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Molecular Epidemiology of *Mycobacterium tuberculosis* Isolated from Pulmonary Patients in Iraq

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Abstract: One hundred sputum samples of referred patients from different regions of Iraq were processed in this study, and 35 samples only were excluded. Based on Auramine O staining, 40 *Mycobacterium* isolates were obtained. Samples were cultured on Löwenstein-Jensen slants for biochemical tests that diagnosed 35 (87.5%) *Mycobacterium tuberculosis*. All strains obtained in cultures were systematically tested for their susceptibilities to four antituberculous drugs; Isonicotinic hydrazide, Ethamptol, Refampicin and Streptomycin. Only three isolates (8.5%) were found harboring multi-resistance to drugs. Basic demographic data including age, sex and location were recorded. Most infections were within the age range of 16-30 years. The sex ratio was 72% males and 28% females. The strains were screened for the presence of IS6110 as a molecular marker. All tested strains were found to contain this insertion sequence (IS). Seventy-two percent of the strains had high copy number of IS (6-10 copies), the rest had low copy number (less than 6 copies). There was no clustering; strains were widely diverse based on location and the number of IS6110. There was low degree of active transmission among patients and most of the infections may be due to reactivation.

Keywords: Epidemiology, Iraq, Mycobacteria, Pulmonary infection.

INTRODUCTION

The ability of *Mycobacterium tuberculosis* to persist in the form of a long-term asymptomatic infection, referred to as latent tuberculosis, is central to the biology of the disease (Stewart et al., 2003).

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The major difference between *M. tuberculosis* and other mycobacterial infections is the transmission from person to person. Thus, it is important to diagnose TB as rapidly as possible (Soini & James, 2001). Initially, the diagnosis of mycobacterial disease based on clinical data, but definitive diagnosis usually includes the isolation and identification of the infecting organism in the laboratory. The traditional laboratory procedure for clinical specimens involves decontamination and digestion of the specimen, microscopic examination for the presence of acid-fast bacilli (AFB), isolation of the organism by culture, and drug susceptibility testing of the recovered organism. Because of the slow growth rate of mycobacteria, these can take several weeks. Molecular methods were developed for direct detection, identification, and susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days (Grange, 1998).

As one of the extra-chromosomal genetic elements, the Insertion Sequence IS*6110*, also known as IS*986* (McAdam et al., 1990; Zainuddin & Dale, 1989) has been established as the standard tool for molecular epidemiology of tuberculosis, exploiting the multiple polymorphism exhibited by most clinical isolates of *M. tuberculosis*. The discriminating ability of IS*6110* depends on the rate of variation being high enough to generate diversity in epidemiologically unlinked isolates, and yet being low enough for related strains to exhibit essentially identical patterns (Hermans et al., 1990). Although most isolates of *M. tuberculosis* contain multiple copies of IS*6110* (typically 10–20 copies), a minority (largely confined to South and South-East Asia) possess only a single copy. Most strains of *M. bovis* and BCG vaccine strains carry either one or two copies (Fomukong et al., 1993).

A crucial aspect of any TB control program is the ability to determine where the transmission is occurring in order to prevent further spread of infection and prevent active disease by identifying newly infected people. Genetic fingerprinting has helped to establish transmission links between individuals and to demonstrate instances in which related people were infected with unrelated strains (Thierry et al., 1990). We present here an IS*6110*-based classification of *M. tuberculosis* isolated from different regions of Iraq.

METHODS AND MATERIALS

Mycobacterial strains

One hundred sputum samples of referred patients from different regions of Iraq were processed in this study. Thirty-five samples

were excluded based on the wrong procedure for sputum collection. The remaining 65 samples were subjected to Auramine O staining; 40 *Mycobacterium* isolates were obtained. These were cultured on Löwenstein-Jensen slants for biochemical tests, and 35 (87.5%) of *M. tuberculosis* were diagnosed by this method. The strains were isolated from outpatients attended the Iraqi Institute for Chest and Respiratory Diseases near the Medical City in the capital Baghdad. However, cases were referred from different provinces of the country to this central facility. The strains were geographically distributed as follows; Baghdad province (7), Babel (4), Dyala (4), Mosul (2), Tikrit (3), Kirkuk (3), Nasiriah (4), Aumara (2), Ramadi (1), Najaf (3) and Basrah province (2) patients.

The mycobacterial samples were cultured on Löwenstein-Jensen medium. Primary isolation and culturing were based on fluorescent staining and biochemical tests (Robert et al., 1991). In accordance with recommendations of the TB Control Program (Murray & Alland, 2002), drug susceptibility testing by the proportion method was performed for all culture-confirmed cases of TB.

DNA amplification

Each bacterial culture was suspended in 1 ml of TE buffer (10 Mm Tris-Hcl & 1Mm EDTA) and placed in oven at 80 ˚C for 60 minutes for killing the cells. DNA was purified as described elsewhere (Van Soolingen et al., 1991). The method was reliable and yielded enough and suitable amounts of DNA for PCR. The diagnostic Primers used for amplification were those described by Willi et al. (1999). These are:

INS-1 (5´-CGTGAGGGCATCGAGGTGGC-3´)
INS-2 (3´-AAACAGTGGCTGCGGATGCG-5´)

The amplification reactions were carried out in a volume of 25 µl containing (25mg); 10 mM Tris-HCl; 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM (each) dATP, dCTP, dGTP and dTTP (Promega). Primers were at 20 pM concentration each plus 2.5 unit of Taq polymerase (Promega). The reaction was carried as: initial denaturation at 96˚C for 3 min; 3 cycles as follows; 95˚C for 50 sec, 70˚C for 1 min, 72˚C for 20 sec; 3 cycles at 95ºC for 50 sec, 68˚C for 1 min, 72ºC for 20 sec; 25 cycles at 94ºC for 1 min, 67ºC for 40 sec and 72ºC for 3 min. The primers used for fingerprinting were the followings (Neimark et al., 1996):

F1 (5´CGCCAGAGACCAGCCGCC-3´).
F1 (5´CCGCACCGCCCGCTCACGCA3´)

The reaction was carried out as 30 cycles; 94ºC for 1 min, 58ºC for 1 min, 72ºC for 5 min and 7- min extension for the last cycles.

RESULTS AND DISCUSSION

Epidemiology

Bacteriological diagnosis

With staining techniques, it is not possible to distinguish between TB and nontuberculus mycobacteria in clinical specimens (Tenover, 1993). Furthermore, the sensitivity of the direct acid-fast smear is lower than that of culture (Nolte & Metchock, 1995). Accordingly, the 40 auramin O-positive samples were cultured on Löwenstein-Jensen slant for 6 weeks until the appearance of typical cream-colored, buff and rough colonies against the green egg-based medium. The followed biochemical tests have confirmed the diagnosis of 35 strains (87.5%). The *M. tuberculosis* strains were tested for their susceptibilities to four first-line antituberculus drugs used in Iraq [Isonicotinic hydrazide (INH), Ethamptol (EMP), Refampicin Rm) and Streptomycin (Sm)]. Only three strains (8.5%) were found to be multi resistant. Drug resistance may be an indication of poor quality of treatment such as improper prescription or irregular taking of medications.

Molecular diagnosis

All strains contained IS*6110* represented by a 245bp PCR product on agarose gel electrophoresis (Fig. 1). Absence of IS*6110*-free strains may encourage the use of this diagnostic approach in Iraq. The method is more accurate and faster than conventional method currently used for TB diagnosis. Early diagnosis of *M. tuberculosis* disease prevents the development of drug resistant *M. tuberculosis* (Willi et al., 1999). It was found that female cases are less than male cases (28.5% versus 71.5%). However, women from rural areas may be unable to attend specialized centers, which may lead to a false low percentage of female infection. Information taken from the patients has indicated that they were under low socioeconomic conditions.

The majority of infections took place among the age group 16-30 years (37.14%), while the percentage among the age group 1-15 years was 14.28%, and this may be attributed to the BCG vaccine given at young ages. The diagnosis of TB disease in children is more difficult owing to the nonspecific signs and symptoms of TB and the presence of fewer mycobacteria, which results in fewer positive bacteriologic tests. Children, especially infants, are at increased risk for progression from latent tuberculosis (LTB) to active TB disease, which may sometimes be as severe as tuberculosis meningitis (Wootton et al., 2005). There may be low percentages of cases discovery among children in general. Understanding TB epidemiology among children is particularly important to the eventual elimination of TB in Iraq. On the other hand, adult cases have been referring from different regions and the Baghdad majority (20%) is only related to the density of population.

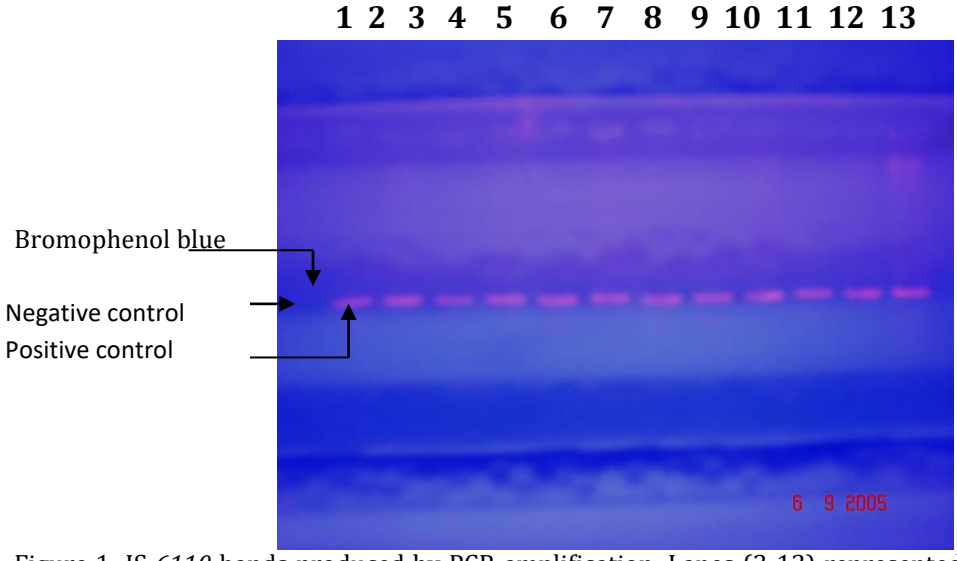

Figure 1: IS *6110* bands produced by PCR amplification. Lanes (3-13) represented amplified DNA from mycobacterial strains. Lane (2) amplified DNA from BCG strain. Lane (1) negative control. Gel electrophoresis was done by using 0.8% agarose gel concentration, 5 V/cm for 2½ hours.

The first described method for differentiating *M. tuberculosis* isolates used mycobacteriophages (Raleigh & Wichelhausen, 1973; Bates et al., 1976) or enzymatic characterization (Roman & Sicilia, 1984). Although useful for studies of specific outbreaks, these techniques had very low

discriminatory power. IS*6110* can be used for both DNA fingerprinting of *M. tuberculosis* and for diagnosis of tuberculosis disease specifically (Willi et al., 1999). Stained DNA visually compared for similarity based on presence or absence of bands on agarose, and variation in bands intensity were taken to constitute strains differences (Niemark et al., 1996). We have found that the numbers of IS6110 per strain are varied from 1-10 copies. The strains can be classified into two groups; group A, contains the strains that have less than six copies of IS*6110*. Group B contains strains with more than six copies of the Insertion Sequence in their chromosomal DNA (Yange et al., 1995a). It was found that 28% of the strains in this study belong to group A, and the rest belong to group B. The mean number of IS*6110* for all strains tested is 6.7.

Figure (2) indicates clear diversity banding while Figure (3) indicates that there was substantial regional diversity of IS*6110* among *M. tuberculosis* strains. Strains isolated from Baghdad province were polymorphic. Two of them contained 10 copies, two contained seven copies and the rest contained six, three, two or one copy. Except for Nasiriah and Tikrit provinces where two strains showed five or seven copies, respectively, no repetition or copy similarity was observed among the rest of the strains. Strains containing 10 copies were distributed in Baghdad, Najaf, Babel, Aumara and Diala provinces, while strains containing five copies of IS*6110* distributed in Mosul, Tikrit, Nasiriah, Kirkuk and Diala provinces. This may refer to low degree of active transmission of tuberculosis. Some cases may have arisen from reactivation of previous infection.

There was no association observed between copy number and drug resistances. Although there were only three multidrug resistant strains recovered, it is unlikely that resistance would strengthen IS*6110*-based sub grouping. Strains with IS*6110* lower than six copies may require the use of additional genetic markers such as polymorphic GC-rich repetitive sequence (PGRS) and direct repeats (DR) to establish the genetic relatedness (Van Soolingen et al., 1993). Nevertheless IS*6110* is the most commonly used genetic marker for typing *M. tuberculosis*. IS*6110* copy numbers and size profiles showed no clustering between the strains in this study. This means that there were no epidemiological link and unlikely represent recent infections. Epidemiological data in most urban populations tested strongly suggest that the rate of clustering reflects the level of recent transmission of TB (Gutierrez, 1998; Ferrazoli, 2000).

With the poor informational background obtained, it is hard to speculate whether the samples were representing primary or secondary infections. Transmission rates vary with host and environmental factors. The general principle that clustering of IS*6110* patterns equates with recent transmission might not always apply in all situations.

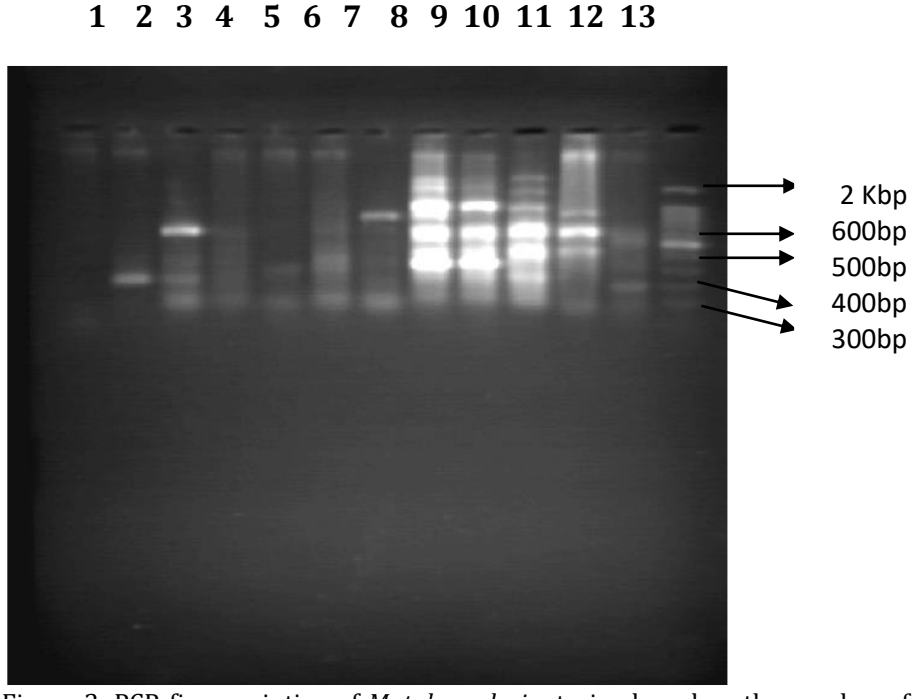

Figure 2: PCR fingerprinting of *M. tuberculosis* strains based on the number of IS*6110* copies: lane (1) negative control; lanes (2-12) IS*6110* bands; lane (13) ladder of molecular weight marker; isolates in lanes (3, 9, 11) are multidrugs resistant. (Gel electrophoresis was done by using 1.5% agarose gel concentration, 5 V/cm for 2½ hours).

For instance, studies have shown that the rate of clustering varies depending on the area under study. In resource-poor countries, clustering rates of 14-41% have been recorded (Dale et al., 1999). These findings were unexpected since the rates of clustering in some of these studies are comparable to or lower than those seen in low incidence countries.

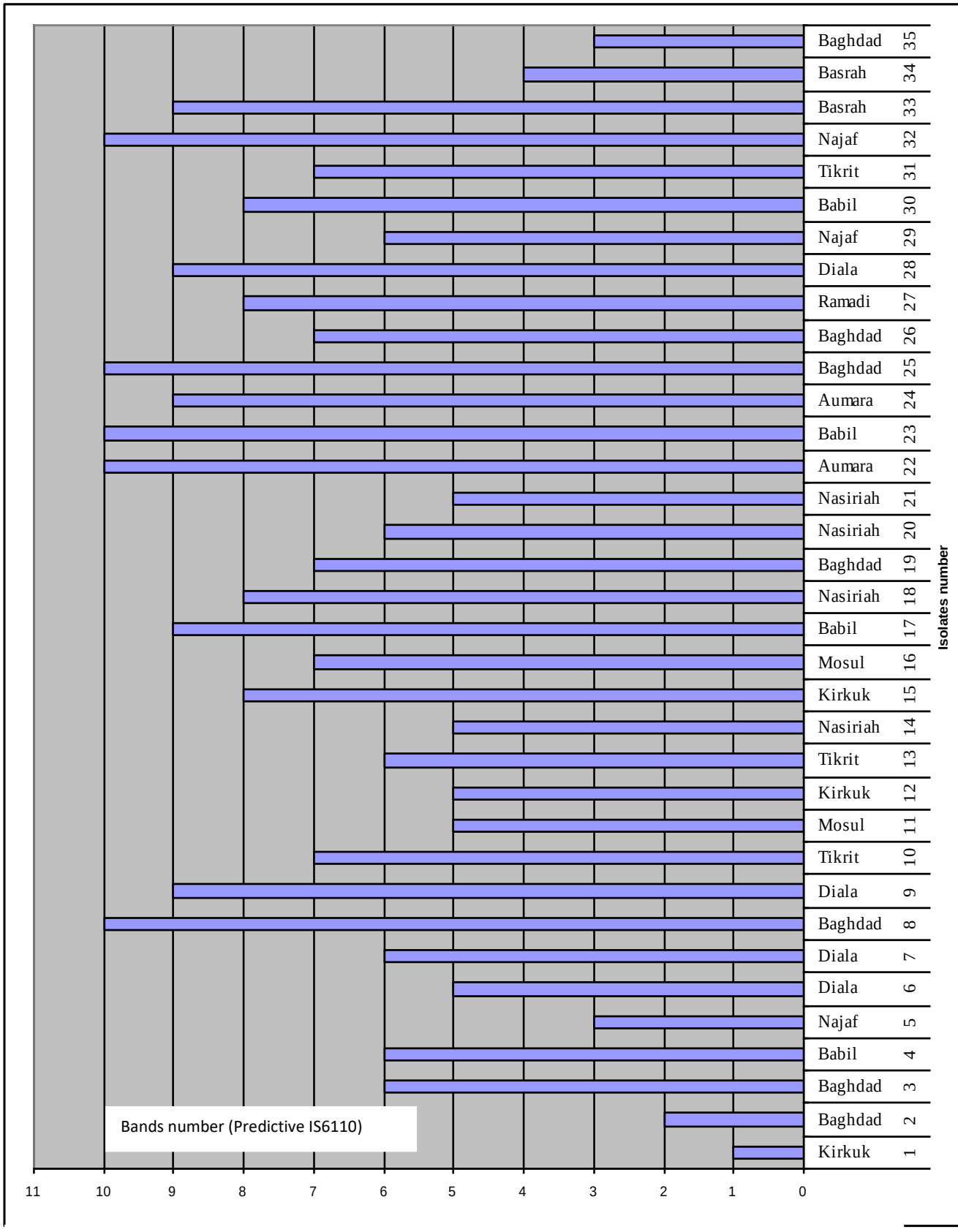

Figure 3: Numbers of IS6110 copies exhibited by 35 *M. tuberculosis* strains isolated from patients in different provinces of Iraq.

In these higher-incidence areas, with poor tuberculosis control, more transmission is expected. However, most of these studies do not report the incidence of tuberculosis and in many cases; it is difficult to ascertain the percentage of samples analyzed in the study of those available in the community, as it is the case in Iraq. This is particularly important because the number of isolates sampled in the study may be small in comparison to the total number of circulating isolates contributing to transmission in the area (Murray & Alland, 2002). For example, samples of this study were taken under risky security situations where outpatients from governorates are reluctant to approach health centers like the TB center in Baghdad.

However, this study has demonstrated that the majority of strains included in the analysis have multiple IS*6110* copies (72%). This is similar to those found in other populations (Yang et al., 1995a; Soini & James, 2001). On the other hand, low copy number are not unusual as exemplified by 21% in Vietnam, 26% in Tanzania (Yang et al., 1995b) , 8% in Denmark (Yang et al., 1994) or 5% in Tunisia (Hermans et al., 1995). IS*6110* has sufficient discriminatory power for DNA fingerprinting of Mycobacterial strains and can be used as the foundation of future molecular epidemiological studies of TB in Iraq.

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