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Characterizing *Mycobacterium tuberculosis* isolates from Karachi, Pakistan: drug resistance and genotypes

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SUMMARY

Objectives: To study the prevalence, risk factors, and genotypes of drug-resistant *Mycobacterium tuberculosis* in Karachi.
Methods: Pulmonary tuberculosis (TB) patients were recruited in a cross-sectional study (2006–2009). Drug susceptibility testing was performed for culture-positive cases (n = 1004). Factors associated with drug resistance were evaluated using logistic regression analysis. Strains were typed using spoligotyping and mycobacterial interspersed repetitive units–variable number tandem repeat (MIRU–VNTR). The associations of genotype and drug resistance were explored using the Chi-square test.
Results: Resistance rates – new and previously treated – were as follows: multidrug-resistant (MDR)-TB, 2.4% and 13.9%, respectively; rifampin (RIF) monoresistance, 0.1% and 0.6%, respectively; any isoniazid (INH) resistance, 8.9% and 28.5%, respectively; and INH monoresistance, 3.0% and 6.3%, respectively. Prior TB treatment was a risk factor for MDR-TB (adjusted odds ratio (AOR) 6.8, 95% confidence interval (CI) 3.5–13.1) and INH monoresistance (AOR 2.4, 95% CI 1.1–5.2). Additional risk factors included low socioeconomic status for INH monoresistance (AOR 3.3, 95% CI 1.7–6.5), and belonging to Balouchi (AOR 9.2, 95% CI 2.5–33.4), Sindhi (AOR 4.1, 95% CI 1.2–13.5), or Pakhtun (AOR 3.4, 95% CI 1.0–11.2) ethnicity for MDR-TB. Although Central Asian strain (55.6%) was the most prevalent genotype, MDR-TB was significantly associated with Haarlem (H) genogroup (crude OR 9.2, 95% CI 3.6–23.8).
Conclusions: An MDR-TB rate of 2.4% is reported in new patients. Low RIF monoresistance supports the use of RIF as a marker for MDR-TB in this population. The need to strengthen TB care in the identified at-risk groups is emphasized. Based on INH resistance rates, a review of national treatment/prevention regimens relying on INH is suggested.

1. Introduction

Emerging drug resistance poses a serious threat to the control of tuberculosis (TB), which remains a leading infectious cause of death worldwide.[1,2] The World Health Organization (WHO) estimated 440 000 (95% confidence interval (CI) 390 000–510 000) cases of multidrug-resistant (MDR)-TB globally in 2008 (3.6% of all incident TB cases), causing an estimated 150 000 deaths.[3] Pakistan ranks eighth on the list of 27 high MDR-TB burden countries, with an estimated 15 000 cases occurring in 2008 (2.9% in untreated and 35.4% in treated patients).[4] A hospital-based study from Pakistan reported an MDR-TB prevalence of 1.8% amongst untreated TB patients.[5] In the absence of a national prevalence survey, information from community-based studies is required to help estimate the burden of MDR-TB in the country. Globally, resistance to isoniazid (overall) is estimated at 13.3% (10.3% in new and 27.7% in treated patients).[6] Resistance to isoniazid (INH) has also been reported from neighboring India (11% in new and 37% in treated patients)[7] and Bangladesh (23% in new patients).[8] A three-drug regimen, i.e., INH, rifampin (RIF), and ethambutol (EMB), is recommended during the continuation phase for new TB patients in populations with high levels of INH resistance.[9] In view of limited local information on INH resistance in untreated patients, the national guidelines in Pakistan currently advocate INH and RIF during the continuation phase.[10] However, local INH resistance data are essential for the selection of an appropriate regimen for such patients. This information will also help in deciding the role of INH prophylactic therapy for patients with latent TB in this area.

Over the last 10 years, molecular typing of *Mycobacterium tuberculosis* (MTB) has emerged as an important tool for estimating

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ongoing TB transmission.[11] Spacer oligonucleotide typing (spoligotyping) of MTB strains from Pakistan has shown a heterogeneous population structure, with a predominance of the Central Asian Strain (CAS) genotype.[12–14] An association between MDR-TB and Beijing strains has also been reported from the country.[12,13] These studies have established the baseline diversity and population structure of MTB isolates from Pakistan. Given a population of over 160 million, an expansive and diverse terrain, and multiple ethnic groups, there is a need to study strains from defined geographic areas within Pakistan.

An earlier interim analysis of this study reported the prevalence of and risk factors associated with MDR-TB.[15] These were reassessed in light of this completed study and a larger sample size. Moreover, the prevalence of INH resistance and risk factors for INH-monoresistant TB were determined. Prevalent MTB genotypes and their associations with drug resistance were also investigated.

2. Methods

This was a cross-sectional study conducted in Karachi from July 2006 to September 2009. Karachi is the economic hub and largest city of Pakistan, with a population of around 17 million. It is divided into 18 administrative units.[16] The study subjects were recruited from 10 field clinics run by the Marie Adelaide Leprosy Centre (MALC) located in 10 of the 18 administrative units of Karachi, as previously described.[15] These clinics are accessible to the population from across the city, including individuals from the low to middle socioeconomic groups. They provide care to a mixed population representing all the major ethnic groups of the city. The clinic patients are either self-referred or are referred by their general practitioners. All patients presenting to the clinics with a clinical suspicion of pulmonary TB during the study period and who consented to participation were recruited into the study and interviewed on their initial visit. A pre-tested structured questionnaire was administered to glean information on socio-demographic and other characteristics. Early morning sputum specimens were collected for smear examination, culture, and drug susceptibility testing.

Drug susceptibility was tested by agar proportion method on enriched Middlebrook 7H10 medium (BBL) using the following drug concentrations: RIF 1 μg/ml and 5 μg/ml, INH 0.2 μg/ml and 1 μg/ml, streptomycin (STR) 2 μg/ml and 10 μg/ml, EMB 5 μg/ml and 10 μg/ml, ofloxacin 1 μg/ml, amikacin 6 μg/ml, kanamycin 6 μg/ml, and capreomycin 10 μg/m. Pyrazinamide (PZA) sensitivity testing was carried out using the BACTEC 7H12 medium pH 6.0 at 100 g/ml (BACTEC™ PZA Test Medium, Becton Dickinson, USA); MTB H37Rv was used as control with each batch of susceptibility testing. Resistance to RIF 1 μg/ml, INH 0.2 μg/ml, STR 2 μg/ml, and EMB 5 μg/ml was used as the cut-off for this study. MDR-TB was defined as a TB isolate resistant to at least INH and RIF.[3] Extensively drug-resistant (XDR)-TB was defined as MDR isolates with additional resistance to a fluoroquinolone (ofloxacin) and to any one of the second-line injectable anti-TB drugs (amikacin, capreomycin, or kanamycin).[3]

2.1. Genotyping methods

2.1.1. Spoligotyping

Culture-positive isolates were typed by spoligotyping using a commercially available kit (Isogen Bioscience BV, Maarssen, the Netherlands).[17] Results were analyzed using BioNumerics software (BioSystematica, UK). Dendrograms were generated using the unweighted pair group method with arithmetic averages (UPGMA) calculation. The spoligotypes were compared with the most prevalent MTB subfamilies as identified by the World Spoligotyping Database SpolDB4.0 of the Pasteur Institute, Guadeloupe.[18]

2.1.2. Mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) typing

MDR-TB isolates were further genotyped by PCR amplification of the 15 MIRU-VNTR loci, as previously described by Supply et al.[19] PCRs were carried out using 40–60 ng of DNA per reaction in 25 μl volume using 0.4 μM specific primers, 0.5 mM dNTPs mix, 1 mM $MgCl_2$, 1× PCR buffer, 4% dimethyl sulfoxide (DMSO), and 1 U of Super Tth Taq DNA polymerase. PCR was performed as follows: 15 min at 95 °C, 35 cycles, 1 min at 94 °C, 1 min at 59 °C, 1 min 30 s at 72 °C, 10 min at 72 °C. The PCR products were electrophoresed on a 3% agarose gel and sized with a 100-bp ladder (Promega). All the reactions were performed in duplicate using standard positive and negative controls supplied with the MIRU-VNTR validation kit. Sizing of the PCR fragments and assignment of the various VNTR alleles were also done using the standard protocol (Philip Supply INSERM U629, Institut de Biologie/Institut Pasteur de Lille; May 2005; http://www.genoscreen.com). Results of the MIRU-VNTR typing were analyzed using BioNumerics software (BioSystematica, UK).

2.2. Data analysis

Data were double-entered in EpiData and compared for errors. Analysis was done on 1004 culture-positive specimens using SPSS version 19 (IBM SPSS, Chicago, IL, USA). Descriptive statistics were computed for all variables. Means (± standard deviation) were calculated for continuous variables, while frequencies with percentages were calculated for categorical variables.

The prevalence of MDR-TB and INH-resistant TB with 95% CI was calculated. Socio-demographic and other factors associated with MDR-TB and INH- monoresistant TB were evaluated using the Chi-square test of independence. Adjusted odds ratios (AOR) and their 95% CI were estimated using logistic regression analysis.

Patients with MDR and INH-monoresistant MTB were compared to patients with drug-susceptible MTB strains. Individuals with resistance patterns other than MDR and INH-monoresistant TB were excluded from the analysis. Variables with a p-value of <0.25 in the univariate analysis were considered for multivariable analysis. The final model was constructed after checking for confounding and interaction. Model adequacy checks were performed. Variables with a p-value of <0.05 were retained in the final model. INH-monoresistant TB was defined as resistance to only INH.

Some independent variables were re-coded into meaningful categories, while a few new variables were developed. A variable for crowding index was constructed by dividing the number of individuals per household by the number of bedrooms. A participant's household crowding was defined as 'low' if they scored an index of 0–1.9, moderate if 2–3.9, and high if $\geq$4. Another variable termed socioeconomic status was constructed based on the possession of household commodities in accordance with the National Health Survey of Pakistan 1990–1994 criteria. Ethnicity was assessed using mother tongue as the proxy variable.

The association of strain types and drug resistance was explored using the Chi-square test of independence. Crude odds ratios (COR) were calculated for significant associations found.

3. Results

3.1. Study population

A total of 1229 subjects with a clinical suspicion of pulmonary TB were recruited. Specimens from 16 cases grew *Mycobacterium* species other than MTB, while 209 samples were culture-negative. These 225 samples were excluded from the analysis. MTB culture-positive patients (n = 1004) were studied. The mean age of the