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New insight into the molecular characterization of isoniazid and rifampicin resistant *Mycobacterium tuberculosis* strains from Saudi Arabia

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ABSTRACT

Data on the genetic variation of isolates of *Mycobacterium tuberculosis* and spectrum of mutations determining resistance to principal anti-tuberculosis drugs isoniazid (INH) and rifampicin (RIF) have not yet been studied in Saudi Arabia. One hundred and fifty-one clinical isolates of *M. tuberculosis* from different regions in the country showing resistance to RIF and INH were subjected to drug susceptibility testing, characterization of mutations conferring drug resistance and genotyping. Phenotypically 17 (11.3%) isolates were resistance to RIF, 75 (49.6%) were resistant to INH and 59 (39.1%) were resistant to both RIF and INH, respectively. Sixteen (10.6%), 74 (49%) and 56 (37.1%) were determined as resistant to RIF, INH and to both by line probe assay. High frequency of *rpoB* 531 mutations (67.1%) in RIF resistant strains and *katG* 315 mutations (65.2%) in INH resistant strains were found. Mutations responsible for INH resistance, *katG 315* (*P* value < 0.001, odds ratio: 1.81, 95% CI [1.51, 2.18]) and *inhA-15* (*P* value – 0.004, odds ratio: 1.48, 95% CI [1.22, 1.8]) were predominant among the newly diagnosed cases. Beijing strains were significantly associated with multi drug resistance and mutations in combination of *rpoB531* and *katG315* (*P* value – <0.001, odds ratio: 6.83, 95% CI [2.65, 17.58]). In addition multi drug resistance was significantly associated with treatment history (*P* value < 0.001, odds ratio: 3.16, 95% CI [2.14, 4.67]). Furthermore, a higher rate (39.3%) of clustering among the multidrug resistant strains particularly with Beijing family (52.9%) was observed. Saudi Arabia harbors highly diverse drug resistant *M. tuberculosis* population with an ongoing transmission which needs to be immediately managed.

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1. Introduction

After years of decline, Tuberculosis (TB) has re-emerged as a serious public health problem world wide, especially with increased drug resistance among *Mycobacterium tuberculosis* strains, which hinders the success of TB control programs (Palomino, 2006). The emergence of multidrug-resistant TB (MDRTB), defined as resistance to at least isoniazid (INH) and rifampicin (RIF), the two principal first-line anti-TB drugs, poses a serious threat to TB control. MDRTB reduces the response to standard short-course chemotherapy with first-line anti-TB drugs, leads to higher mortality and treatment failure rates, and increases periods of transmissibility of the disease (Schluger, 2000; Schneider and Castro, 2003). The development of drug resistance in *M. tuberculosis* isolates is the result of genetic mutations in particular genes (Zhang and Telenti, 2000). At least nine genes are generally known to be linked with resistance to first line anti TB drugs; *katG*, *inhA, ahpC* and *kasA* for isoniazid resistance, *rpoB* for rifampicin resistance, *rpsL* and *rrs* for streptomycin resistance, *embB* for ethambutol resistance and *pncA* for pyrazinamide resistance (Laurenzo and Mousa, 2011). There is mounting evidence that the genetic variability among clinical isolates may have dramatic consequences on the outcome of infections (Barczak et al., 2005). It is also believed that the genetic diversity of *M. tuberculosis* contributes to the wide spectrum of tuberculosis clinical presentations, including acute primary TB (localized or disseminated), latent disease and

Abbreviations: MIRU-VNTR, Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats; INH, isoniazid; RIF, rifampicin; MDRTB, multi drug resistant Tuberculosis.

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reactivation (Manabe et al., 2003; Marquina-Castillo et al., 2009). However, the molecular mechanisms for such variations in *M. tuberculosis* pathogenesis and host adaptation have been identified in only a few cases (O'Brien et al., 1994; Reed et al., 2004).

Saudi Arabia has a moderate incidence of active tuberculosis as per the recent World Health Organization (WHO) data: 24 cases per 1,00,000 populations. The new cases of MDRTB are reported on a rate of 1.2% and among previously treated pulmonary cases are 9% (WHO, 2011). An alarming rate (20%) of high prevalence of MDRTB was reported from the Western region of Saudi Arabia during 2001 (Khan et al., 2001). The annual influx of pilgrims, as well as a high proportion of migrant workers in the country, the majority of whom are from TB endemic countries, increases the opportunities of TB transmission (Al Kassimi et al., 1993; Milaat et al., 1994). In 2007, a nationwide epidemiological study conducted showed a rate of drug resistance of 19.7% and existence of 4.5% MDRTB (Al-Hajoj et al., 2007). The prevalence of mutations conferring drug resistance to *M. tuberculosis*, their genetic diversity and risk factors are still unknown in Saudi Arabia.

In this study we investigated a total of 151 clinical isolates of *M. tuberculosis* obtained from nine regions of Saudi Arabia, which showed resistance to INH and RIF. All isolates were subjected to drug susceptibility testing and characterization of mutations by line probe assay followed by genotyping using MIRU-VNTR (Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats) and spoligotyping. We studied the prevalence of mutations associated with INH and RIF resistance and determined their association with phylogenetic lineages and treatment history.

2. Materials and methods

2.1. Study samples

The study samples were selected from a collection of 1505 clinical isolates obtained from nine provinces of Saudi Arabia during 2002–2005 as part of a previous study (Al-Hajoj et al., 2007). Of the total, 151 clinical isolates which showed resistance to INH and/or RIF were enrolled into the study. These isolates were recovered from residents and citizens regardless of site of infection. The preliminary selection of samples was carried out based on the primary drug susceptibility results received from the TB referral laboratories of the provinces.

2.2. Identification and drug susceptibility testing

Identification of all the isolates was carried out by Genotype MTBC® Assay (Hain Life Science, Nehren, Germany) as per manufacturer's instructions. The drug susceptibility testing (DST) for INH and RIF were carried out by using BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems, Sparks, MD, USA) according to the manufacturer's protocol.

2.3. Extraction of genomic DNA and characterization of mutations

The genomic DNA was extracted from all isolates by using the QIAamp DNA Mini spin column (Qiagen, Hilden, Germany) method as per the manufacturer's instructions. Identification of mutations in *rpoB, katG* and *inhA* genes associated with resistance to RIF and INH was performed by using Genotype MTBDR *plus*® kit (Hain Lifescience, Nehren, Germany), a PCR based reverse blot hybridization method according to the manufacturer's protocol. Isolates with mutations in the *rpoB* gene were considered to be RIF resistant, and mutations in the *katG* and/or *inhA* genes to be INH resistant. Strains possessing mutations in both *rpoB* and *katG*/*inhA* were regarded as MDRTB.

2.4. Spoligotyping and MIRU VNTR typing

Of the 151 isolates, 146 with interpretable mutation results were subjected to MIRU-VNTR typing and spoligotyping. Spoligotyping was performed as previously described (Kamerbeek et al., 1997) with the commercial kit (Ocimum Biosolutions, Hyderabad, India). Multi locus (24 loci) VNTR typing was carried out by using the commercial kit (Genoscreen, Lille, France) according to the manufacturer's recommendations. Eight sets of triplex PCR were carried out to cover all 24 loci and the PCR products were subjected to capillary electrophoresis using DNA analyzer 3730 (Applied Biosystems, CA, USA).

2.5. Data analysis

The primary data of MIRU typing were analyzed for assigning the alleles by using the software Gene Mapper version 4.0 (Applied Biosystems, CA, USA). These results were transferred to the MIRU-VNTR Data Manager Version 1.0 (Genoscreen, Lille, France) and finally presented as numerical codes representing the alleles for each 24 loci. The membranes with the spoligo typing results were scanned and finally converted into octal numerical codes. The Spoligo and MIRU patterns were submitted to MIRU-VNTR Plus database (www.miruvntrplus.org) (Allix-Beguec et al., 2008). The lineages were assigned based on the strategies described previously (Allix-Beguec et al., 2008; Weniger et al., 2010). As spoligotyping results frequently show regular homoplasy issues they were not used to create the phylogenetic tree. Instead analysis was conducted only with MIRU-VNTR data. A phylogenetic tree was made separately for MDRTB to study the pattern of clusters. A cluster is defined when "two or more isolates exhibit identical MIRU alleles for all the 24 loci". The statistical analysis of the data was carried out by SPSS, version-13.0 (IBM Corporation, New York, USA). Pearson's Chi square test was carried out to prove the statistical associations and a difference of P value <0.05 was considered statistically significant.

3. Results

3.1. Demographics of study samples

The proportion of males was higher in this study (68.2%) compared to females (32.8%). The younger generations (age groups 16–29 and 30–44) showed the highest number of cases (34.4% and 41%), respectively. Cases of Saudi citizens were higher (57.6%) than the non Saudis (42.4%). A clear dominance of pulmonary infection was also observed (77.5%) in comparison with extra-pulmonary cases (22.5%). Majority (68.2%) of the study samples belonged to "New" cases compared to "previously treated" cases (31.8%) (Table 1).

3.2. Identification and drug susceptibility testing

All isolates (151) were tested and identified as *M. tuberculosis* by using Genotype® MTBC Assay (results not shown). Phenotypic drug susceptibility test results showed that 17 (11.3%) isolates were resistant to RIF, 75 (49.7%) to INH and 59 (39.1%) were MDRTB, respectively. Mono drug resistance was predominant among newly diagnosed cases. MDRTB cases were high among the patients with more than 1 month treatment history (Table 1). The line probe assay obtained 148 interpretable results. Mono resistance of RIF was found in 16 (10.6%) isolates. On the other hand INH mono resistance was observed in 74 (49%) isolates and MDRTB cases were 58 (38.4%) (Tables 1 and 2).

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