spacer region-specific primers, spacer region 33 specific (5′ACACCGACATGACGGCGG3′) and spacer region 34 specific (5′CGACGGTGTGGGCGAGG3′); IS6110 (5′GGACAACGCCGAATTGCG′3 and 5′TAGGCGTCGGTGACAAAGGCCAC′3), and Mycobacterium genus-specific TB11 (sequence 5′ACCAACGATGGTGTGTCCAT3′) and TB12 (sequence 5′CTTGTCGAACCGCATACCCT3′). Expected PCR products are 550, 439, and 172 and 99 bp, respectively.

PCR mixtures contained 20 µl of 2× PCR mix, 10 µL of primer mix with each primer at 0.66 pmol/µL, 0.2 µL of Taq polymerase enzyme (Roche Diagnostics), and 10 µL of extracted DNA. The PCR conditions were 95°C for 3 min; 30 cycles of 95°C for 20 s, 65°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. After PCR, the products were analyzed by electrophoresis in agarose gel.

3.4 Typing methods

For ribotyping, the standardization of PCR was done using three sets of primers to amplify the 16S region (Table 1). Note: The primers 16S and 16S R F were used for amplification of 16S Mycobacterium.

Primer	Sequence	Size (nt)	Tm (°C)	Reference
R1 R2	5′-TTGTACACACCGCCCGTCA-3′ 5′-GAAACATCTAATACCT-3′	19 16	62.3 46.5	Sechi A, et al, 1998.
16S F 16S R	5′AGAGTTTGATCCTGGCTC-3′ 5′-CGGGAACGTATTCACCG-3′	18 17	57.62 59.61	Strom et al, 2002.
P13P F P11P R	5′-GAGGAAGGTGGGGATGACGT-3′ 5′-AGGCCCGGAACGTATTCAC-3′	20 19	64 60	Sorrell et al, 1996.

Table 1. Primers used for amplification of 16S Ribosomal DNA of Mycobacterium.

A set of chosen primers, which amplified for desired sequences are shown below (Table 1). (Strom et al, 2002).

Ribotyping by PCR was performed with two primers complementary to conserved regions. The sequences of the primers were described on Table 1. Amplifications were carried out in a final volume of 25 µl. Twenty five cycles of amplification were performed, with each cycle consisting of 2 min of denaturation at 94°C, 45 seconds of annealing at 62°C, and 1 min at 72°C. The last cycle consisted of a 7 min extension at 72°C. The amplification products were visualized after electrophoresis at 90 V for 90 min in a 2% agarose gel, and the gel was stained with SYBER Gold (Invitrogen).

3.5 ERIC-PCR

For ERIC-PCR, a pair of primers (Sechi et al, 1998) used and their characteristics are described below (Table 2).

Amplification reactions were performed in a volume of 50 µl with final amounts of 1 U of Taq polymerase, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 200 µM of deoxynucleoside triphosphate (Gibco, BRL, Life Technology, Paisley, United Kingdom). The reaction mixtures were then incubated for 5 min at 95°C, followed by 35 cycles of 94°C for 30 s, Touch-down (47-57°C), and 65°C for 4 min and a final extension at 70°C for 7 min. The amplification products were visualized after electrophoresis at 90 V for 90 min in a 2% agarose gel, and the gel was stained with SYBER Gold (Invitrogen).

Primer	Nucleotide sequence	Size (nt)	Tm(°C)
ERIC 1R	5′-ATGTAAGCT CCT GGGGATTCAC-3′	22	62.7
ERIC 2	5′-AAGTAAGTGACT GGGGTGAGCG-3′	22	64.5

Table 2. Primers for ERIC-PCR

3.6 Gene drug resistant analysis

Eight pairs of PCR primers (PR1 to PR16) were used to simultaneously amplify regions of eight genes associated with resistance to six antituberculosis drugs. In addition, eight pairs (PR17 to PR32) of internal PCR primers were then used to determine the DNA sequences of these genes (Table 3 and 4)

3.7 Sequencing

PCR products obtained from only 36 out of 100 bacterial strains for ERIC-PCR and 15 bacteria drug resistant were purified with an EXO-SAP. Components were supplemented with gold buffer (Applied Biosystem) and sequenced on an Applied Biosystem 310 Genetic analyzer (ABI Prism 310 Genetic analyzer), using big dye terminator cycle sequencing Ready Kit (Applied Biosystem).

For Drug resistant, the purified samples were analyzed with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The DNA sequences are collected and edited with Data Collection software version 1.01 and Sequencing Analysis version 3.7 (Applied Biosystems)

and compared with those of M. tuberculosis H37Rv (GenBank access no. NC_000962) with the program Geneious version 4.5.4 (Software Development Biomatters Ltd).

Additionally, for ribotyping, sequence of 16S Ribosomal DNA of mycobacterial determined in an ABI Prism® 3130. Obtained sequences were analyzed in NCBI database using BlastN analysis (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Alignment editing of 16S sequences was performed by Chromas Lite 2.0, BioEdit Sequence Alignment Editor Version 7.0.4.1 and CLC Sequence Viewer Version 6.1 software.

Finally, the comparison between isolated Mycobacteria and reported Mycobacterium tuberculosis strains were made by a microbial identification and phylogenetic analysis of obtained data using MEGA4 Software Version 4.0.2. Tree topologies were determined by methods of Minimum evolution criterion and Maximum Parsimony, with a value of reliability, "Bootstrap" of 100 replications for phylogenetic analysis.

4. Results and conclusion

Male population in Tamaulipas is the most affected by TB with 61% of the isolates evaluated in this work. Geographical distribution of infected people represented a greater proportion in Central and South of the State with 52% and 45% of isolates evaluated, respectively.

Region	Sequences	Position	Size (bp)
rpoB	PR1 (forward) 5-CCGCGATCAAGGAGTTCTTC-3 PR2 (reverse) 5-ACACGATCTCGTCGCTAACC-3	1256–1275 1570–1551	315
katG	PR3 (forward) 5-GTGCCCGAGCAACACCCACCCATTACAGAAAC -3 PR4 (reverse) 5-TCAGCGCACGTCGAACCTGTCGAG-3	1–32 223–2200	2,223
mabA	PR5 (forward) 5-ACATACCTGCTGCGCAATTC-3 PR6 (reverse) 5-GCATACGAATACGCCGAGAT-3	-217 a -198 1145–1126	1,362
embB	PR7 (forward) 5-CCGACCACGCTGAAACTGCTGGCGAT-3 PR8 (reverse) 5-GCCTGGTGCATACCGAGCAGCATAG-3	640–665 3387–3303	2,748
pncA	PR9 (forward) 5-GGCGTCATGGACCCTATATC-3 PR10 (reverse) 5-CAACAGTTCATCCCGGTTC-3	-80 a -61 590–572	670
rpsL	PR11 (forward) 5-CCAACCATCCAGCAGCTGGT-3 PR12 (reverse) 5-GTCGAGAGCCCGCTTGAGGG-3	4–23 575–556	572
rrs	PR13 (forward) 5-AAACCTCTTTCACCATCGAC-3 PR14 (reverse) 5-GTATCCATTGATGCTCGCAA-3	428–447 1756–1737	1,329
gyrA	PR15 (forward) 5-GATGACAGACACGACGTTGC-3 PR16 (reverse) 5-GGGCTTCGGTGTACCTCAT-3	1–19 397–379	398

Table 3. Primers for multiplex-PCR (Sekiguchi et al. 2007)

Thirty-seven out of 40 samples were analyzed by 16S gene sequences, 34 of them were grouped in the MTC, and the 3 remaining sequences were integrated into the NMTC (Figure 1). Moreover, from 37 sequences analyzed, only 12 of these showed polymorphisms on a

segment of 250 nucleotides with an average size of 750 nucleotides for each sequence. Of these 12 sequences, only 3 isolates were grouped in NMTC, the 9 remaining isolated strains show polymorphism in their nucleotide sequences belong to the MTC. Two sequences of isolates tested showed 100% and 98% identity respectively with the species of *M. fortuitum* according to our analysis in the NCBI database (strain *M. fortuitum* 16S gene with accession number DQ973806.1 and strain *M. fortuitum* 16S gene with accession number AY457066.1, respectively).

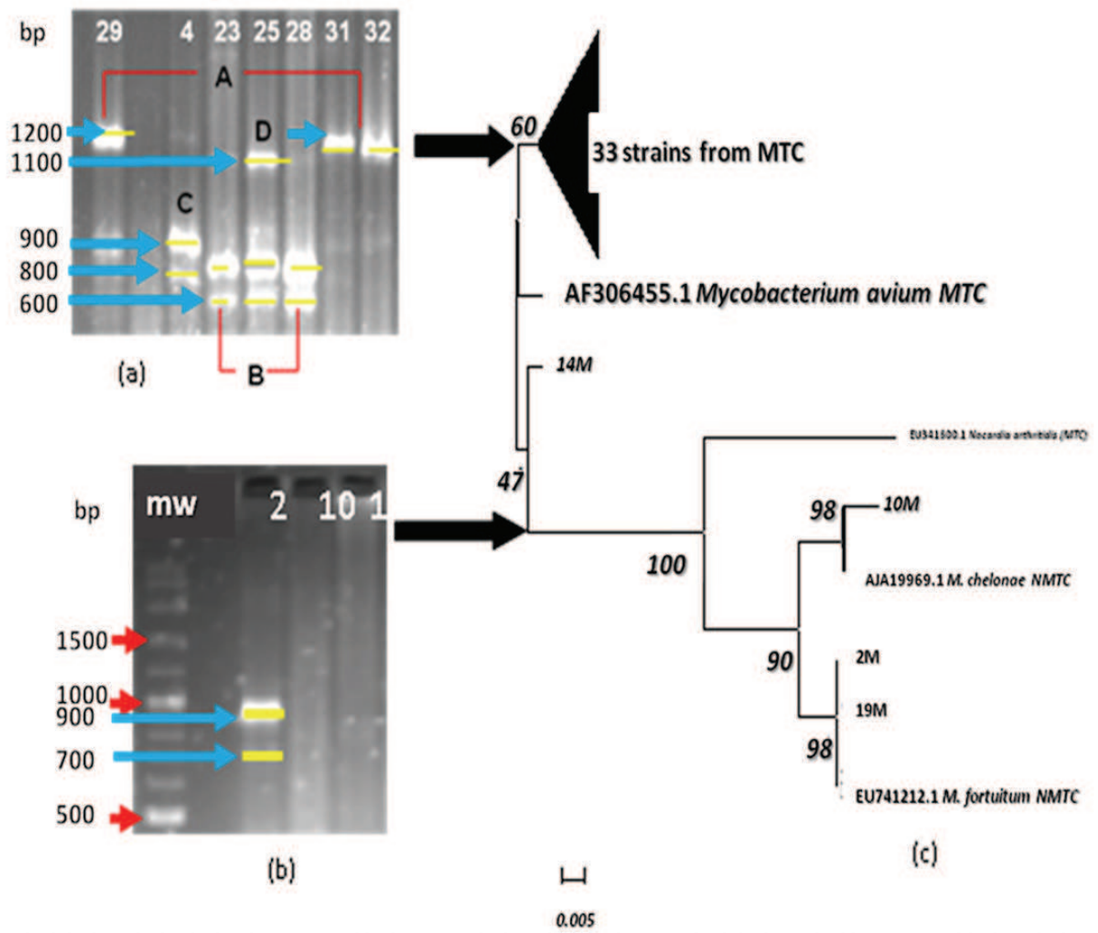


Fig. 1. Comparison of genetic profiles from isolated mycobacteria by ERIC-PCR VS Ribotyping (a) In picture, letters A, B, C, and D show four different genetic profiles of ERIC-PCR grouped in 7 isolates of mycobacteria. Arrow on direction of figure (c) indicates 7 isolated strains are part of the MTC. (b) Image shows ERIC-PCR amplifications of 3 isolates (samples 2M, 10M, 19M) clustered in NMTC. (c) Phylogenetic tree based on comparison of 16S gene mycobacterium species from MTC and NMTC. *Mycobacterium spp* sample.

A third sequence of one isolated strain showed 100% identity with *M. chelonae* (*M. chelonae* strain T9 with AM884324.1 access number). In this sense, it is useful to mention that LESPT identified these strains as *M. tuberculosis* based only on their microbiological and biochemical results. It is important to mention that LESPT does not conducted molecular analysis to identify their samples.

One out of the two identified strains was *M. fortuitum*, made by sequencing, but no for microbiology, since this was identified as *M. tuberculosis* by LESPT. The other isolated itself coincided with both techniques.This gives us a different result, although microbiology diagnostic and taken at this time, we do believe strongly that this is due to *M. fortuitum.*

Gene	Sequences	Position
rpoB	PR17 5-TACGGCGTTTCGATGAAC-3 (complementary strand)	1529–1512
katG	PR18 5-ACGTAGATCAGCCCCATCTG-3 (complementary strand)	689–670
	PR19 5-GAGCCCGATGAGGTCTATTG-3	574–593
	PR20 5-CCGATCTATGAGCGGATCAC-3	1162–1181
	PR21 5-GAACAAACCGACGTGGAATC-3	1729–1748
mabA	PR22 5-ACATACCTGCTGCGCAATTC-3	-217 a -198
embB	PR23 5-ACGCTGAAACTGCTGGCGAT-3	646–665
	PR24 5-GTCATCCTGACCGTGGTGTT-3	1462–1481
	PR25 5-GGTGGGCAGGATGAGGTAGT-3 (complementary strand)	1596–1577
	PR26 5-CACAATCTTTTTCGCCCTGT-3	2007–2026
	PR27 5-GCGTGGTATCTCCTGCCTAAG-3	2581–2601
pncA	PR28 5-GGCGTCATGGACCCTATATC-3	-80 -61
rpsL	PR29 5-CCAACCATCCAGCAGCTGGT-3	4–23
Rrs	PR30 5-CAGGTAAGGTTCTTCGCGTTG-3 (complementary strand)	979–959
	PR31 5-GTTCGGATCGGGGTCTGCAA-3	1291–1310
gyrA	PR32 5-GATGACAGACACGACGTTGC-3	1–19

Table 4. Primers for sequencing (Sekiguchi et al. 2007)

The use of the reference strain H37Rv of *M. tuberculosis* sequence served as a reference or guide for clustering of the isolates studied, since in the phylogenetic analysis, the type strain H37Rv was integrated with the 34 isolated MTC, thus reaffirming the phylogenetic relationship of isolates tested with the species *M. tuberculosis* (Figure 1). On the other hand, the reference sequence of *Nocardia arthritidis* (No. Access EU841600) showed no relation with the 37 evaluated strains in phylogenetic analysis, being totally separated from the two complexes formed, MTC and NMTC. This comparison is done, because of *Nocardia* is also acid-resistant and can be confused with *Mycobacteria* on microscopic analysis, hence the importance of making the comparison. Then, It should be mentioned that the identification of mycobacteria isolated from the 16S sequences was proved to be an appropriate strategy to establish the level of genetic relatedness among isolates studied and know how related isolates were isolated or if these could be separated into MTC and NMTC.

ERIC-PCR technique gave 4 different genetic profiles for mycobacteria (Figure 1). It should be emphasized that three of these genetic profiles are consistent with those reported in molecular epidemiology studies by amplifying sequences ERIC (Sechi et al, 1998). From 34 isolates clustered in the complex of M. tuberculosis, seven of these 4 make up the genetic profiles obtained by ERIC-PCR. Then they expect the rest of the isolates (27 isolates) that make up this complex terms grouped in the 4 genetic profiles (A, B, C and D) obtained by ERIC-PCR.

However, one aspect to consider in the results obtained in this work is that of obtaining the 4 different genetic profiles amplified by ERIC-PCR, they did not allow to discriminate among species of MTC and NMTC as it was expected, those profiles or genetic patterns of 3 isolated strains have been totally different from other profiles of 7 isolated strains (Figure 1), all of 10 isolated strains are in MTC.

Regarding to genetic relatedness of 40 isolates of mycobacteria studied, phylogenetic analysis of 16S gene sequences showed 37 sequences, which formed two groups. The first group of MTC consisting of 34 isolates and the second group resulted in NMTC consisting of 3 isolates. The percentages of identity were from 98% to 100% for isolates clustered in both complexes.

The analysis on relationship between the isolates studied and their geographical origin revealed that the Mycobacterium tuberculosis complex is distributed both in the central and south of the state of Tamaulipas, MX. Meanwhile, species such as *M. fortuitum* and *M. chelonae* are only found circulating in the central region of Tamaulipas.

It should be noted that the isolates studied are only samples originating from the central and southern Tamaulipas. No isolated strains were obtained from northern part of Tamaulipas, which would have complemented the results of this research. For example, as mentioned before in those border states (US-Mexico border) there is a great number of people and cattle to move from different parts of country and abroad, which could suggest existence of different strains of *M. tuberculosis* in Tamaulipas, and even the presence of other species found in the central and southern Tamaulipas, MX, allowing us to know not only that isolated strains in each region are preferably, but also known how those isolated strains are circulating all around Tamaulipas.

Note this work was limited to samples of LESPT; however the proposal will be to analyze samples from all health districts in Tamaulipas, and analyze samples of other states, such as Veracruz and/or Coahuila. Additionally, we will seek to refine the ERIC-PCR and implementing the MIRU-VNTR and spoligotyping for more complete diagnosis. In addition, arrangements are made between the County LESPT and McAllen, Texas, United States, to have samples (about 14,000 isolates) identified and stored in the United States from Tamaulipas.

Finally, the preliminary results were shown (Table 5), where mutations, insertions, transversions, and transitions were found. In general, the mutations obtained did not alter the chemical or structural composition of proteins that confer resistance to an antibiotic to the mycobacteria and their regions sequenced. In these particular cases, we selected to work with isolated strains were resistant to antibiotics commonly administered in Mexico, the results obtained for the case of pyrazinamide, a silent mutation was found, so that the resistance exhibited by the bacteria should be caused by mutations on the sequenced region. In the case of isoniazid *mab*A gene, we found an insertion within the gene that could be the cause of resistance exhibited.

These results indicate that DNA sequencing-based method was effective for detection of MDR strains. However, when novel mutations in drug resistance-related genes are detected by the method, it is essential to also perform drug susceptibility testing, because novel mutation may not be associated with drug resistance.

Gene	Number of sample	Changes	
		Nucleotide	Protein
*rpo*B	2	CGG-TGG	R476W
rrs	2	TGG-AGG	W193R
*rps*L	2	AAA-AAG	K121K
*pnc*A	2	GGT-GGC	G75G
*mab*A	2	702 T insertion -15 C-T upstream	
*gyr*A	2	GAG-CAG	E21Q

Table 5. Relationship of changes found in the sequences of the genes of interest.

5. Conclusion

In conclusion, two strategies were carried out to study the samples isolated from patients with TB diagnostic of LESPT from Tamaulipas, MX. The first was the identification of isolates and determine if these isolates belonged or not to MTC. Second, to determinate if mutations in primary sequences of genes related to resistance to major antibiotics used to kill mycobacteria in Tamaulipas, could be detected.

For the first part of the study, there were used 3 strategies, a multiplex-PCR, ERIC-PCR, and ribotyping. For the second direct amplification of 16S DNA region was performed.

Multiplex-PCR for 99% of the samples coincided with the microbiological results, identifying *M. tuberculosis*, primary. In the case of ERIC-PCR, the samples could be grouped into 4 different groups; however it could differentiate between MTC and NMTC. Finally, ribotyping produced promising results by discriminating the isolated strains and identifying 99% as *M. tuberculosis*.

Finally, the results indicate that DNA sequencing-based method was effective for detection of MDR strains. However, when novel mutations in drug resistance-related genes are detected by the method, it is essential to also perform drug susceptibility testing, because novel mutations are not always associated with drug resistance.

6. Perspectives

This kind of work will answer other questions: is it necessary ribotyping before or after ERIC-PCR or multiplex-PCR and it is important to recognize each species of Mycobacteria to understand if TB strains would circulate all around Tamaulipas and if those ones would be or get in USA too? In a few years we will understand this phenomenon; meanwhile this chapter makes the first approach to understand how TB strains are moving and if those strains are or not drug resistant on a border State between USA and Mexico. The present investigation continues, pending to sequence regions of resistance to pyrazinamide and ethambutol, which are largest genes.

7. Acknowledgments

Financing of this research was by Instituto Politécnico Nacional (projects SIP 20090679 and SIP 20100504). Narváez-Zapata J. A. and Reyes-López M. A. are fellows of CONACYT-SNI,

COFAA, and EDI from Instituto Politécnico Nacional. Authors are also grateful for the support received from Network of Drug Development and Diagnostic Methods (RED FARMED) from CONACyT. Finally, authors really appreciated the samples submitted for QFB. Norma Alicia Villareal Reyes from LESPT.

8. References

Alam S., Brailsford S., Whiley R. & Beighton D. (1999). PCR-Based Methods for Genotyping Viridans Group Streptococci. Journal of Clinical Microbiology. Vol. 37, No. 9. p. 2772-2776.

Borrell S, Gagneux S. (2011). Strain diversity, epistasis and the evolution of drug resistance in Mycobacterium tuberculosis. Clin Microbiol Infect. Jun;17(6):815-20

Cadmus SI, Falodun OI, Fagade OE. (2011). Methods of sputum decontamination with emphasis on local tuberculosis laboratories. Afr J Med Med Sci. 40(1):5-14

Connell DW, Berry M, Cooke G, Kon OM. (2011).Update on tuberculosis: TB in the early 21st century. Eur Respir Rev. 20(120):71-84

Ferrer G, Acuna-Villaorduna C, Escobedo M, Vlasich E, Rivera M. (2010). Outcomes of multidrug-resistant tuberculosis among binational cases in El Paso, Texas. Am J Trop Med Hyg. 83(5):1056-8

Fitchett JR, Vallecillo AJ, Espitia C. (2011). Tuberculosis transmission across the United States-Mexico border. Rev Panam Salud Publica. 29(1):57-60

Gillings, M. and Holley, M. (1997). Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. The Society for Applied Bacteriology, Letters in Applied Microbiology. No. 25. p. 17-21.

Havlir, D.V. and Barnes, P.F. (1999) Tuberculosis in patients with human immunodeficiency virus infection. N. Engl. J. Med. 340, 367–373

Kent, P. T., and G. P. Kubica. (1985). Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

Marhöfer, RJ, Oellien F, Selzer PM. (2011). Drug discovery and the use of computational approaches for infectious diseases. Future Med Chem. 3(8):1011-25

Pang Y, Zhou Y, Wang S, Lu J, Lu B, He G, Wang L, Zhao Y. (2011). A novel method based on high resolution melting (HRM) analysis for MIRU-VNTR genotyping of Mycobacterium tuberculosis. J Microbiol Methods. 86(3):291-7

Pavlic M, Griffiths MW. (2009). Principles, applications, and limitations of automated ribotyping as a rapid method in food safety. Foodborne Pathog Dis. 6(9):1047-55

Pérez-Lago L, Herranz M, Martínez Lirola M; on behalf of the INDAL-TB Group, Bouza E, García de Viedma D. 2011. Characterization of Microevolution Events in Mycobacterium tuberculosis Strains Involved in Recent Transmission Clusters. J Clin Microbiol. 49(11):3771-3776.),

Rodwell TC, Kapasi AJ, Moore M, Milian-Suazo F, Harris B, Guerrero LP, Moser K, Strathdee SA, Garfein RS. (2010). Tracing the origins of Mycobacterium bovis tuberculosis in humans in the USA to cattle in Mexico using spoligotyping. Int J Infect Dis.;14 Suppl 3:e129-35

Roetzer A, Schuback S, Diel R, Gasau F, Ubben T, di Nauta A, Richter E, Rüsch-Gerdes S, Niemann S. 2011.Evaluation of Mycobacterium tuberculosis typing methods in a

four-year study in Schleswig-Holstein, Northern Germany. J Clin Microbiol. Oct 12 [Epub ahead of print])

Sala C, Hartkoorn RC. (2011). Tuberculosis drugs: new candidates and how to find more. Future Microbiol. 6(6):617-33

Sechi A., Zanetti S., Dupré I., Delogu G. and Fadda G. (1998). Enterobacterial Repetitive Consensus Sequences as Molecular Targets for Typing of Mycobacterium tuberculosis Strains. Journal of Clinical Microbiology. Vol. 3, No. 1. p. 128-132.

Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopeć E, Zwolska Z, Kirikae F, Toyota E, Kobayashi I, Morita K, Kudo K, Kato S, Kuratsuji T, Mori T, Kirikae T. (2007). Detection of multidrug resistance in Mycobacterium tuberculosis. J Clin Microbiol. 45(1):179-92.

Sonnenberg, P. et al. (2005) How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. J. Infect. Dis. 191, 150–158

Sorrell TC, Chen SC, Ruma P, Meyer W, Pfeiffer TJ, Ellis DH, Brownlee AG. (1996). Concordance of clinical and environmental isolates of Cryptococcus neoformans var. gattii by random amplification of polymorphic DNA analysis and PCR fingerprinting. J Clin Microbiol. 34(5):1253-60.

Soudani A, Hadjfredj S, Zribi M, Messaadi F, Messaoud T, Masmoudi A, Zribi M, Fendri C. Genotypic and phenotypic characteristics of tunisian isoniazid-resistant Mycobacterium tuberculosis strains. 2011. J Microbiol. 49(3):413-7).

Sougakoff W. (2011). Molecular epidemiology of multidrug-resistant strains of Mycobacterium tuberculosis. Clin Microbiol Infect. 17(6):800-5

SSA. (2009). Secretaría de Salud. Informe México 2009.

Ström K, Sjögren J, Broberg A, Schnürer J. (2002). Lactobacillus plantarum MiLAB 393 Produces the Antifungal Cyclic Dipeptides Cyclo(L-Phe-L-Pro) and Cyclo(L-Phe-trans-4-OH-L-Pro) and 3-Phenyllactic Acid. Appl Environ Microbiol. 68(9):4322-7.

Struelens, J., and Members of the European Study Group on Epidemiological Markers (MESGEM). (1996). Consensus guidelines for Appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiology and Infect, 2 (1): 2-11.

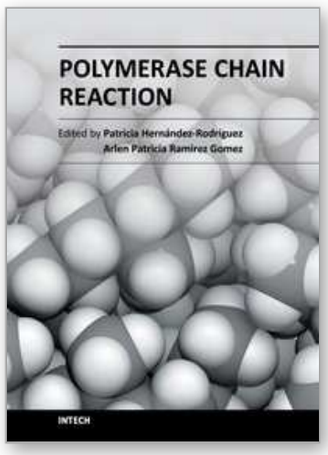
Varma-Basil M, Kumar S, Arora J, Angrup A, Zozio T, Banavaliker JN, Singh UB, Rastogi N, Bose M. 2011. Comparison of spoligotyping, mycobacterial interspersed repetitive units typing and IS6110-RFLP in a study of genotypic diversity of Mycobacterium tuberculosis in Delhi, North India. Mem Inst Oswaldo Cruz. 106(5):524-35)

Venkatesh KK, Swaminathan S, Andrews JR, Mayer KH. (2011). Tuberculosis and HIV co-infection: screening and treatment strategies. Drugs. 71(9):1133-52

Wagner KD, Pollini RA, Patterson TL, Lozada R, Ojeda VD, Brouwer KC, Vera A, Volkmann TA, Strathdee SA. (2011). Cross-border drug injection relationships among injection drug users in Tijuana, Mexico. Drug Alcohol Depend. 113(2-3):236-41

Yeboah-Manu D, Yates MD, Wilson SM. (2001). Application of a simple multiplex PCR to aid in routine work of the mycobacterium reference laboratory. J Clin Microbiol. 39(11):4166-8.

Yew WW, Sotgiu G, Migliori GB. (2010). Update in tuberculosis and nontuberculous mycobacterial disease Am J Respir Crit Care Med. 2011 Jul 15;184(2):180-5



Polymerase Chain Reaction

Edited by Dr Patricia Hernandez-Rodriguez

ISBN 978-953-51-0612-8

Hard cover, 566 pages

Publisher InTech

Published online 30, May, 2012

Published in print edition May, 2012

This book is intended to present current concepts in molecular biology with the emphasis on the application to animal, plant and human pathology, in various aspects such as etiology, diagnosis, prognosis, treatment and prevention of diseases as well as the use of these methodologies in understanding the pathophysiology of various diseases that affect living beings.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen