Egyptian Journal of Chest Diseases and Tuberculosis (2014) **63**, 369–375

The Egyptian Society of Chest Diseases and Tuberculosis

Egyptian Journal of Chest Diseases and Tuberculosis

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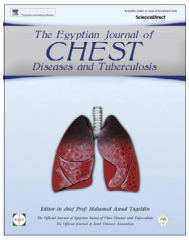

ORIGINAL ARTICLE

DNA fingerprinting and drug resistance patterns of active pulmonary *Mycobacterium tuberculosis* in Mansoura hospitals, Egypt

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Received 5 January 2014; accepted 26 January 2014
Available online 22 February 2014

KEYWORDS

Mycobacterium tuberculosis;
PCR;
RFLP;
Egypt;
DNA fingerprinting

Abstract Increased application of DNA fingerprinting has advanced the understanding of the dynamics of TB epidemiology. Typing of MTB is important for case tracing, and identifying community outbreaks.

Objective: We aim to detect pattern of drug resistance and molecular genotypes of MTB at Mansoura hospitals using PCR-RFLP.

Methodology: 123 sputum samples obtained from ZN smear positive cases were cultivated on Lowenstein Jensen (LJ) medium, out of them 67 specimens (54.5%) were positive culture. Genotypic analysis was done by the RFLP method after DNA extraction and PCR amplification. The susceptibilities to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol (EMB) were determined by the indirect nitrate reductase assay.

Results: The identified restriction patterns yielded 3 bands with different sizes and revealed 3 genotypes only. Restriction patterns are equal to 245/125/100 bp, and 245/125/80 bp fragments for Bst EII digests and 155/140/60 bp (MTC), 155/110/70 bp (intracellulare) and 160/140/70 bp (malmoense) fragments for Hae III digest. Genotyping of MTB detected that MTC was the commonest genotype

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Peer review under responsibility of The Egyptian Society of Chest Diseases and Tuberculosis.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejcdt.2014.01.014

among studied cases 49/67 (73.1%), followed by *Mycobacterium intracellulare* 14/67 (20.9%), while *Mycobacterium malmoense* had the least incidence 4/67. There was significant increased risk of resistance to STM, RIF, ETH and INH with *M. intracellulare* ($p = 0.021$, $p < 0.001$, $p = 0.001$, $p < 0.001$) while MTC showed lower resistance to STM, RIF, ETH and INH and all *M. malmoense* isolates showed no resistance.

Conclusion: PCR-RFLP was found to be a simple and reproducible method for genotyping of MTB strains and for early detection of *Mycobacterium tuberculosis* infection.

Introduction

Tuberculosis is more prevalent in developing countries, in which up to 95% of cases occur [1]. It remains a major challenge to global public health in the 21st century, especially with the steady increase in drug resistant TB [2]. The key of controlling the spread of tuberculosis includes proper case finding, rapid diagnosis, immediate initiation of effective therapy and contact tracing to arrest further transmission [3]. Recent developments in DNA technology and molecular biology methods have led to rapid detection of mycobacterial DNA by nucleic acid amplification [4]. Genotyping methods of TB include DNA fingerprinting, RFLP based on the polymorphism of the insertion sequence IS6110 among MTB complex strains, fingerprinting based on the polymorphic GC rich sequence (PGRS), Spoligotyping and variable-number tandem repeats (VNTR) typing based on the analysis of polymorphisms in the DR locus. Among these methods, the IS6110 fingerprinting is the recommended standard primary genotyping method and has been used routinely worldwide [5].

The increased application of DNA fingerprinting has advanced the understanding of the dynamics of TB epidemiology. The typing of *Mycobacterium tuberculosis* strain is important for case tracing, distinguishing between relapse and reinfection by an exogenous strain and identifying nosocomial, institutional and community outbreaks. Also it can be applied to confirm recent transmission of tuberculosis in a linked population or lab cross contamination [6]. Clinical isolates of MTB obtained from patients infected by the same strain of bacillus usually exhibit identical or very similar fingerprint patterns, and the patients are more often found to be epidemiologically linked [5]. Restriction fragment length polymorphism (RFLP) techniques have been developed for several mycobacterial genes but the one most investigated and validated is heat shock protein (hsp65). The restriction patterns are highly distinct by this method and can be identified visually [7]. Moreover the reliability of PCR-RFLP results with one enzyme is augmented if the results are confirmed with a second restriction enzyme [8].

Restriction fragment length polymorphism (RFLP) techniques have been developed for several mycobacterial genes but the one most investigated and validated is heat shock protein (hsp65). The restriction patterns are highly distinct by this method and can be identified visually [7]. Moreover the reliability of PCR-RFLP results with one enzyme is augmented if the results are confirmed with a second restriction enzyme [8]. The 65 kDa hsp65 is one of the major immunoreactive proteins of the mycobacteria. In 1993, a method for differentiating among slow growing mycobacterium species by hsp65 gene based restriction fragment length polymorphism (RFLP) analysis was developed using simple DNA extraction followed by a PCR step based on amplification of a 439 bp fragment of hsp65 gene involving genus specific primers [9].

Aim of work

The aim of this study is to detect pattern of drug resistance and molecular genotypes of *M. tuberculosis* at Mansoura hospitals using restriction fragment length polymorphism RFLP.

Subjects and methods

This study was carried out on 123 ZN positive sputum samples collected from pulmonary TB patients either new patients never treated or were previously treated after permission from human ethics committee at the university and the relevant authorities during the approval of the proposal. All cases were cultivated on Lowenstein Jensen (LJ) medium, out of them 67 specimens (54.5%) were culture positive. They were 51 males and 16 females and their age ranged from 15–70 years. Patients were selected from Mansoura University Hospitals and Mansoura Chest Hospital.

The patients were subjected to – full history taking especially for past history of similar conditions and intake of anti-TB drugs From all patients, sputum samples were collected and subjected to the following microbiological examinations:

1. Mycobacterial cultures on Lowenstein-Jensen (LJ) media.
2. Antimycobacterial susceptibility tests (AST) were done by indirect nitrate reductase assay (indirect NRA) for streptomycin (STM), isoniazid (INH), rifampicin (RIF) and ethambutol (ETH).

Genotypic analysis

It included three main steps:

1- DNA extraction to release DNA from mycobacterial cells.
2- Amplification of the target part of gene by PCR and detection by agarose gel electrophoresis.
3- RFLP analysis of the amplified DNA.

1-DNA extraction [10]

d. DNA extraction

Chromosomal DNA was extracted from growth harvested from surface of Lowenstein Jensen (LJ) medium by the simple boiling method. In short, few colonies were removed and suspended in 500 µl of sterile double distilled water and was boiled for 10 min. After centrifugation at 12000*g* for 3 min, 5 µl of supernatant was used for the PCR.

2-DNA amplification [11]

439 bp fragment of hsp65 gene was amplified by using Tb11 genus specific primers and restriction enzyme analysis for digestion of products with HaeIII & BstEII enzymes was employed.

Primer sequence

Forward primer: Hsp (5′ ACC AAC GAT GGT GTG TCC AT 3′).
Reverse primer: HSP (5′ CTT GTC GAA CCG CAT ACC CT 3′).

3- rflp [12]

Materials

(1) External DNA markers (50 bp).
(2) Restriction enzymes (HaeIII, BstEII) and digestion buffer (Biolabs, New England).
(3) Amplified PCR product.Restriction enzyme analysis: Bst EII and Hae III restriction enzymes
(4) The interpretation of results was done according to the following diagram

Results

Demographic data, smoking, and history of treatment of the 67 culture positive pulmonary TB patients were 51 (67.1%) males and 16 (23.9%) females, represent 27 (40.3%) new cases, and 40 (59.7%) previously treated cases, with age ranged from 15 to 70 years (mean age was 41.1 years). Cough was the commonest clinical presentation in 50/67 (74.6%) followed by fever in 39/67 (58.2%), then weight loss in 36/67 (53.7%), and chest pain in 27/67 (40.3%). Hemoptysis was the least presenting feature in the studied patients in 19/67 (28.4%). Mean tuberculin test diameter was 18.6 mm in all cases while Mean ESR was 39.1 mm at first hour and 70.94 mm at second hour (data not shown). Table 1 shows MTB genotypes and restriction of amplified DNA product that were done by BST E II enzyme which yielded 2 patterns of bands 245/125/80 bp and 245/125/100 bp and Digestion by Hae III enzyme gave 3 patterns of restriction bands 160/140/70 bp, 155/110/60 bp and 155/140/70 bp. For more clarification:

1. BST EII enzyme yield 245/125/80 bp and Hae III enzyme yield 160/140/70 bp that reflect (*M. tuberculosis complex*) (*MTC*) in 73.1% (49/67) of studied cases
2. BST EII enzyme yield 245 /125/100 bp & Hae III enzyme yield 155/140/60 bp pattern that reflect (*Mycobacterium intracellulare*) in 14/67 (20.9%) of cases while
3. BST EII enzyme yield 245/125/100 bp & Hae III enzyme yield 155/110/70 bp pattern that reflect (*Mycobacterium malmoense*) in 4/67 (6.0%) of cases.

Moreover, there were no significant differences between each of Mtb genotypes (*MTC, M. intracellulare and M. malmoense)* and sex, residence, smoking habit, or clinical presentation in the tuberculous patients (data not shown). Table 2 *shows* isolated genotypes in new cases and previously treated tuberculous cases. There was an insignificant association between history of treatment and isolated genotypes. Table 3 shows drug susceptibility patterns according to previous treatment in tuberculous patients. Risk of resistance to 4 drugs and 3 drugs was insignificantly higher in previously treated than new cases ($p = 0.084, 0.217$). Table 4 shows separate drug susceptibility patterns of first line anti-TB drugs in relation to history of treatment. Previously treated cases had significantly higher resistance to INH versus new cases ($p = 0.040$), however there is no significant difference to STM, RIF or ETH resistance between both groups. Table 5 shows drug susceptibility patterns in relation to isolated genotypes. There were significant relations between various genotypes and *drug susceptibility patterns* ($p = 0.014$); *M*. All cases of *M. malmoense* were sensitive to 4 drugs (100%) of cases, while all 4 drug resistance cases were of *M. intracellulare* genotype ($p < 0.001$), Table 6 *shows separate drug susceptibility patterns in relation to isolated MTB genotypes*. There was significant increased risk of resistance to STM, RIF, ETH and INH with *M. intracellulare* genotype ($p = 0.021$, $p < 0.001$, $p = 0.001$, $p < 0.001$). MTC showed lower resistance to STM, RIF, ETH and INH while all *M. malmoense* isolates were sensitive to all the 1st line anti-TB drugs.

Discussion

Tuberculosis as one of the oldest recorded human infections, is one of the biggest killers among the infectious diseases, despite the use of a live attenuated vaccine and antibiotics [13]. The development of drug resistance in the population has increased concern that TB again became an incurable disease [14].

Table 1 Genotyping and restriction bands of the isolated MTB.

Genotypes	Restriction Enzymes and Bands	Total ($n = 67$)	
		No	%
Mycobacterial tuberculosis complex (MTC)	BST EII → 245/125/80 bp Hae III → 160/140/70 bp	49	73.1
M. intracellulare	BST EII → 245/125/100 bp Hae III → 155/140/60 bp	14	20.9
M. malmoense	BST EII → 245/125/100 bp Hae III → 155/110/70 bp	4	6.0

Table 2 Isolated genotypes in relation to new cases and previously treated tuberculous patients.

	MTC	*M. intracellulare*	*M. malmoense*	*p*
New cases ($n = 27$)	23 (85.2%)	3 (11.1%)	1 (3.7%)	0.111
Previously treated cases ($n = 40$)	26 (65.0%)	11 (27.5%)	3 (7.5%)	

Table 3 Anti-TB susceptibility patterns of first line treatment by indirect nitrate reductase assay in new versus previously treated cases.

Drug resistance profile		Previously treated cases (40)		New cases (27)		*p*
		No.	%	No.	%	
Sensitive to 4 drugs ($n = 32$)		16	50.0	16	50.0	0.122
Resistance to any drug ($n = 35$)	Total ($n = 35$)	24	68.6	11	31.4	
	One drug resistance ($n = 10$)	6	60.0	4	40.0	0.582
	Two drug resistance ($n = 6$)	3	50.0	3	50.0	0.769
	Three drug resistance ($n = 11$)	8	72.7	3	27.3	0.217
	Four drug Resistance ($n = 8$)	7	78.5	1	12.5	0.084

Table 4 Separate drug susceptibility patterns of first line anti-TB drug in relation to history of treatment.

	Previously treated cases ($n = 40$)				New cases ($n = 27$)				*p*
	Sensitive		Resistant		Sensitive		Resistant		
Total cases	16	40.0	24	60.0	16	59.3	11	40.7	0.122
Streptomycin (STM)	24	60.0	16	40.0	19	70.4	8	29.6	0.385
Rifampicin (RIF)	23	57.5	17	42.5	21	77.8	6	22.2	0.086
Ethambutol (ETH)	26	65.0	14	35.0	23	85.2	4	14.8	0.068
Isoniazid (INH)	23	57.5	17	42.5	22	81.5	5	18.5	0.040

Table 5 Drug susceptibility patterns in relation to isolated genotypes.

Drug susceptibility patterns		MTC	*M. intracellulare*	*M. malmoense*	*p*
Sensitivity to 4 drugs ($n = 32$)		25 (78.1%)	2 (6.3%)	4 (12.5%)	0.014
Resistance to drugs ($n = 35$)	Total ($n = 35$)	24 (68.6%)	11 (31.4%)	0 (0%)	
	One drug resistance ($n = 10$)	10 (100.0%)	0 (0%)	0 (0%)	0.322
	Two drug resistance ($n = 6$)	5 (83.3%)	1 (16.7%)	0 (0%)	0.490
	Three drug resistance ($n = 11$)	9 (81.8%)	2 (18.2%)	0 (0%)	0.273
	Four drug resistance ($n = 8$)	0 (0%)	8 (100.0%)	0 (0%)	< 0.001

Clinical isolates of MTB obtained from patients infected by the same strain of bacillus usually exhibit identical or very similar fingerprint patterns, and the patients are more often found to be epidemiologically linked [5].

The aim of this study is to detect molecular genotypes and drug resistance patterns of *M. tuberculosis* at Mansoura hospitals. To achieve this aim 123 sputum samples obtained from ZN smear positive cases were cultivated on Lowenstein Jensen (LJ) medium, out of them 67 specimens (54.5%) were positive culture that were divided into two groups; 27/67 (40.3%) newly diagnosed cases & 40/67 (59.7%) previously treated cases.

This study revealed that *M. tuberculosis complex* (MTC) had the commonest incidence in 49/67 (73.1%), followed by *M. intracellulare* in 14/67 (20.9%) while *M. malmoense* had the least incidence in 4/67 (6.0%). Compared with others studies who found that 97.5% of isolates were *M. tuberculosis* complex (MTBC) and 2.6% were Mycobacteria other than tuberculosis (MOTT), While 22 isolates were *Mycobacterium africanum* [15].

Increased prevalence of MTC genotype could be explained as this strain is highly virulent causing extensive transmission among patients. Moreover, geographic proximity of patients to one another might have increased opportunities for TB exposure and supported transmission through casual contact [16]. As the incidence of NTM infections has increased over the past couple of decades, the most common clinical manifestation is pulmonary disease. *M. malmoense* and *M. intracellulare* are two of the most important members of non tuberculous mycobacteria [17]. *Mycobacterium avium* complex (MAC) consists of several closely related slow-growing nonchromogen mycobacteria, including *M intracellulare*. MAC accounts for the largest portion of all NTM infections in most epidemiologic series. They are saprotrophic organisms that enter into hosts via the gastrointestinal tract usually, but also can

Table 6 Separate drug susceptibility patterns in relation to isolated Mtb genotypes.

Drug susceptibility		MTC		*M. intracellulare*		*M. malmoense*		*p* value
		No	%	No	%	No	%	
STM	Sensitive ($n = 43$)	34	79.1	5	11.6	4	9.3	0.021
	Resistant ($n = 24$)	15	62.5	9	37.5	0	0	
RIF	Sensitive ($n = 44$)	37	84.1	3	6.8	4	10.8	<0.001
	Resistant ($n = 23$)	12	52.2	11	47.8	0	0	
ETH	Sensitive ($n = 49$)	40	81.6	5	10.2	4	10.0	0.001
	Resistant ($n = 18$)	9	50.0	9	50.0	0	0	
INH	Sensitive ($n = 45$)	38	84.4	3	6.7	4	8.9	<0.001
	Resistant ($n = 22$)	11	50.0	11	50.0	0	0	

be via the lungs [17]. *M intracellulare* can colonize the respiratory tract of patients and it may cause disease indistinguishable from tuberculosis. *M. malmoense* is one of the slow growing mycobacteria belonging to the Runyon group III. *M. malmoense* is also reported to be the second most common non tuberculous mycobacterium recovered from sputum [18].In this study restriction of amplified DNA product was done by BST EII and Hae III restriction enzymes that yielded;

BST EII $\rightarrow$ 245/125/80 bp and Hae III $\rightarrow$ 160/140/70 bp (*M. tuberculosis* complex).

BST EII $\rightarrow$ 245/125/100 bp and Hae III $\rightarrow$ 155/140/60 bp (*M. intracellulare*).

BST EII $\rightarrow$ 245/125/100 bp and Hae III $\rightarrow$ 155/110/70 bp (*M. malmoense*).

Azar and Abdulrazagh [16] study was done on 145 clinical isolates (96.6%) which showed identical restriction patterns equal to 160/145/72 bp fragments for Hae III and 250/120/82 bp fragments for Bst EII digests but diverse restriction patterns were observed for five clinical isolates in Hae III digest only, while their Bst EII digestion patterns showed no variation and were similar to other isolates. Two strains showed different Hae III patterns as 180/100/80 bp and 194/72 bp. The third different Hae III digest pattern was seen in three strains as 160/145 bp.

In our study the isolates had less than five copies. This pattern is similar to that of other countries in the Asian and Ocean regions, such as Malaysia, Oman, Hong Kong, and Madagascar [16,17]. While in Das et al. [18] study from south India, a large number of isolates of *M. tuberculosis* with low copy numbers or no copies of IS 6110 element have been observed. In Magana-Arachchi et al. [19] RFLP analysis of the 131 isolates, the copy number of IS 6110 element in *M. tuberculosis* strains varied from 1 to 7, the majority having 3 to 5 copies which agrees with our study.

In this study as well as in Ida et al. [20] study there was no statistically significant association between any clinical, epidemiological parameter and strain clustering.

In this study also, as the susceptibilities to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol (EMB) were determined by the indirect nitrate reductase assay we found that 47.8% of cases were sensitive to the four anti-tuberculous drugs. All patients received streptomycin, rifampicin, ethambutol and isoniazid. ETH showed higher sensitivity 73.1% followed by INH 67.2%, RIF 65.7% then STM 64.2%.

These results were in concordance with those reported by Abbadi et al. [5] who found that 56.8% of cases were susceptible to the four anti-tuberculosis drugs. Similarly Abdelaal et al. [21] in the same locality reported that the sensitivity of Mtb was highest for STR (64%) and EMB (56%) and lowest for RIf (54%) and INH (48%) while Mashaly, (2011) [22] found that the sensitivity of Mtb was higher for STM and ETH (74.4% for each) followed by RIF (55.8%) and INH (34.9%). On the other hand the order of the observed susceptibility pattern of Mtb was different from reported in the same locality by Zaghloul [23]. He found that the highest sensitivity was to RIF (79%) and STM (71%) followed by INH (64%) and the lowest sensitivity was encountered with ETH (14%).

The significant decrease in the sensitivity of Mtb to INH and RIF in the last 10 years could be attributed to poor compliance and wide use of these drugs for non specific infections as well as for certain infectious diseases like brucellosis. While the significant increase in sensitivity of Mtb to ETH was attributed to the restriction of its use in the management of TB patients [16].

In our study of Anti-TB susceptibility patterns of first line treatment by indirect nitrate reductase assay we found that 47.8% of cases were sensitive to the four anti-tuberculous drugs while 52.2% cases were resistant to one or more drugs. Resistance varied according to number of drugs; 11 cases (16.4%) showed 3 drug resistance followed by 10 cases (14.9%) showed one drug resistance, 8 (11.9%) cases showed 4 drug resistance then 6 cases (9%) showed 2 drug resistance. Badran [24] found that among total 153 TB cases, 50 cases (32.7%) were resistant to one or more of the first line antituberculous drugs as 48% were mono drug resistance and 52% showed multi resistance. Similarly, Mashaly [22] showed 41.9% monodrug resistance and 46.5% cases with multi drug resistance.

This study showed that 2 ry resistance to 1st line anti TB drugs was higher than 1 ry resistance. This agrees with Al-Akhali et al. [25] and Surucuoglu et al. [26] in other different localities that found a higher prevalence of resistance among previously treated than newly diagnosed TB patients (17.4% vs. 9.8% and 45.5% vs. 25%, respectively). Also Mashaly [22] showed 2 ry resistance to 1st line anti TB drugs was higher than 1 ry resistance (93.3% and 85.7%) in the same locality. The high resistance pattern detected in this study might be explained by previous anti-TB treatment in 40/67 patients which has been identified as an important risk factor for the acquisition of drug resistant TB [27] Resistance against 2 or 3 drugs is difficult to treat and often results in treatment failure [28].

Multi drug resistance (resistance to at least RIF and INH) was found in 25/67 (37.3%) cases. It is comparable to those

reported in other different studies conducted at Mansoura hospitals as reported in Mashaly [22] who found 14/43 (32.6%) MDR isolates, Badran [24] and El-Moursy et al. [29] 26% and 46%, respectively.

Regarding drug susceptibility patterns in relation to isolated genotypes, there was a significant difference between various genotypes and drug susceptibility patterns ($p = 0.014$); *M. intracellulare* showed higher resistance in 11/14 of cases (78.6%) while *M. malmoense* showed higher sensitivity in 100% of cases to the 4 antituberculous drugs. In Griffith et al. [30] study most strains of *M. malmoense* are sensitive to ethambutol, and some are sensitive to rifampicin. As for other NTM species the clinical response to treatment does not correlate well with standard in vitro testing for antimicrobial susceptibility. Although overall cure rates remain below 50%, the best responses have been observed with combinations of isoniazid, rifampin, and ethambutol [31].

Conclusion

Our results showed that *M. tuberculosis complex* (*MTC*) genotype had the commonest incidence followed by *M. intracellu*lare while *M. malmoense* had the least incidence. Moreover *M. intracellulare* showed the higher resistance while *M. malmoense* showed the higher sensitivity to antituberculous drugs. We recommend PCR-RFLP as a simple and reproducible method for genotyping of mycobacterium tuberculosis strains and for early detection of *M. tuberculosis*. This method could also differentiate MBT complex and non tuberculous mycobacteria directly from clinical samples, that could help clinicians to select appropriate chemotherapy early which would reduce morbidity.

Conflict of interest

None.

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