RAPD analysis and antibiotic susceptibility for *Mycobacterium tuberculosis* strains isolated from different locations in Egypt

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ABSTRACT

The routine identification of mycobacterial strains isolated from patients in different locations in Egypt was confirmed by specific DNA fragment amplification. The susceptibilities of 72 *Mycobacterium tuberculosis* strains against the four antibiotics used in tuberculosis treatment (Isoniazid, INH; Rifampicin, Rif; Streptomycin, St and Ethambutol, E) were examined. Our results indicated that, multi drug resistant tuberculosis (MDR-TB) represents about 19.5% of the tested strains, whereas sensitive strains represented 26.4%. The genetic polymorphism of the tested strains was examined using RAPD analysis. Six selected strains represent the different antibiotic susceptibility groups were examined using RAPD fingerprinting. No difference between the strains was recorded using the RFLP analysis of amplified specific fragment. The discrimination power of RAPD analysis was inadequate to clarify the genetic correlation between the tested strains. MDR-TB was approximately double time in 2008 compared with the value in 2007. Most of the new MDR-TB was correlated with resident dense population regions.

Keywords: *Mycobacterium tuberculosis*; MDR-TB; RAPD; antibiotics susceptibility

INTRODUCTION

Tuberculosis is a major cause of morbidity and mortality in the world from a single infectious agent (Murray *et al.*, 1992). It was estimated that mycobacteria have infected one third of the global population with approximately 8.8 million new cases in the year 2005 (WHO, 2007). Despite of tuberculosis is a global epidemic, it affects mainly the undeveloped and poor countries with dense population and few hygiene care where 98% of all TB deaths occur (Grange and Zumla, 2002).

Tuberculosis began to be treated with antibiotics in 1940, since streptomycin (STR) and *para*-aminosalicylic acid (PAS) were discovered and then used for TB treatment separately, but by the late 1940's, combination treatment of both STR and PAS were used to overcome the developing resistance problem (Iseman, 2002). Now, several antibiotics are used for TB treatment to overcome the resistance problem. Isoniazid (INH), Ethambutol (E), and Rifampicin (RIF) were implemented in TB treatment (Murray, 2004), then pyrazinamide (PZA) was reintroduced during the 1980's and came to replace STR in the regiment in several places (Iseman, 2002). Since that time, no new TB drug has been discovered (Saltini, 2006).

The increase in antibiotic resistance has generated considerable medical problems in TB treatment, since the increased death rate is attributed to the emergence of new resistant strains of *M. tuberculosis*. Multidrug-resistant TB (MDR-TB), associated with high death rates of 50% to 80%, in a relatively short time (4 to 16 weeks) from diagnosis (Dooley *et al.*, 1992).

MDR-TB are the strains that are resistant to at least two of the best anti-TB drugs, Isoniazid and Rifampin. The treatment success rate for strains resistant to both RIF and INH varied between 44-77%, compared with > 90% for fully susceptible strains (Loddenkemper *et al.*, 2002).

The WHO and the International Union Against Tuberculosis and Lung Diseases (IUATLD) publish yearly reports which estimate and analyze the prevalence of drug resistance trends (WHO 2003; WHO 2008). The most important conclusion obtained was to identify TB drug resistant hot spot regions where specific efforts from the global society should be focused.

The tragedy of tuberculosis treatment is that, 50 years after the introduction of effective specific chemotherapy, the number of cases and the more threatening is higher worldwide. There are an increasing number of infections with organisms which are resistant to the major anti-tuberculosis agents (Pablos-Mendez *et al.*, 1998; Dye, 2000; Dye and Espinal, 2000).

The epidemiology of TB can be monitored by screening the different genotypes present in the different areas. Molecular markers reveal differences of natural sites at the DNA level. These variations might be a single nucleotide difference in a gene or a piece of repetitive DNA (Johns *et al.*, 1997).

The random amplified polymorphic DNA (RAPD) as a one of DNA fingerprinting technique has been used for differentiation between various kinds of microorganisms (Williams *et al.*, 1990; Bazzicalupo and Fani, 1996),

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including *M. tuberculosis* isolates (Harn *et al.*, 1997; Richner *et al.*, 1997; Rodriguez *et al.*, 2000; Vázquez-Marrufo *et al.*, 2008). Moreover, since it samples the overall diversity of genomes and corresponds therefore to a generalist marker (Tibagrenc, 1995), this method allows the comparison of genetic diversity between different microorganisms.

Molecular typing of *M. tuberculosis* can facilitate investigation into the transmission of TB. In the present study, we planned to investigate the prevalence of drug resistant mycobacteria at different locations in Egypt, and to clarify the genetic correlation using RAPD analysis between the antibiotic resistant strains to get information about transmission of MDR-TB within regions with epidemic TB.

The aim of our study is also to support the national and global efforts exerted in fighting tuberculosis diseases by studying of the percentages of multi drug resistant bacteria in two subsequent years in different governorates in Egypt. Also the genetic correlations between the isolates were also examined.

MATERIALS AND METHODS

Bacterial strains

We analyzed 72 strains of *M. tuberculosis* isolated from different specimens (newly infected) belonged to 20 governorates in Egypt. Mycobacteria were isolated from pathological samples at the Tuberculosis reference lab, ministry of health central labs, Cairo, Egypt. The strains were collected and grown on fresh Loewenstein-Jensen slants at 37 °C for 8 weeks.

The samples were collected in 2007 and 2008. Thirty five strains were isolated in 2007 and 37 Mycobacterial strains were isolated in 2008. Of these, 7 strains were collected from patients in Cairo; 5 from both Giza and Beny-Swief; 4 from each of Alexandria, Suez, Menofya, Sharkia, Gharbia, El-Menia; 3 strains from Dakahlia, Demiat, Kafr El-Sheekh, Ismailia, Assuit, Sohag, Quina, Aswan, Red Sea; while 2 strains from each of Matrouh and Sewa, thus all regions in Egypt were covered (Figure 1).
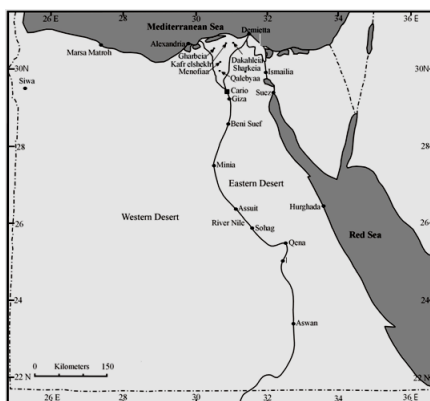
Figure 1: Locations where the Mycobacteria were isolated

Bacterial identification

The bacteria were identified using routine bacteriological procedures (Baron and Finegold, 1990). Identification was then confirmed by a specific gene amplification using Genekam kits for *Mycobacterium tuberculosis* identification (Genekam Biotechnology AG, Duisburg, Germany).

RFLP analysis

The amplified fragments were then digested with restriction endonulease *Hae* III enzyme (Boehringer Mannheim) from *Haemophilus aegptius*. *Hae* III recognizes the sequence `5GG↓CC 3 and generates blunt end fragments. The amount of enzyme and DNA, the buffer and ionic concentrations were calculated for a final volume of 50 µL reaction (Ausubel *et al.*, 1995).

Antibiotic susceptibility

The antibiotic susceptibilities of all *M. tuberculosis* strains were tested (Fadda and Rose 1984) (data not shown).

RAPD Fingerprinting

Bacterial DNA was prepared from fresh culture using Cetyltrimethylammonium bromide (Merck, Darmstadt, Germany) (van Embden *et al.*, 1993).

A total of 20 primers (Table 1) were initially screened for the ability to produce discriminatory polymorphism and reproducible results. As slight variations in banding patterns were noted even when the same DNA controls were analyzed simultaneously, isolates were routinely assayed in duplicates.

Table 1: Primers (10-mers) used in the pilot study to type six *M. tuberculosis* strains represents the antibiotics resistant groups.

Primer	Sequence	GC content
OPA-02	TGCCGAGCTG	70 %
OPA-03	AGTGAGCCAC	60%
OPA-04	AATCGGGCTG	60%
OPA-06	GGTCCCTGAC	70%
OPA-08	GTGACGTAGG	60%
OPA-13	CAGCACCCAC	70%
OPA-15	TTCCGAACCC	60%

Plasmid screening

Plasmids were extracted from bacterial colonies using two methods, (Brinbiom and Doly, 1979) and using mini-prep QIAGEN kits for plasmid extraction.

RESULTS

Identification of strains

The results of routine bacteriological methods for bacterial identification indicated their affiliation to *Mycobacterium tuberculosis*. These results were confirmed on the molecular level, since our strains and the positive control (*Mycobacterium tuberculosis*) showed a band of 317 bp according to Genekam TB kits protocol (Figure 2).

RFLP analysis of PCR products

Two visible fragments were observed after digestion of the amplified fragment with restriction endonuclease *Hae* III in each strain (Figure 3).
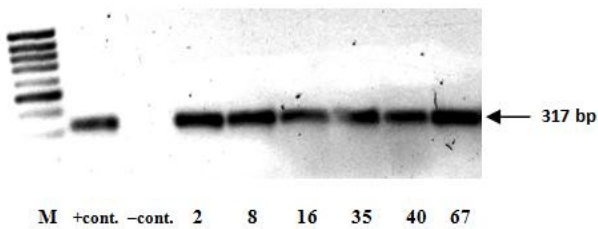
Figure 2: Amplification of Tb specific fragment (317 bp) according to genekam kits. M: 100 bp DNA ladder, lanes labeled 2 – 67: Representative of our strains.
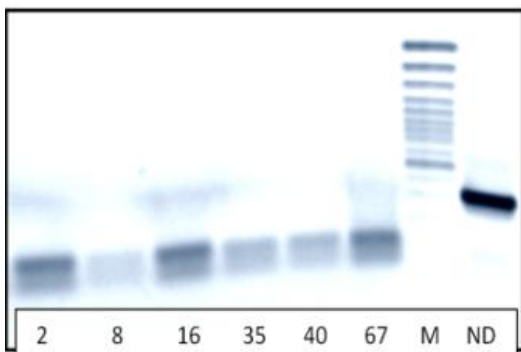

Figure 3: Representative RFLP profile of amplified specific 317 bp fragment. Lanes labeled 2 – 67: Representative of our strains. M: 100 bp DNA ladder. ND: not digested amplified fragment.

Antibiotic susceptibility

As shown in Figure 4, multidrug resistant strains represent about 19.4% of tested strains while sensitive strains represent 26.4 % of the strains tested. The multi-drug resistant strains were analyzed as shown in Figure 5. Most of these strains were resistant to INH, Rif and St (64.3% of total MDR-TB strains). The results showed also that, 21.4% of MDR strains were resistant to INH, Rif, St, while these resistant to each of (INH, Rif, St, E) and (INH, Rif) were only 7.1% of each group.

Of the 14 MDR-TB strains, 4 strains (28.5%) were isolated in 2007, while 10 (71.4%), and were isolated in 2008 (Figure 6). Most of the MDR-TB strains isolated in 2008 where from densely populated regions (ranged from 184 – 1602 residents/Km^2.)

Since seventy two isolates may be considered inadequate for analysis, Chi square test ($\chi2$) was applied to ensure the benignity of experimentation and hypotheses. The results of this analysis relied in the acceptance region in the ($\chi2$) reference table.
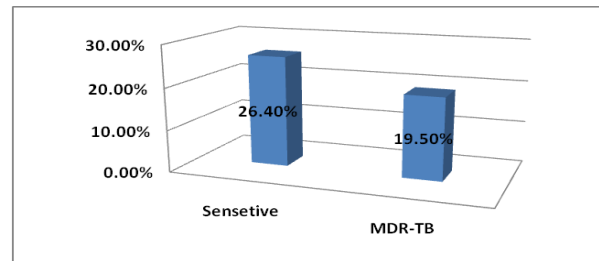
Figure 4: The susceptibility of *Mycobacterium tuberculosis* strains towards used antibiotics.
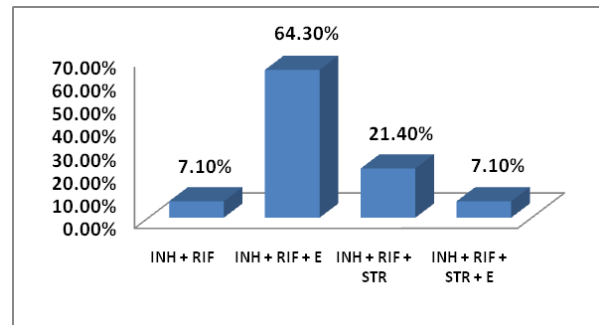
**Fig. 5:** Multi-drug resistant groups.
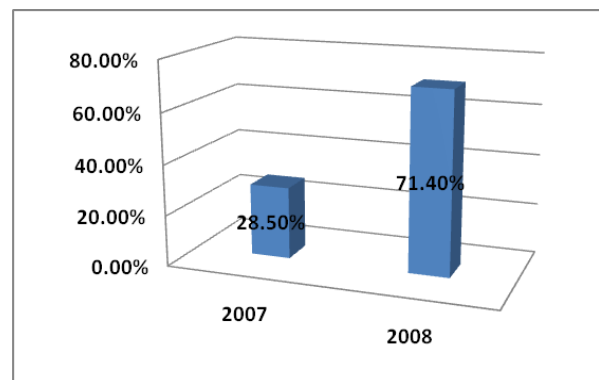
Fig. 6: MDR-TB strains during studying period.

RAPD-PCR

To show the reproducibility of the RAPD-PCR method, three different experiments were performed using 8 different primers, each of which was carried out on 3

different days (Figures 7 A – H). By comparing the anding patterns visually, slight differences between the strains could be recorded. Applying the bands distribution to statistical programs (SPSS) had generated a dendrogram (Figure 8) constructed on the basis of the different patterns for the 6 *M. tuberculosis* which represent the different antibiotic susceptibility groups. The dendrogram shows the genetic relationships among the different representative strains. The strains separated into two main clusters, at about 76 % similarity, the first one contains the strains sensitive to E and INH (G1), whereas the other group (G2) contains the strains resistant to E and INH (G2B). The sensitive strain separated as a distinct sub-cluster in the second group (G2A).
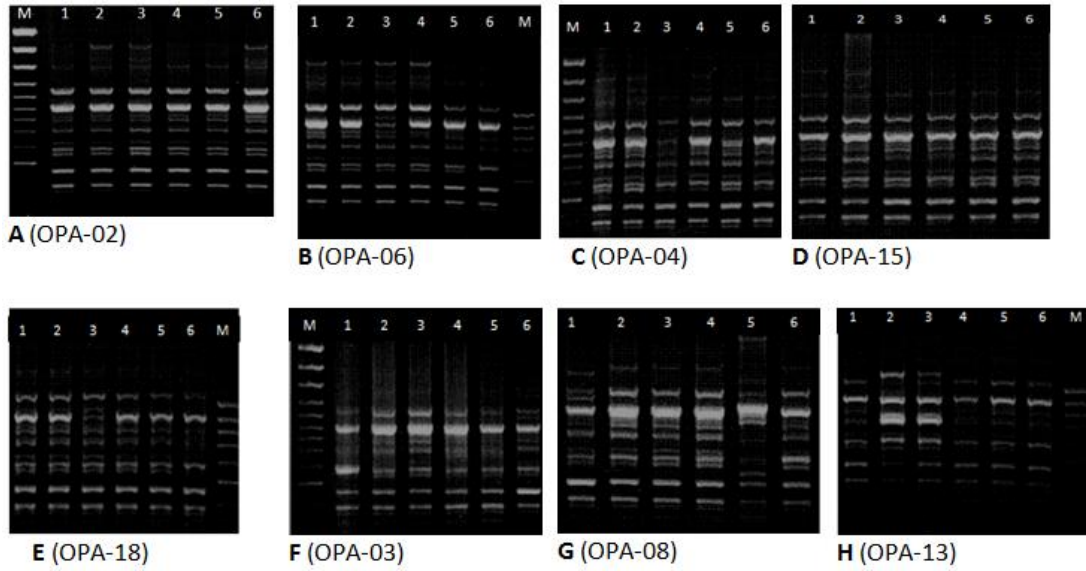
Figure 7: Fingerprint patterns of RAPD analysis performed with *M. tuberculosis* strains. Lanes: 1 (strain no. 2); 2 (strain no. 8); 3 (strain no. 16); 4 (strain no. 35); 5 (strain no. 40); 6 (strain no. 67); M (DNA molecular weight marker 100 bp ladder.
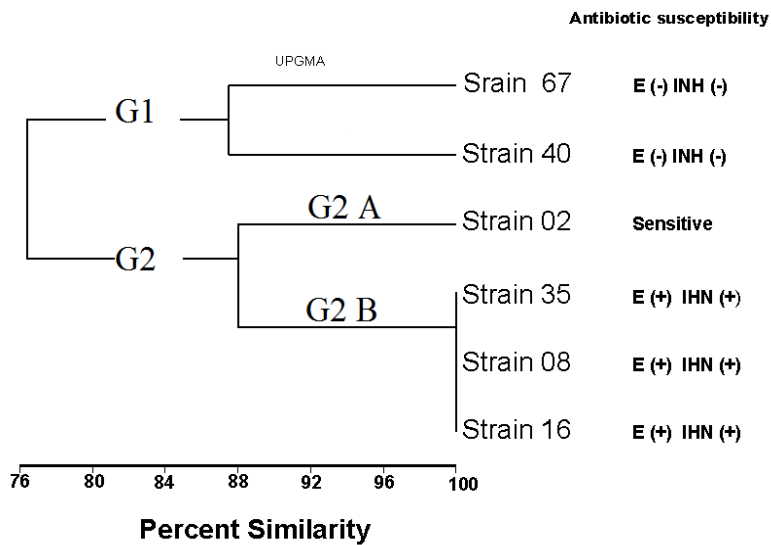
Figure 8: Dendrogram constructed on the basis of the patterns and the genetic relationships of six representative strains obtained with the RAPD primers.

Plasmid screening

No plasmids were detected in any of the tested strains by using the described methods.

DISCUSSION

Tuberculosis is a common and often deadly infectious disease caused by mycobacteria. The recent appearance of multi drug-resistance (MDR-TB) has necessitated the combination of two or more drugs be used to help delay the emergence of resistant strains. The delayed initiation of effective therapy is one of the major factors contributing to MDR-TB outbreaks. (Edlin *et al.*, 1992; Pearson *et al.*, 1992).

Several studies and reports discussed the *Mycobacterium tuberculosis* resistance and outbreak, when accompanied with the Immuno deficiency diseases (HIV). Tuberculosis is particularly insidious problem to those who have AIDS. In these patients the T-lymphocytes that normally mount a response to *M. tuberculosis* are being destroyed by virus and patients cannot response to therapy. Usually the naturally rates between these patients raises to 70 – 90% within 4 months from developing the symptoms (WHO, 2008). Malcolm (2000) reported that, the incidence of TB is increasing markedly, since TB infections increased from 91 cases (2.1 per 100,000 populations) in 1988 to a peak of 165 cases (3.7 per 100,000) in 1992. Similarly, Bloom and Murray (1992), reported that the occurrence of TB recorded extra 20% between 1985 and 1992 according to the Centers of diseases control and prevention, 1993. In 1995, Sepkowitz *et al.* calculated that the antibiotic resistant rate of TB was as high as 36 % from the previous years in Minnesota, USA.

Christopher *et al.* (2002) have studied the Worldwide Incidence of Multidrug-Resistant tuberculosis. They surveyed 64 countries for the MDR-TB, and they found that the incidence of these MDR-TB strains has increased by 3.4%. The highest rates were in Estonia (14%), Henan Province, China (11%), and Latvia (9%). Rifampin resistance for example has been shown to be associated with genetic alterations in an 81-bp region of the rpoB gene encoding the DNA-dependent RNA polymerase b-subunit (Musser, 1995).

Our results (Figure 4), showed that 19.5% of the 72 tested strains were resistant to each INH and Rif (MDR-TB), whereas 26.4% of these strains were susceptible. This indicated that these MDR-TB strains are initially resistant strains and are usually transmitted through droplet which enters the respiratory tract of people usually living in urban get toes with generally poor quality of life. This contributes to the establishment of the disease and increase the concentration in the air. More than 25% of the strains still susceptible. 71.4% of these MDR-TB strains were isolated in 2008 while 28.5% isolated in 2007 (Figure 6). These results indicate that MDR-TB strains increased during period of study (2007, 2008). Chi square test ($\chi 2$) was applied to ensure this hypothesis according to our available 72 samples. The results of this analysis

lied in the acceptance region in the ($\chi 2$) reference table and this hypothesis (raise of MDR-TB) is accepted which is consistent with the global observations of increasing the MDR-TB strains.

Our results (Figure 5) showed that most of the MDR-TB (64.3% of MDR-Strains and 6.5% of total strains tested) was resistant to Ethambutol in addition to INH + RIF. This proportion, however was 0% and 0.9% in the newly and retreated patients according to last survey of tuberculosis drug resistance done in Egypt during 2002 whereas a total number of 849 patient enrolled 632 new and 217 old patients (Programmatic Management of Drug Resistant-TB, 2007). In this report also, the resistance to four first line antibiotics (INH, RIF, E, STR) was 1.4% and 25% in new and retreated patients respectively, while in our study is 7.1% among new patients, which supports again the global conclusions of increasing the MDR-TB strains. Despite the use of the directly observed treatment short-course strategy for controlling the disease since 1991, the incidence is still increasing in some countries, e.g Morocco (Ottmani *et al.*, 1998).

Despite the efforts exerted in fighting TB in Egypt and all over the world, the outbreak of this infectious disease still continued. That is may be due to that the efforts are made only in one direction, the clinical and health care angle. Since the socioeconomic and demographic factors play a major role in the spread of disease, several precautious should be taken in consideration. HIV epidemic as well as these demographic and socioeconomic factors such as poor quality of life also serve to maintain substantial reservoir for potential infections (Bloom and Murray, 1992).

Sepkowitz *et al.*, (1995), have supported this opinion; they reported that more than twice the number of cases in 1988, and a 25% increase from 1998, in the number of TB cases. However national reports indicated that infectious have declined steadily since 1993. These authors analyzed these data to explain such unusual situation and they found that the most notable trend in the epidemiology of TB in Minnesota is the large and increasing number of cases among foreign-born persons. From 1995 to 1999, the number of foreign-born TB cases in Minnesota doubled while the number of cases among U.S.-born persons decreased about 42%.

WHO (2007), reported that, data from countries of the Eastern Mediterranean showed that MDR-TB among new cases was higher than previously estimated with the exception of Morocco and Lebanon which showed 0.5% and 1.1%, respectively. However, MDR-TB among new cases were 5.4%, and 2.9% in Jordan and Yemen, respectively. The Americas, Central Europe and Africa, reported the lowest proportions of MDR-TB; with the notable exceptions of Peru, Rwanda, and Guatemala, with 5.3%, 3.9%, and 3.0% MDR-TB among new cases, respectively.

Not only the MDR-TB is rising with time throughout the world, but, a new definition in October 2006 was announced, namely extensively drug resistant tuberculosis (XDR-TB) which is relatively a rare type of MDR-TB. XDR-TB is defined as TB which is resistant to

isoniazid and rifampin, and also resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). XDR-TB is of special concern for persons with HIV infection or other conditions that can weaken the immune system. These persons are more likely to develop TB disease once they are infected with a higher mortality rate once they develop TB.

Several researchers have used RAPD fingerprinting for genotyping of *Mycobacterium* strains besides these specific markers for *M. tuberculosis* typing. Tazi *et al.,* (2004), reported that, the values obtained with the RAPD data were almost always higher than those obtained with the MIRU data, which suggests that the RAPD technique allows detection of more mutational events than the MIRU technique. However, for both genetic markers, the diversity was notable and almost equivalent in all the subgroups they investigated. The polymorphism rate showed that all the RAPD primers gave polymorphic patterns in all the groups, and for the MIRU VNTR loci only 1 out of the 12 loci (locus 24) was totally monomorphic. The phylogenetic analysis showed that the Moroccan *M. tuberculosis* isolates were not separated from the other *M. tuberculosis* stocks, regardless of the analysis used (Wagner or neighbor-joining analysis based on MIRU or RAPD data). In 1995, Abed *et al.* used RAPD analysis but not on the whole genomic DNA but on 16S-23S spacer region. They demonstrated that the discriminatory power of 16S-23S spacer region-based RAPD analysis was higher than when whole genomic DNA was used as a RAPD template. According to results of our RAPD analysis, we suggest that all strains belong to very close genotypes, since the similarity coefficient was more than 75 % according to the primers used. It would be much helpful if another techniques in use. To discriminate the genetic relation and the epidemiology status, this will be done in further studies. Our results of plasmid(s) screening agree with the results concluded by other investigators, since no reports are available about extracting plasmid from *Mycobacterium tuberculosis*, but from other *Mycobacterium* species. Jucker and Falkinham, (1990), demonstrated that most of plasmids are found in environmental mycobacterial species, such as *Mycobacterium fortuitum* and *Mycobacterium avium.* They reported that approximately 50% of *M. avium* strains harbour plasmids and same *spp*. with multiple plasmids as demonstrated by Kibry *et al.* (2002). However, thus far there is no evidence the species belonging to *Mycobacterium tuberculosis* have natural plasmids (Zainuddin and Dale, 1990).

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