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Molecular Epidemiology of Tuberculosis

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http://dx.doi.org/10.5772/59751

1. Introduction

Tuberculosis (TB) is one of the infections with major impact worldwide and it is produced by members of the *Mycobacterium tuberculosis* complex, this bacteria mainly affects the lungs. The TB is considered the second cause of death for an infectious disease after of the Human Immunodeficiency Virus(HIV)in the world [1]. It is one of the oldest diseases and has accompanied mankind during its evolution. Evidence of the etiologic agent for the disease was found in skeletons 4000 years old in Europe and the Middle East [2].According to the Global Tuberculosis Report 2013 of the World Health Organization, in the 2012, an estimated 8.6 million new cases of diseased and 1.3 million deaths from this cause occurred. This data shows that despite the many efforts of government organizations to control the disease, it remains an important public health public worldwide.

In the last years the biology molecular methodologies have led many advances which allow the analyses of genetic material of different microorganisms such as *M. tuberculosis*, with the analysis of the Insertion element IS6110, the polymorphism within the direct repeat (DR) locus and analysis using mycobacterial interspersed repetitive units, this methodologies allow the molecular typing of the mycobacteria and the knowledge of the different lineages of *M. tuberculosis*, contributing to the development of the molecular epidemiology and the control politics of the disease in the different cities and countries.

2. General aspects of disease and etiologic agent

2.1. Epidemiology

TB is a global disease. In 2012, the World Health Organization(WHO) estimated in its annual report about 8.6 million new cases of the disease (100,000 less than in 2011) which represents

an incidence rate of 122 cases per 100,000 inhabitants in average worldwide: 5.7 million cases were diagnosed as new cases, and it is estimated that 1.1 million of the total cases showed co-infection infection with HIV; and 450,000 cases of multi-drug-resistant tuberculosis (MDR-TB), being South East Asia, Africa, and the Pacific region the most affected areas. Additionally, an estimate of 1.3 million deaths were caused by the disease, and despite of noticing a reduction in the mortality rate, the increase of deaths in populations such as children and women is remarkable.

On the other hand, approximately 2.9 million cases were lost, that is to say, they were not diagnosed or they were not notified to the national tuberculosis programmes. Regarding treatment, 56 million people were successfully treated from 1995 to 2012, saving 22 million lives among the countries which adopted the Directly Observed Treatment, Short-course (DOTS) strategy, proposed by the WHO.

According to the WHO report, the Americas and the Western Pacific regions are the only ones, out of the six regions, which have achieved goals with regards to the decrease of mortality, prevalence, and incidence by the year 2015.Prevalence of the disease was approximately 31 – 41 cases and incidence was 27 – 31 cases per 100,000 inhabitants in the Americas. Out of the 280,000 incident cases, about 31,000 showed co-infection with HIV; however only 219,349 cases were reported. Mortality of the disease reached a rate of 1.9 deaths per 100,000 inhabitants, that is to say 20,000 deaths among people suffering the disease.

In Colombia, 11990 confirmed cases of tuberculosis in all of its forms were confirmed for the same period. Out of these, 10956 corresponded to new cases for an incidence of 23.5 cases per 100,000 inhabitants. According to the clinical presentation of the disease, 80.4% of cases were pulmonary tuberculosis and 19.6% were extra pulmonary TB [3].

The departments with the largest number of cases in Colombia correspond to Antioquia, Cundinamarca concentrating its high number of cases in the city of Bogota, Valle del Cauca, Atlántico, Risaralda, and Santander.

2.2. Clinical forms

2.2.1. Pulmonary tuberculosis

It is the most common type since it is the manner in which the disease can be spread to other persons. The disease is directly transmitted from person to person; when a diseased person coughs, sneezes, or spits, bacilli are expelled to the air and they will be inhaled by people around the patient [4].

It is estimated that one third of the world population has latent TB; that is to say, they are infected by the bacillus but they have not gotten sick, nor they can spread the infection. People infected with the tuberculosis bacillus have a 10% risk of getting TB throughout their lives. However, this risk is higher for people who have a deficiency or compromise of their immune system, as in cases of infection with the human immunodeficiency virus (HIV), malnutrition, diabetes, or tobacco users [5].

When the disease appears, the symptoms (coughing, fever, night sweats, and weight loss, among others) may be mild for several months. As a result, patients delay seeking medical attention and they transmit the bacteria to other people. During one year, a tuberculosis diseased may infect approximately 10 to 15 people by close contact. Up to two thirds of tuberculosis patients die if they do not receive appropriate treatment [6].

2.2.2. Extra pulmonary tuberculosis

Even though it represents only a small proportion of TB cases, approximately 10 – 15%, patients develop the most severe forms of the disease. Since the disease may affect any organ, the diagnosis is often more difficult and delayed [7]. Populations most affected by extra pulmonary TB are children and persons with immunosuppression, such as those who have leukemia, diabetes and living with HIV.

In accordance with the WHO criteria, the forms of extra pulmonary TB are classified into severe and less severe. Meningitis, milliary, pericarditis, peritonitis, extensive or bilateral pleural, intestinal, spinal, genital urinary are considered to be severe forms. TB of lymph nodes, unilateral pleural, bones (except for the spine), and skin are considered to be the less severe [8].

2.3. Etiologic agent

The *Mycobacterium* genus is taxonomically located in the *Mycobacteriacea* family (table 1) and comprises 150 species [9], among which there are species of the *M. tuberculosis* complex, made up of *M. tuberculosis, M. bovis, M bovis BCG, M. africanum, M microtti, M caprae, M. pinipedii, M. canetti, M. mungi, M. orygis* [10].

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	Mycobacterium

Table 1. Taxonomic classification

2.3.1. Microscopic characteristics

The dimensions of the bacillus are approximately 1-10 µm long (usually 3-5 µm) and 0.2 – 0.6µm wide (Figure 1) [11]. It has a complex cellular envelope composed of a cellular membrane and cellular wall; the latter provides mechanical support for the bacteria and gives its characteristic in acid-fast staining due to the large content of lipids [12]. The morphologic feature of

presence of cord in the Zielh Neelsen staining is presumptive of mycobacteria of the *M. tuberculosis* complex; formation of the cord is attributed to the trehalose glycolipid 6, 6-dimycolate or cord factor composed of mycolic acids molecules [13].
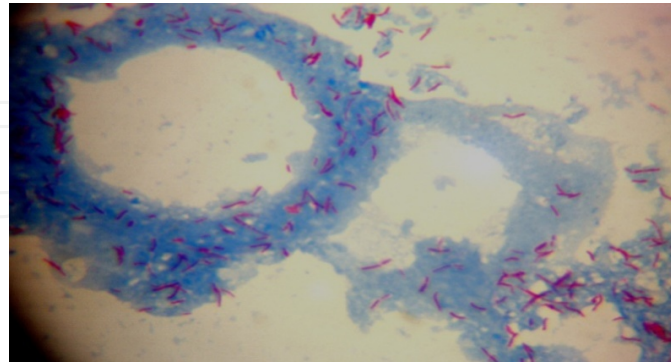

Figure 1. Acid-fast bacilli in a bacilloscopy.

2.3.2. Morphologic characteristics in culture

M. tuberculosis is characterized by having slow growth in synthetic media since its generation time ranges from 18 to 24 hours. Low growth rate is associated to the wall permeability which limits the entry of nutrients [14] and additionally, it has been observed that one or two operons rm are found in the mycobacteria managed by the P1 and P2 promoters. The rmA operon located in the *mur*A gene is found in all of the species, including *M. tuberculosis* and *M. leprae.* Nevertheless, in some species such as the fast-growth ones, an additional *rm*B operon is found, located in the *tyr*S gene [15, 16]

Synthetic media use for the isolation of *M. tuberculosis* contain nutrients such as glycerol, carbon source, asparagine, and ammonia ions as source of nitrogen and micronutrients besides albumin incorporated in the culture media with the aggregate of hen eggs or bovine seroal-bumin. Colonies characteristic of *M. tuberculosis* developed in a solid medium based on eggs are often rough, without pigment, and dry (Figure 2) [17]

Figure 2. *M. tuberculosis* culture

3. Genome of *Mycobacterium tuberculosis*

With the complete genome sequencing of *M. tuberculosis*, H37Rv strain, more genetic information of the bacterium was generated. In total, a sequence of 4,411,539 pairs of bases which contain about 4000 genes and a high content of guanine + cytosine (G+C) of 65.6%. The point for the beginning of numbering was the initiation codon for the dnaA gene, a marker for the origin of replication oriC. The G+C content is relatively constant and several regions showing larger content of G+C were detected, corresponding to sequences that belong to a large family of genes which include the polymorphic GC-rich repetitive sequence (PGRS) [18].

Genome is rich in repetitive DNA, particularly in insertion sequences and in new multigene families; an example of these is the presence and distribution of insertion sequences (IS). IS6110, a sequence of the IS3 family, is of particular interest. It is widely used for typing of strains and molecular epidemiology due to its variation in the insertion site and the number of copies [19]. In the H37Rv *M. tuberculosis* genome, sixteen copies of IS6110 were identified; some sites of these copies were grouped in sites with the insertion name of hot spots[20].

Recently another repetitive region highly preserved within the chromosome of *M. tuberculosis* was described: the DR locus [21] [22], which is a member of the Clustered Regularly Interspersed Palindromic Repeats – CRISP sequences [23]. After the discovery of the DR region, the variable number of tandem repetitions (VNTR) was found [24], subsequently, the mycobacterial intergenic repetition units (MIRU) were identified [25] that are also listed as VNTR multiple locus analysis. Multilocus sequences typing (MLST) was introduced as an alternative method [26]; and recently, the single nucleotide polymorphism (SNP) was described [27] followed by large sequence polymorphism (LSP). The latter is carried out either by micro arrangements or polymerase chain reaction (PCR) in real time [28]

4. Molecular markers of *M. tuberculosis* genotyping

4.1. IS6110

Insertion element found within species of the *M. tuberculosis* complex, and was shown to be related to the IS3 family of insertion sequences which were discovered in members of the family Enterobacteriaceae [29].

IS6110 is 1,361 bp long and contains 28-bp, imperfect inverted repeats at its extremities with three mismatches and 3-bp direct repeats that probably result from repetition of the target sequence [30] are present in different copy numbers and are integrated at various chromosomal site [31]. The number of IS6110 copies present in the genome is species- and strain-dependent. Most strains of *M. tuberculosis* carry between eight to 15 copies in different positions of the genome although single copy strains are common. [32]The polymorphism of restriction fragments generated by breaking the IS6110 fragment with the PvuII enzyme, has been used as a method for genotyping of *M. tuberculosis* complex species

4.2. IS1081, direct repeat and major polymorphic tandem repeat

It is a 1324-bp insertion sequence found in *M. tuberculosis* complex. It has a lower degree of polymorphism than IS6110 because of its low transpositional activity [33, 34]

4.3. Polymorphic GC-rich repetitive sequence

It has numerous copies [35- 37] and consists of many tandem repeats of a 96 bp GC rich consensus sequence. PGRS elements are present in 26 sites of *M. tuberculosis* chromosomes [38] and have been detected in mycobacteria not belonging to the *M. tuberculosis* complex [39].

4.4. DR locus

It contains multiple repeated DR regions of 36 pb, interspersed with non-repetitive spacer sequences of 34 to 41 pb which constitute DVR (Figure 3); the size of the DR locus varies from 6 DVR (06 kb) to 56 DVR (6 KB), and both the DR region and the spacers have shown little variation in the order of presentation among strains.

The DR region has been identified as an integration hot-spot of the IS6110 insertion element [40].
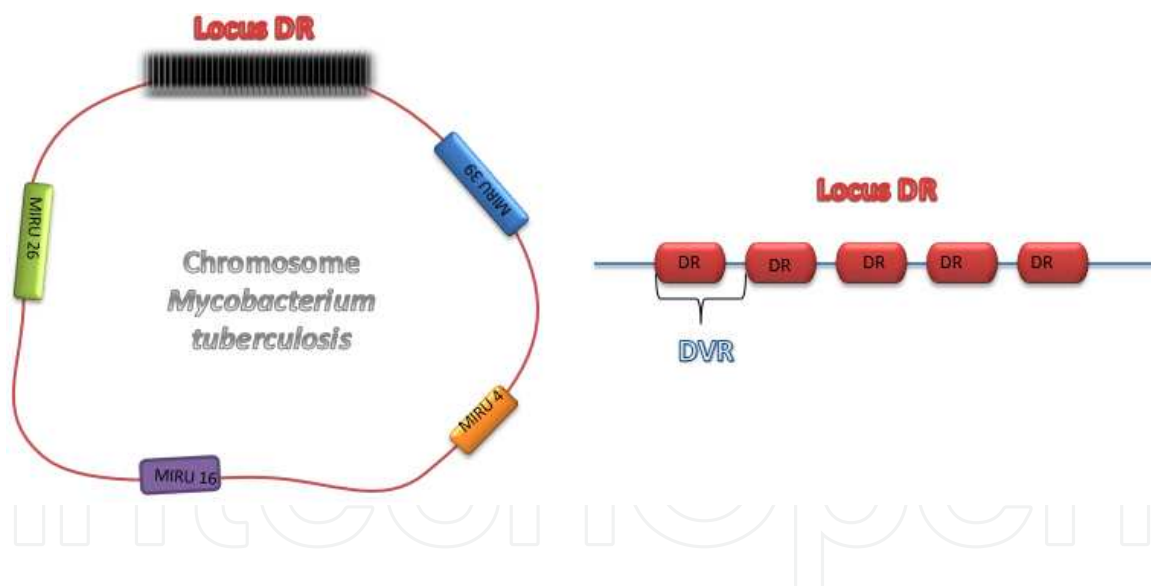

Figure 3. DR locus

Deletion of one or more DVR [41] and duplication of DVR are the mechanisms which generate the variation among different strains. These deletions and duplications are likely to be mediated by the homologous recombination among neighboring or distant DRs, or by Splicing processes during DNA replication [42]. Nevertheless, the process in which the deletion of a DR occurs due to transposition of the IS6110 has also been described [43]. All of these changes generated in the DR locus are the foundation for the development of the Spoligotyping methodology [44].

4.5. VNTR

DNA segments containing "tandem repeated" sequences in which the number of copies of the repeated sequence varies among strains. Variable Number Tandem Repeat (VNTR) sequences have emerged as valuable markers for genotyping. MIRUs are a class oftandem repeated sequences. There are a total of 41 MIRU loci[45].

5. Genotyping methodologies

The following are the genotyping methodologies based on repetitive regions of the *M. tuberculosis* chromosome mostly used worldwide:

• IS6110-based restriction fragment length polymorphism (RFLP) analysis.

• Spoligotyping.

• Mycobacterial interspersed repetitive units (MIRU) analysis

5.1. IS6110-RFLP methodology

Identification of IS6110 was a great break through for epidemiology of tuberculosis [46]. IS6110 is an insertion sequence made up by 1361 bp, capable of making copies of themselves and then inserting the copies in a genome locus in a process known as transposition [47]. IS6110-based restriction fragment length polymorphism analysis has been considered the golden standard method for *M. tuberculosis* genotyping [48-49]. Strains of *M. tuberculosis* differ in the number of IS6110 copies and their distribution is highly variable in the genome [50].

The first step in conducting RFLP is purification of DNA from a culture of *M. tuberculosis*; then the *Pvu*II restriction enzyme cuts the DNA on specific sequences in hundreds of different fragments. The fragments are separated per size in an agarose gel and transferred to a membrane. A probe is used in order to detect fragments containing IS6110; the picture is caught on a film, and each IS6110 copy produces a band. RFLP patterns with seven or more bands provide more specificity for discrimination of isolations, contrary to strains with patterns of six or less bands in which there is lower discrimination. Usually, there are eight to 18 copies per strains, but the number may vary from zero to 25 [51].

There is a small number of *M. tuberculosis* strain that do not contain the IS6110 sequence, so a print without bands will be obtained in the analysis, or those which contain a low number of copies will show less polymorphism than the ones containing a large number of copies. The main disadvantages of this methodology is the complexity of the procedure which has a length of approximately five days, and the amount of DNA necessary for the digestion with restriction enzymes; this requires obtaining cultures with sufficient growth in order to perform DNA extraction unlike PCR based methods such as MIRU and spoligotyping [52].

5.2. Spoligotyping

It is a PCR-based method for detection and typing of the *M. tuberculosis* complex using a single chromosome locus with high polymorphism, the direct repeated region DR. It consists on a sequence of 36 pb which are separated by 43 non-repeated spacers, each of which contains 36 to 41 pairs of bases [53].

The amplification of the DR locus is carried out by means of PCR, using primers, one of which is marked with biotin. The PCR products are hybridized perpendicularly to the membrane that contains 43 oligonucleotides of known sequence. The membrane is incubated with streptavidine-peroxydase conjugate which links to biotin of the PCR products. The detection of hybridization signals is made by means of a chemiluminiscence system (ECL). When DR regions of several strains are compared, the order of the spacers is almost the same in all of the strains, but deletions of the spacers may be found which generate the differences among each one of the strains [54].

In order to compare the patterns obtained with the results published, it is necessary to make the conversion of the obtained bands pattern. Each one of the 43 spacers produces either a dark box (indicating the presence of the spacer) or a clear box (indicating the absence of the spacer), and a binary numeric code 1 -0 is assigned respectively.

To simplify this numbering, the 43-digit binary code is converted to a 15-digit octal code (i.e. digits 0 to 7). Each 3-digit binary set is converted to its octal equivalent, and ultimately the remaining digit shall be either 1 or 0. The translation of the binary numbers to octal numbers is as follows: 000 = 0; 001 = 1; 010 = 2; 011 = 3; 100 = 4; 001 = 5; 110 = 6; 111 = 7. Each octal designation is unique, thus representing a specific brands pattern [55]. Finally, this code is entered in the SITVIT WEB international database which presents information on the diverse lineages and its world distribution. It contains information about the genetic diversity of the *M. tuberculosis* complex based on 62,582 clinical isolates from 153 countries [56].

Spoligotyping offers great advantages such as its usefulness in genotyping strains of *M. tuberculosis* that contain little IS6110 sequences [57]; the use of the methodology directly on clinical samples [58] which would allow simultaneous diagnosis and typing of *M. tuberculosis*, *M. bovis*, and differentiation of species comprising the *M. tuberculosis* complex. Studies conducted with clinical samples showed unique hybridization pattern, whereas strains of an outbreak shared the same pattern. Patterns obtained from direct examination of the clinical samples were identical to the ones obtained by means of using DNA from cultures of these samples [59].The use of a second generation spoligotyping membrane was proposed with the aim of increasing the test´s power of discrimination using 51 oligonucleotides of spacers [60].

By using spoligotyping, species and subspecies comprising the *M. tuberculosis* complex can be classified based on the patterns obtained on hybridization, and at the same time the diverse lineages of the *M. tuberculosis* species [61].

5.3. MIRU-VNTR methodology

Mycobacterial interspersed repetitive units are loci in the genome of *M. tuberculosis* containing a number of variable tandem repetitions [62]. The length of MIRU is in the range of 50 – 100 pb and belong to the category of VNTR "mini satellites" [63]. Forty-one loci of this type have

been identified in *M. tuberculosis*, among them, 12 loci have shown that they vary in the number of tandem repetitions and most of them among the repeating units.

It is a PCR-based method that uses these 12 different interspersed units for genotyping. The estimation to determine the number of repetitions is based on the size of the amplicon. The results are reported as 12 numbers, each one corresponding to the number of repetitions. The power of discrimination of the 12 MIRU-VNTR regions is much greater than the one of spoligotyping and close to IS6110 RFLP for typing of *M. tuberculosis* strains. Recently a system was proposed which includes typing of 24-loci MIRU-VNTR combining multiplex PCR analysis or a DNA analyzer based on fluorescence with the computer automation of genotyping was proposed [64].

6. Species and lineages of *M. tuberculosis*

6.1. *M. tuberculosis*

It was the first member of the complex to be described by Doctor Robert Koch in 1882. It is the species most frequently involved in the development of pulmonary TB in humans [65].*M. tuberculosisis* by far the most important pathogen of the complex in terms of the number of infected hosts and its public health implications.

6.2. *M. bovis*

It causes TB in a wide range of wild and domestic animals. *M. bovis* is naturally resistant to pyrazinamide, a first-line anti TB drugs[66]. Its epidemiologic importance lies in the zoonotic transmission of *M. bovis* to humans due to contact with infected animals, or to consumption of products from these animals.

6.3. *M. africanum*

It is the species that causes more TB in humans in Western Africa [67]. There are two large variants of *M. africanum*: the African Variation I, isolated in the east of Africa, and variant II, to the west [68]. Besides, M. africanum type I has been recently subdivided into *M. africanum* type I, eastern African I (MAF1), prevalent around the Gulf of Guinea; and *M. africanum* type I, eastern African 2 (MAF2), prevalent in southeast Africa. *M. africanum* type II has been reclassified into *M. tuberculosis* and indicated as the "Uganda" genotype [46].

6.4. *M. bovis* **BCG or Bacillus Calmette-Guerin (BCG)**

It is the vaccine's strain, and it is a live attenuated variant of *M. bovis*. Its spoligotype is characterized by the absence of spacers 3, 10, 17, 22, and 39 to 43 [47].The efficacy of this vaccine varies in different populations, with a consistently low efficacy in many tropical regions of the world [48], [49]. High efficacy when BCG is used to vaccinate newborns. Neonatal vaccination with BCG imparts protection against the childhood manifestations of TB (in particular, meningitis) [50], [51].

6.5. *M. microti*

It causes the disease in voles,wood mice and shrews. Its isolation is rare in human clinical samples, but recently, isolations from humans have been characterized. The spoligotypes analysis in isolates reveals the single presence of spacers 37 and 38 [53].

6.6. *M. canetti*

It was added to the list of *M. tuberculosis* complex in 1997. It isolated in 1993 from a 2-year-old Somali child with lymphadenitis, this isolate exhibited an unusually smooth and glossy colony morphology. To this date there have been few reports of TB cases due to this subspecies, and it is thought that animals are natural hosts of the bacterium [52].

6.7. *M. caprae*

This subspecies has been isolated mainly from goats in Spain [44], but it has also been found in boars, pigs, and in some cases of persons related to goat breeding [54]. The genetic footprint obtained by spoligotyping is characterized by the absence of spacers 1, 3 to 16, 10 to 33, and 39 to 43 [54].

6.8. *M. pinnipedii*

It was originally isolated from TB cases in pinnipeds such as sea lions and seals. Recently, cases have been described in terrestrial animals such as the Brazilian tapir. Its spoligotype has only the spacers 25 to 38 [55].

6.9. *M. mungi*

Identified in 2010 as a pathogen of the *M. tuberculosis* complex as TB causal agent in banded mongooses that live close to humans in the district of Chobe, Botswana, because these animals live in human-made structures and scavenge human waste, including feces.TB has been identified in only humans and mongooses. Strain assessment of human TB has not been conducted; the full host spectrum and transmission dynamics of this pathogen, currently unknown [56].

The spoligotype pattern of *M. mungi* isolates is characterized by absence of spacers 3, 7, 9, 12 to 36, and 39. It was determined that this pattern is preserved in the described isolates, but it is not included yet in international databases [56].

6.10. *M. orygis*

Species described in 2012 by Van Ingen and collaborators. It has been isolated from members of the Bovidae family such as oryx, gazelles, antelopes, cows, rhesus monkeys and waterbucks, although their exact host range remains unsettled, however cases have been described in humans [57]. Its spoligotype pattern is characterized by the absence of spacers 4-9, 14-24. 35, 36, and 39.The most common spoligotype (ST587) is present in the SITVIT WEB database and labeled *M. africanum* [58].

6.11. *M. tuberculosis* lineages (Fig. 4)

The lineages of *M. tuberculosis* are distributive a worldwide.

6.12. Haarlem (H)

Characterized by a pattern with absence of spacer 31 and the presence of at least one spacer between 1 and 30. It is highly prevalent in Northern Europe, while it is less extended in the Caribbean and Central Africa, where it is thought to be introduced by the European colonization [59].

6.13. Latin America and Mediterranean (LAM)

Characterized by the absence of spacers 21 to 24, 33 to 36, and the presence of at least one spacer between 1 and 30. It is frequent in Mediterranean and Latin American countries. Some genotypes have shown strong geographic associations, for instance LAM10-Cameroon or LAM7-Turkey which were initially catalogued as LAM although there has not been phylogenetic association with other spoligotypes [60, 61].

6.14. T Lineage

Comprised by modern strains of TB. This lineage is characterized "by default" and it includes strains which are difficult to classify into other groups [11] It has absence of spacers 33 to 36 and presence of at least one spacer between 1 to 30 besides the presence of the spacers 9 or 10, and 31; there is also presence of one spacer between 21 to 24 [62].

6.15. X lineage

Its pattern has absence of spacers 18 and 33 to 36. It was identified in Anglo-Saxon cities, and it is highly prevalent in South Africa, less in Latin America. However, there is high presence of this genotype in Mexico, which can be explained by its closeness to the United States. The X lineage was the first group identified in Guadeloupe [63] and the French Polynesia [64].

6.16. East African-Indian (EAI)

Absence of spacers 29 to 32 and 34; and presence of at least one spacer between 1 and 30. It is frequent in South East Asia, India, and Western Africa [62].

6.17. Central Asian (CAS)

Absence of spacers 4 to 27, and 23 to 34. It is highly prevalent in sub-Saharan countries and Pakistan. This linage has also proved to be endemic in Sudan, sub-Saharan countries, and Pakistan. This spoligotype has numerous variants and subgroups such as CAS1-Kili (Kilimanjaro), CAS-Dar (Dar-es-Salaam), and CAS-Delhi [62].

6.18. Beijing

This genotype has absence of spacers 1 to 33 and presence of spacers 34 to 43; in terms of public health it continues to be a serious problem for TB control due to its high virulence and association with multi-drug resistance [11].

6.19. MANU

It may be the ancestral clone of the strains in genetic group 1. It was subdivided into Manu 1 (absence of spacer 34), Manu 2 (absence of spacers 33-34), and Manu 3 (absence of spacers 34 to 36). It is a new family from India [62].

Figure 4. World distribution of lineages *M. tuberculosis* (Modified of Demay,2012)

7. Molecular epidemiology support in controlling tuberculosis

Molecular epidemiology has become in recent years an essential tool in the study of cases of tuberculosis, together with classical epidemiology, thus making the analysis of situations such as:

- Early detection and rapid control of outbreaks.

- Set transmission cases restricted communities

- Establish the geographic origin of the strains

- Monitoring of cross-contamination in the laboratories of TB

- Detection of genotypes associated with drug resistance

- Differentiation of cases of relapse and reinfection

In the analysis of data obtained by molecular epidemiology is essential to know definitions created from the development of molecular epidemiology, as they are

Cluster: A genotyping cluster is two or more *M. tuberculosis* isolates that share matching genotypes or the same DNA fingerprinting. An epidemiologic cluster is two or more persons with TB who share known epidemiologic links [38].

Matching genotypes: two or more *M. tuberculosis* isolates that share the same genotype.

Nonmatching genotype: an isolate that has a unique genotype (i.e., a genotype pattern that does not match the pattern of any other isolate in a TB program's database) [38].

7.1. Early detection of outbreaks

Analysis of clinical isolates of certain areas can help determine if isolates share the same genotype, and they form a cluster. But additionally be analyzed factors such as place of residence, work and time spent on them [38].

Genotypic guide Atlanta CDC studies raises three criteria for an outbreak [38].

1. An increase in the expected number of TB cases.

2. Transmission continues despite adequate control efforts by the TB program.

3. The contact investigation has grown to a size that requires additional outside help.

7.2. Transmission between cases of restricted communities

In conducting the study epidemiologically linked contacts and TB patients can be established [38]:

- Epidemiologic links between two TB cases that were identified during contact investigations and later confirmed by subsequent cluster investigations.

- New epidemiologic links that were identified during cluster investigations but not discovered during previous contact investigations.

7.3. Establishment of the geographical origin of the isolates

The analysis of several clinical isolates obtained worldwide, has established partnerships lineages with specific geographical areas, and in the case of LAM lineage or Latin American and Mediterranean, which as its name suggests occurs at high frequency in these geographic areas. The EIA or East African Indian [41].

7.4. Detecting genotypes associated drug resistance

The main genotype associated with drug resistance is the Beijing genotype, these strains exhibit high virulence and ease of propagation, but additionally the majority of clinical isolates

belonging to this lineage with mutations that confer drug resistance, so its eradication much more difficult in the population, was first identified in China in 1995 [65], but has quickly spread to other countries, and has been responsible for outbreaks of MDR-TB [66, 67]:

7.5. Differentiation of relapses and reinfections

Initially, we must take into account in terms of TB programs, it only considers the situation of relapse which is defined as a patient previously treated for TB who has been declared cured or completed treatment and is newly diagnosed with bacteriologically positive tuberculosis. But the genotypic analysis classifies this situation relapse and reinfection, which are defined as, a case of relapsed TB represents a worsening of an infection after a period of improvement and is caused by the same strain of *M. tuberculosis*. TB that represents a reinfection is caused by a second infection with a strain that is different from the strain that caused the initial infection [38].

7.6. Cross contamination in the laboratories of TB

With the genotypic analysis of clinical isolates processed in a laboratory during the same time period can be set if cross-contamination occurred between crops.

Genotyping can help identify instances of incorrect TB diagnoses that are based on false positive cultures. Incorrect diagnoses can result from laboratory cross-contamination of cultures during batch processing, pipetting, transfer of bacilli from a broth-culture system, work in a faulty exhaust hood, and species-identification procedures, mislabeling of patient specimens, clinical equipment contaminated [38].

The identification of cross-contamination in a laboratory setting allows control measures to prevent patients are misdiagnosed.

7.7. Incorporating genotyping methodologies in TB diagnostic laboratories

Since the development of the genotyping methodologies, especially those that are based on nucleic acid amplification, it has been observed their advantage for use with isolated genetic material directly from clinical samples. Examples of these methodologies is spoligotyping, described by Kameberck, which allows simultaneous detection and typing of *M. tuberculosis*. Rapid identification and genotyping of *M. tuberculosis* families through the methodology implemented in multibacillary cases of TB, causing the rapid spread of the disease in community, reduce the time between suspicion of disease and treatment. Additionally, the application of the methodology of Spoligotyping in paucibacillary cases allows for quick diagnosis in cases of extrapulmonary tuberculosis, and rapid identification of *M. tuberculosis* complex species involved in the infection, supporting the clinician in addressing treatment.

8. Conclusion

The methods most commonly used in developing countries via bacteriological diagnoses were smear and culture show the presence of the causative agent of the disease. Currently being

implemented new, more sensitive diagnostic technologies based on the detection of deoxyri-bonucleic acid (DNA) mycobacterial [68] and with which results are obtained in less time. Some of these methods provide the additional advantage of performing genotyping processes allowing the identification of family or families of *M. tuberculosis* present in the region, some of these methods are the analysis of the insertion sequence IS6110, polymorphism of the spacer sequences of Direct repeat region DR and analysis of the intergenic repeat units mycobacterial MIRU, and thus support the strategies of disease control with the detection of cases, relapse, and reinfection of new strain circulation, thus contributing to the development of molecular epidemiology.

9. Application exercise

9.1. Exercise 1: analyses of cases

Case 1

Patient presented an episode of TB in 2010, he completed his treatment but with some disruption, then in 2013, he presents a new case of TB. The following are spoligotypes patterns identified for Spoligotyping in each of the isolated (table 2).
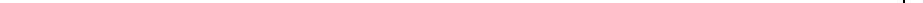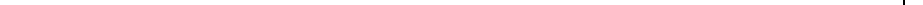
| 2010 | ■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■■□□□■■■■■■■ | LAM9 |
| 2013 | ■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■■□□□■■■■■■■ | LAM9 |

Table 2. Spoligotypes case 1

Case 2

Patient presented an episode of TB in 2012, he completed his treatment in June of this year. In October 10th 2013, the patient was diagnosed with TB. The analyses for spoligotyping of the strains showed the following patterns (table 3).

| 2012 | ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | T1 |
| 2013 | ■■□■■■■■■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■ | LAM2 |

Table 3. Spoligotypes case 2

Questions

1. Define the situation of each case, justified your response

2. Which other diagnosis methodologies you can use for confirmation the case.

9.2. Exercise 2

Complete the gaps on the table 4

Number	Binary code	Octal code	SIT	Lineage
001	110111111111011111110000111111100001111111			
002	111111111111111111111111111111100001111111			
003	111111111111111111100001111111100001111111			
004			273	
005	111111111111111111111111000000100001110111	777777774020731	62	
006			91	
007	111111111111111111100001111111100001111111			
008	111111111111111111111111000000100001110111			
009		777777777720771		
010	000000000000000000000000000000000111111111	000000000003771	1	
011	111111111111111111111111111110100001111111			
012			447	
013	111111111111111111111101111111100001111111			
014	111111111111111111111111111111100001111111			
015	110111111111011111110000111111100001111111			
016	111000011111111111111000000000000111111111			
017	100111111111111111111111111000010110001111		11	
018		777777607760771		
019	111111111111110111111111111111111100001111111			
020	111111111111111111100001111111100001111111			
021			373	
022			42	
023			64	
024		777777477760771		
025	111111110011111111111111000000000000001110111			
026	000011111111111111100000111101100001110111			
027	111111111111111111101111111111100001110111			
028	111111111111111111100001111111100001111111			
029			33	
030			53	

Table 4. Exercise 2

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