Patterns of Drug Resistance and RFLP Analysis of Mycobacterium tuberculosis Strains Isolated from Recurrent Tuberculosis Patients in Sri Lanka

Article *in* The Southeast Asian journal of tropical medicine and public health · May 2010

Source: PubMed

CITATIONS
10

READS
82

4 authors, including:

Dhammika Nayoma Magana-Arachchi
National Institute of Fundamental Studies - Sri Lanka
54 PUBLICATIONS **139** CITATIONS

SEE PROFILE

Naduviladath Chandrasekharan
University of Colombo
114 PUBLICATIONS **2,245** CITATIONS

SEE PROFILE

PATTERNS OF DRUG RESISTANCE AND RFLP ANALYSIS OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS ISOLATED FROM RECURRENT TUBERCULOSIS PATIENTS IN SRI LANKA

DN Magana-Arachchi[1], AJ Perera[1], V Senaratne[2] and NV Chandrasekharan[3]

[1]Department of Microbiology, Faculty of Medicine; [2]Central Chest Hospital, Welisara, Ragama; [3]Department of Chemistry, Faculty of Science, University of Colombo, Colombo, Sri Lanka

Abstract. The aim of the study was to determine drug sensitivity and DNA fingerprints of *Mycobacterium tuberculosis* strains from retreatment cases of pulmonary tuberculosis. The study population consisted of 131 culture positive, retreatment tuberculosis patients admitted to the Chest Hospital, Welisara, Sri Lanka who had taken anti-tuberculosis drugs previously. Forty-eight percent of the isolates were susceptible to all 12 drugs tested. Twenty isolates were resistant to first line drugs, 28 to both first and second line drugs and 17 to second line drugs. Forty-six percent were resistant to a single drug, 23% to two and 19% to 3 drugs, respectively. Resistance to *p*-aminosalicylic acid (15%) was most common followed by ethambutol (14%), isoniazid and pyrazinamide (12%). Multi-drug resistance was present in four isolates. Using RFLP analysis the copy number and IS *6110* element in *M. tuberculosis* strains varied from one to seven, the majority having 3 to 5 copies. The prevalence of acquired drug resistance to individual drugs was comparatively lower except resistance to ethambutol. The majority of retreatment patients belonged to the defaulter category and this stresses the importance of implementing directly observed treatment short course and susceptibility testing of isolates in retreatment TB patients to prevent the spread of drug resistance. By using the IS *6110* genetic marker it was possible to differentiate most of the *M. tuberculosis* isolates. However, for an unambiguous confirmation of the identities of strains, additional genetic markers should be employed in strain typing such as spoligotyping.

Key words: *Mycobacterium tuberculosis*, drug resistant patterns, RFLP analysis, recurrent TB patients, Sri Lanka

INTRODUCTION

Mycobacterium tuberculosis is an extremely successful pathogen that kills nearly 2 million people in the world each year (WHO, 2007). The study of tuberculosis (TB) epidemiology and transmission, traditionally involving patient contact tracing, has been improved by the use of molecular strain typing (Mathema *et al*, 2006). Molecular epidemiological studies have added much-needed accuracy and precision to the study of transmission

Correspondence: DN Magana-Arachchi, Institute of Fundamental Studies, Hantana Road, Kandy, KY20000, Sri Lanka.
Tel:+94- 081-2232002; Fax: 94-081-2232131
E-mail: nayomam@yahoo.com

dynamics, and have allowed previously unresolved issues to be newly addressed, such as the classification of recent-versus-reactive disease, the extent of exogenous reinfection and the detection of unsuspected transmission events (Mathema *et al*, 2006). Advances in molecular typing have led to the identification of highly transmissible *M. tuberculosis* complex strains in the last few years. Although TB has been treated effectively, the emergence of drug resistant TB strains has become a global threat to TB prevention and control efforts. Currently, the major threat to TB control is the transmission of extensively drug-resistant (XDR) strains (Gandhi *et al*, 2006; Samper and Martin, 2007). The successful treatment of TB depends upon the selection of an effective chemotherapeutic regimen. Therefore, the aim of the study was to determine the patterns of drug resistance and the DNA fingerprints of *M. tuberculosis* isolated from re-treatment TB patients in Sri Lanka.

MATERIALS AND METHODS

One hundred seventy sputum smear-positive TB patients admitted for re-treatment to Chest Hospital, Welisara, Sri Lanka were enrolled in the study. There were 24 patients among the chest clinic attendees having a history of imprisonment before being diagnosed as having TB (ex-prisoners). The study population consisted of 131 culture-positive re-treatment TB patients. The remaining patients were excluded, as their cultures were negative. Of the 131 patients, 38 cases were relapses after WHO category 1 or category 2 treatment, 92 were defaulters and the remaining patient was a treatment failure. Sputum culture for acid-fast bacilli and DST were carried out at the Department of Microbiology, Faculty of Medicine of the University of Colombo, Sri Lanka and the ethical clearance for the study was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo.

Sputum samples were decontaminated using the standard sodium hydroxide - sodium citrate - N acetyl - L - cysteine method and were inoculated on Lowenstein-Jensen (LJ) medium and Middle Brook 7H-10 agar medium to isolate the *M. tuberculosis* strains (Kubica and Dye, 1963). The strains of *M. tuberculosis* obtained from these media were used for antibiotic sensitivity testing and restriction fragment length polymorphism (RFLP) analysis.

Antibiotic sensitivity testing

Criterion for resistance was based on 1% survival level of the organism in comparison with a control medium without the drug. Resistance was defined as survival of the tubercle bacilli at the following drug concentrations (μg/ml), isoniazid (H), 0.2; rifampin (R), 1.0; streptomycin (S), 2.0; ethambutol (E), 5.0; pyrazinamide (Z), 25.0; *p*-amino salicylic acid (PASER), 2.0; ethionamide (Et), 5.0; cycloserine (Cs), 30.0; kanamycin (Km), 5.0; viomycin (Vm), 5.0; ciprofloxacin (Cx), 2.0 and rifabutin (Rb), 2.0.

IS*6110*-RFLP

RFLP analysis of the 131 isolates by Southern blotting and DNA hybridization with IS*6110* probe was performed according to the standard fingerprinting method (van Embden *et al*, 1993).

RESULTS

Among the 131 patients 63 (48%) were susceptible to all 12 drugs tested and drug resistance was observed in 68 (52%) of the isolates. Eighty-three (63%) isolates were

susceptible to all five first line drugs H, R, E, Z and S of the WHO re-treatment course. Among the resistant isolates, 20 were resistant to first line drugs, 28 to both first and second line drugs of Et, Cs, PASER, Km and Cx while 17 were resistant to second line drugs only. The remaining 3 were resistant to Vm (2) and Rb (1).

Table 1
Resistance to anti-tuberculosis drugs of 131 *M. tuberculosis* isolates.

Drug[a]	Number of resistant isolates	Percentage resistance
H	16	12.2
R	10	7.6
E	19	14.5
S	13	9.9
Z (pH-5.5)	16	12.2
PASER	20	15.3
Et	15	11.4
Cs	15	11.4
Km	9	6.8
Cx	7	5.3
Vm	2	1.5
Rb	1	0.7

[a]The full names of the drugs are indicated in the text.

Pattern of resistance to first line and second line anti-TB drugs between defaulters and relapses were not significantly different. Among the isolates, 31 (46 %) were resistant to a single drug, 16 (23%) to two drugs and 13 (19%) to three drugs. Four isolates (6%) were resistant to four drugs, another two (3%) were resistant to five, and from remaining two isolates (3%) one was resistant to seven and the other to eight drugs.

In tested isolates, resistance to PASER (15.3%) was the most common followed by E (14.5%), H and Z (12.2%) (Table 1). Multidrug resistance was present in 4 (3%) isolates. Among the 48 strains resistant to first line drugs, 30 (23%), 12 (9%), and five (4%) isolates were resistant to one, two and three drugs of the five drug combination, respectively. A single isolate was resistant to all five first line drugs. Among the 83 isolates that were susceptible to all first line drugs there were 20 strains that showed resistance to second line drugs.

Table 2 summarizes the numbers of IS copies found in the strains investigated. The copy number of IS *6110* element in *M.tuberculosis* strains varied from 1 to 7, the majority having 3 to 5 copies. Among

Table 2
Comparison of IS element IS *6110* copy numbers in *M. tuberculosis* isolates.

Number of IS *6110* copies	Number of strains (*N* =130)	
	Ex-prisoners *n* = 24 (%)	General population *n* = 106 (%)
1	6 (25)	13 (10)
2	5 (21)	11 (9)
3	5 (21)	24 (19)
4	2 (8)	22 (17)
5	4 (17)	20 (16)
6	2 (8)	10 (8)
7	0 (0)	6 (5)

Table 3
Comparison of drug resistant pattern and IS element (IS *6110*) copy numbers in *M. tuberculosis* isolates.

Drug[a]	Copy number						
	1	2	3	4	5	6	7
H	1		5	1		2	2
R		1			3	1	
Z	1	1	2	2	4	2	
S	1	1	2			3	1
E	1	4	5	4			1
PASER	2	3	5	4		4	1
Et	1	4	4			1	1
Km			1	3		1	
Cs		1	4	1	3		
Rb					1		
Vm				1	1		
Cx			1	1	3	1	

[a]The full names of the drugs are indicated in the text.

the strains tested one strain from the general population lacked the IS *6110* element.

Pattern of drug resistance and IS *6110* copy number are shown in Table 3. According to the RFLP patterns of the isolates, five sets of isolates had similar banding patterns. However, in antibiotic sensitivity testing in one set, one strain was resistant to drug Z while the other was sensitive to all tested drugs. In the second set, one was sensitive to all drugs while the other strain was resistant to Et. The remaining three pairs of isolates, which had identical RFLP patterns, were sensitive to all drugs tested.

DISCUSSION

In this study, sputum direct smear and culture positive patients in Sri Lanka who had received prior treatment for TB were examined to determine DNA fingerprinting and pattern of drug resistance developed against the first and second line drugs treatments. It was found that acquired drug resistance was high (52%). However, the incidence of multidrug resistant TB was low (3%). The prevalence of acquired drug resistance to individual drugs was comparatively lower in Sri Lanka compared to other countries except E resistance (14.5%). R resistance has been observed most frequently in association with resistance to H (Bloch *et al*, 1994) but the resistance to R and susceptibility to H isolates has also been recognized through surveillance (Ridzon *et al*, 1998). Resistance pattern observed for R in our study is similar, where six out of the 10 R resistant strains did not show resistance to H. Another interesting factor is the low incidence of cross-resistance between R and Rb. There were nine isolates, which were resistant to R but susceptible to Rb, while a single isolate showed resistance to both drugs. This is comparable to a study in Turkey (Cavusoglu *et al*, 2004), where Rb remains active against R resistant strains

with certain genetic alterations. A limitation to our study was that we were unable to test the drugs H, S and E at two different concentrations.

The other notable factor in our study was the presence of significant numbers of bacterial strains resistant to second line drugs without being previously exposed to them. Furthermore, there were strains that were resistant to second line drugs without showing resistance to first line drugs. This is similar to the report by CDC (2006), which recorded multiple cases of TB with resistance to virtually all second line drugs. Therefore, it is difficult to predict the response to treatment without performing an antibiotic sensitivity test. According to the WHO guidelines for the management of drug resistant tuberculosis (Croffton *et al*, 1997) recurrent TB patients can be subdivided into three groups. They are patients excreting bacilli susceptible to all anti-TB drugs, patients excreting bacilli resistant to H but susceptible to R and those excreting bacilli resistant to at least H and R. Therefore, theoretically it is possible for 97% of our study population to recover from TB by using the WHO retreatment regimen under supervision. In this study, although the number of patients with MDR strains was few, the mono-drug and poly-drug resistance to first line drugs were quite high. Hence, in the evaluation of drug resistance, consideration of resistance to drugs other than H and R is recommended.

According to the findings 14% of the isolates were resistant, at least to two of the five-drug regimen, and therefore such patients require the use of second line anti-TB drugs in their chemotherapy. WHO recommends that the drugs prescribed in re-treatment (which the patients have not had previously in the initial regimen) should consist of at least three drugs, preferably 4 or 5, to which the bacilli are likely to be fully sensitive. Wide variation in drug sensitivity patterns in our study indicates the necessity to have an antibiotic sensitivity test before instituting treatment for recurrent TB patients.

The presence of a strain resistant to eight drugs, which was similar to the extensively drug resistant (XDR) strains, signals the possibility of untreatable TB. Although the use of PASER and Et in primary tuberculosis treatment had been discontinued a decade ago in Sri Lanka, the study showed that some of the patients had strains, which are resistant to these drugs. A similar observation was recorded in Peru (Saravia *et al*, 2005), in which more than half of Category I failures with MDR-TB had strains also resistant to S, a drug they had never received. In Sri Lanka up to 1989, treatment regimen of pulmonary tuberculosis consisted of 18 months therapy with the daily administration of H, Et and S for 2-3 months and then H and Et for 16 months or bi-weekly admission of H, Et and S or PASER for 3 months followed by H and S for next 15 months. Although this treatment regime may have attributed to the decline in TB in the country, it may also have contributed to the drug resistant strains and relapses, due to poor patient compliance. Of the patients tested, 43% had initial treatment prior to 1989.

In this study, patients with documented positive culture results and not those with negative culture results who continued to have clinical features suggestive of pulmonary tuberculosis were included. However, non-viable specimens are more likely to be resistant to H, R and other first line drugs (Mitike *et al*, 1997); thus, the actual proportion of patients with resistant isolates is likely to have been higher. The present study reveals that the

majority of the re-treatment TB patients belong to the defaulter category and therefore this stresses the importance of implementing directly observed treatment short course (DOTS) in the island, to prevent recurrence of infection. Effective tuberculosis control programs using standardized regimens and directly observed therapy can decrease the level of acquired drug resistance in a community.

In Sri Lanka drug sensitivity testing on initial isolates of *M. tuberculosis* is not routinely practiced. In the case of category 2 treatments, sensitivity testing is carried out for first line drugs only. However, due to the wide variation in patterns of drug resistance observed in this study, it is recommended that sensitivity tests for both first and second line drugs be carried out before instituting treatment for re-treatment TB patients. Despite its greater initial cost (Croffton *et al*, 1997) it is important to implement DOTS and carry out DST in the case of recurrent TB patients to prevent the spread of drug resistance for which an effective national TB program is necessary.

RFLP analysis showed that the majority of circulating *M. tuberculosis* strains in Sri Lanka belong to a limited number of families, the degree of IS *6110* DNA polymorphism among strains was high with a low copy number. In this study, 68% of the isolates had less than five copies. This pattern is similar to that of other countries in the Asian and Indian Ocean regions, such as India, Malaysia, Oman, Hong Kong, Madagascar and Mozambique (van Soolingen *et al*, 1996; Razanamparany *et al*, 2009). However, in the absence of DNA sequencing analysis, conclusions could not be made whether these isolates were genetically different or they underwent any genetic changes within a given time. In our study, there were five pairs of isolates, which had identical banding patterns. However, the pattern of drug resistance in the two strains of two sets was different. These isolates were collected from patients coming from different districts but in the same province. Although they came to the same hospital for treatment, the strains were unlikely to be epidemiologically related. The findings of Gillespie *et al* (2000) showed that non-random association of IS *6110* with *M. tuberculosis* could result in false positive clustering in unselected collections of isolates. Among the strains tested, one strain lacked the IS *6110* element. Previous studies showed that *M. tuberculosis* strains carrying one or few IS *6110* copies are often difficult to differentiate by IS *6110* standard RFLP analysis because of a site specific preference for insertion of the IS element. Therefore, to differentiate the strains other genetic markers, such as polymorphic rich GC repetitive sequence and direct repeats have been used (van Soolingen *et al*, 1993; Bauer *et al*, 1999). In this study for DNA fingerprinting restriction enzyme *Pvu* II was used to digest chromosomal DNA of the mycobacterial strains. The enzyme cleaves the 1.35 - kb IS *6110* element at a single site. The 541 bp DNA probe used for the hybridization corresponds to a piece of the IS *6110* element and the *Pvu* II site is located within that region. By using this DNA probe all of the possible IS *6110* containing restriction fragments are visualized and when analyzing the fingerprints two bands were considered as a single copy. Therefore, the DNA probe used could differentiate the *M. tuberculosis* strains carrying one or few IS *6110* copies.

This is the first study in Sri Lanka in which the RFLP pattern of *M. tuberculosis* strains in a population has been examined along with the drug sensitivity patterns. By using the genetic marker of IS *6110* it

was possible to differentiate most of the *M. tuberculosis* isolates. However, for an unambiguous confirmation of the identities of strains, additional genetic markers should be employed in strain typing such as spoligotyping. Therefore, establishment of this method of analysis in Sri Lanka will not only be useful to study strain variations in epidemiological analysis but may be used beneficially in treatment of patients as well as identifying patients carrying drug resistance strains.

REFERENCES

Bauer J, Andersen AB, Kremer K, Miorner H. Usefulness of spoligotyping to discriminate IS *6110* low copy number *Mycobacterium tuberculosis* complex strains cultured in Denmark. *J Clin Microbiol* 1999; 37: 2602-6.

Bloch AB, Cauthen GM, Onorato IM, *et al*. Nationwide survey of drug-resistant tuberculosis in the United States. *JAMA* 1994; 271: 665-71.

Cavusoglu C, Karaca-Derici Y, Bilgic A. *In-vitro* activity of rifabutin against rifampicin - resistant *Mycobacterium tuberculosis* isolates with known *rpo B* mutations. *Clin Microbiol Infect* 2004; 10: 662-5.

Centers for Disease Control. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second line drugs worldwide, 2000-2004. *Morbid Mortal Weekly Rep* (*MMWR*) 2006; 55: 301-5.

Croffton J, Chaulet P, Maher D. Guidelines for the management of drug resistant tuberculosis. Geneva: WHO, 1997.

Gandhi NR, Moll A, Sturm AW, *et al*. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 2006; 368: 1575-80.

Gillespie SH, Dickens A, McHugh TD. False molecular clusters due to non - random association of IS6110 with *Mycobacterium tuberculosis*. *J Clin Microbiol* 2000; 38: 2081-6.

Kubica GP, Dye WE, Cohn ML, *et al*. Sputum digestion and decontamination with N-acetyl L-cysteine as a sputum digestant for the isolation of mycobacteria. *Am Rev Respir Dis* 1963; 89: 284-6.

Mathema B, Kurepina NE, Bifani PJ, *et al*. Molecular epidemiology of tuberculosis: current insights. *Clin Microbiol Rev* 2006; 19: 658-85.

Mitike G, Kebede D, Yeneneh H. Prevalence of antituberculosis drug resistance in Harar Tuberculosis Centre, Ethiopia. *East Afr Med J* 1997; 74: 158-61.

Razanamparany VR, Ramarokoto HH, Vololonirina EJ, *et al*. RFLP clusters of *Mycobacterium tuberculosis* strains from the Indian Ocean Region: local and South Asian characteristics. *Mem Inst Oswaldo Cruz* 2009; 104: 441-3.

Ridzon R, Whitney CG, McKenna MT, *et al*. Risk factors for rifampin mono-resistant tuberculosis. *Am J Respir Crit Care Med* 1998; 157: 1881-4.

Samper S, Martin C. Spread of extensively drug-resistant tuberculosis. *Emerg Infect Dis* 2007; 13: 647-8.

Saravia JC, Appleton SC, Rich ML, Sarria M, Bayona J, Becerra MC. Retreatment management strategies when first-line tuberculosis therapy fails. *Int J Tuberc Lung Dis* 2005; 9: 421-9.

van Embden JD, Cave MD, Crawford JT, *et al*. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; 31: 406-9.

van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *M.tuberculosis*. *J Clin Microbiol* 1993; 31: 1987-95.

World Health Organization. Global tuberculosis control: surveillance, planning, financing. WHO report 2007. *WHO/HTM / TB/2007.376*. 2007.