

## Drug Resistant *Mycobacterium tuberculosis* Complex Isolates among Patients with Tuberculous Lymphadenitis

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### Abstract

**Objectives:** Seventy five patients with lymphadenopathy who were referred to the Clinic of Lymphadenopathy at the Institute of Endemic Diseases, University of Khartoum were enrolled in our study to detect gene mutation that associated with drug resistance to streptomycin and rifampicin.

**Materials and methods:** Seventy five patients of lymphadenopathy were enrolled for Fine needle aspiration cytology and polymerase chain reaction (PCR). PCR-single strand confirmation polymorphism (PCR-SSCP) for gene mutation was used to detect gene mutation for tuberculous lymphadenitis group.

**Results:** Cytomorphologically, thirty cases showed necrotizing granulomatous tuberculous lymphadenitis (40%) while twenty cases (26%) showed reactive lymph nodes changes. The remaining twenty five cases (34%) had secondary lymph nodes deposits. All cases of tuberculous lymphadenitis group showed similar patterns to H37 Rv with a fragment size of 123 bp when amplified with IS6110 gene primers specific for *M. tuberculosis* complex, while other groups (reactive and malignant nodes) were negative.

For rps1 43 gene detection, twenty seven cases (27/30) had DNA band patterns identical to H37 while three cases (3/30) were identical to mutant strain that were associated with drug resistance to streptomycin. For the rpoB, all isolates gave identical patterns to H37 Rv strain.

**Conclusion:** PCR is useful to detect *Mycobacterium tuberculosis* isolates in tuberculous lymphadenitis cases. PCR-SSCP is useful for detection of gene mutation targeting drugs for tuberculosis cases; however, it needs more Supporting tools such as sequencing method to confirm the resistance Tb cases.

### Introduction

Anti-tuberculosis (TB) drug resistance is a major public health problem that threatens progress made in TB care and control worldwide. Drug resistance arises due to improper use of antibiotics in chemotherapy of drug-susceptible TB patients [1].

Multi-drug resistance-TB (MDR-TB) is defined as resistance to isoniazid and rifampicin, with or without resistance to other first-line drugs (FLD). Extensive drug resistance-TB (XDR-TB) is defined as resistance to at least isoniazid and rifampicin, and to any fluoroquinolone, and to any of the three second-line injectable amikacin, capreomycin and kanamycin [2,3].

The diagnosis of MDR-TB and XDR-TB is hampered by the absence of effective and affordable rapid diagnostic techniques for drug sensitivity. Several approaches, phenotypic and molecular, have been explored to develop rapid, reliable and accurate methods for the rapid detection of drug resistance in *M. tuberculosis* [4]. The usefulness of PCR-SSCP for determination of rifampicin and isoniazid resistance in *M. tuberculosis* and *M. bovis* cultures from human and animal origin was investigated and worldwide used for detection of point mutation.

It provides a rapid screening tool for the majority of resistant isolates [5,6].

The GenoType MTBDR test is able to detect mutations in the rpoB gene for RIF resistance, and the most frequent mutation at codon 315 of the katG gene for INH resistance, either in isolates or clinical specimens [7,8].

### Material and Methods

Seventy five sequential patients with provisional diagnosis of tuberculous lymphadenitis of different ages, genders and ethnic groups were enrolled in the study. Demographic, clinical data was collected from the patients. The past history of patients with TB infection and whether he/she had received the anti-tuberculosis treatment previously or not is well reported.

### Fine needle aspiration cytology (FNAC) and PCR amplifications

Fine Needle Aspiration (FNAC) was done by experienced pathologist. FNAC findings were classified into four groups [9-10]. Granulomatous, granulomatous/necrotizing tuberculous lymphadenitis, reactive lymphadenopathy and secondary malignant deposit. DNA was extracted from lysates using the phenol chloroform method (PCI) as described by Maniatis et al. [11-15]. Following DNA extraction, all lysate samples were subjected to PCR amplification to identify *M. tuberculosis* complex from other bacteria using a set of forward and reverse primers were used to amplify the target gene IS6110 (Table 1).

PCR amplification was done using protocol Shrafeldin et al. [16-21]. PCR products were electrophoresed. Agrose (1.2 g) was added to 67 ml D.W and 8 ml of 10X TBE, using ethidium bromide staine 1.0 µl. Electrophoresis run at 100 v Gave good results for 45 minutes. Negative and positive controls with 100 bp DNA marker were used in each run. The gel was visualized using Syngene Documentation System (Syngene Ltd., USA).

### PCR-SSCP

Positive samples from patients with lymphadenitis using PCR test were subjected to PCR using two sets of primers (Table 1) to amplify regions rpsl 43 and rpoB 531 that confer drug resistance to streptomycin and rifampicin respectively. The *M. tuberculosis* reference strain and one isolate previously characterized by gene sequencing as mutant (resistant) strain for each region was also used.

Gene	Sequences	Size
Gene IS6110	IS6110-F: CCTGCGAGCGTAGGCGTCGG IS6110-R CTCGTCCAGCGCCGCTTCGG	123 bp
rpo B 531	TR8 TGCACGTCGCGGACCTCCA TR9 TCGCCGCGATCAAGGAGT	157 bp
rpsl 43	STR52 GTCAAGACCGCGGCTCTGAA SRT 34 TTCTTGACACCTGCGTATC	272 bp

**Table 1:** PCR amplify regions rpsl 43 and rpoB 531

4 µl of lysed bacterial cells were used as template for amplification in 20 µl reaction mixture containing 2 µl 10X buffer, 1.6 of magnesium chloride 25 mM; 1.6 of dNTPs 10 Mm, (Promega, Madison WI, USA), 0.2 µl of 10 pmol 5' primer (Forward) and 3' primer (reverse) each, (Inqaba Biotechnical Industries (Pty) Ltd., South Africa) and 1 Unit of 5 Unit/µl Taq polymerase, (Promega, Madison WI USA). Reaction mixture were heated in thermal cycler (Flexigene) as follows: 93°C for 3 minutes followed by 35 cycles at 93°C for 1 minute, annealing at Tm (58°C for rpoB531; 61°C for rpsl 43) for 1 min and extension step at 72°C for 2 minutes. Final extension was done at 72°C for 10 minutes.

Efficient amplification was confirmed by 1.5% agrose gel electrophoresis. A DNA marker (50 bp) was used in each electrophoresis run with H37Rv and negative controls.

For acrylamide gel Five µl PCR products was added to 10 µl "stop" solution (formamide 95%, 20 mM EDTA and 0.005% each of bromophenol blue and xylene-cyanol) Were mixed and heat-denatured at 95°C for 5 minutes, The PCR tubes with denatured products were rounded with soft ice then the denatured products were electrophoresed

on non-denatured PAGE 12% or PAGE 10%. 30% Acrylamide was prepared as 29 gram Acrylamide with 1 gram bis Acrylamide in 100 ml DW. For 12% Acrylamide Gel 4 ml of 30% Acrylamide was added to 3.3 DW, 1.7 10X Buffer, 210 µl of Ammonium per Sulphate, 2 ml glycerol and 5 µl TEMED. For PAGE 10% preparation, 3.3 ml 30% Acrylamide, 4.3 ml D.W, 1.7 10X Buffer, 210 µl of Ammonium per Sulphate, 2 ml glycerol and 5 µl TEMED. The denatured product of samples were loaded with H37RV strain and mutant strain in each trial.

Electrophoresis at constant 80 V in 0.6 X TBE buffer for 4-5 h, the gels were fixed for 6 minutes in buffer containing acetic acid 0.5% and ethanol 10%, pour off, then was washed with D.W. The gel was placed in a sliver nitrate 0.1% using a horizontal orbital shaker, after 20 minutes pour off and washes with D.W then placed in staining buffer for 10 minutes or more, until bands were visible. The staining buffer consisted of 0.1 M NaBH<sub>4</sub>, 0.1 M NaOH and formaldehyde 0.004%. Fixing was done in NaCO<sub>3</sub> 0.75% buffer for 10 minutes. When the bands was clear visible, the buffer pour off and the gel was washed again and placed on the buffer containing Sodium Carbonate 0.75% for 5-10 minutes to stop the reaction. The gel was read against white light and results were photographed.

### Results

All patients (75) tested serologically were found negative for HIV. Forty per cent reported a history of contact with patients with pulmonary diseases [chronic cough], either within the family or in the neighborhood. The main symptoms reported were swellings mainly in the cervical region and nocturnal fever. Cervical lymph node groups were the commonest affected, where it was reported in 66.7% of cases, while other lymph node groups (inguinal, submandibular, and pre-auricular) were reported in 33.3% of cases, this in agreement with findings from the region and globally [9-11]. Anterior cervical nodes were reported in 23.4% of cases, posterior cervical in 20.0% of cases, anterior and posterior cervical (either left or right) were seen in 23.3% of patients.

Mantoux test positivity was universally (100%) reactive in all patients with tuberculous lymphadenitis with a mean of 22.6 ± 6.0 mm in diameter. Mean hemoglobin levels, ESR and total white cells count for these patients were 11.7 ± 2.0 grams/dL and 87 ± 31 mm/first hour and 5.3 ± 1.6 × 10<sup>3</sup>/µL, respectively. Patients with granulomatous tuberculous lymphadenitis were significantly old (p<0.05) and had significantly marked mantoux induration (p=0.001). More females were affected in the cytomorphological group, where five were affected compared to one.

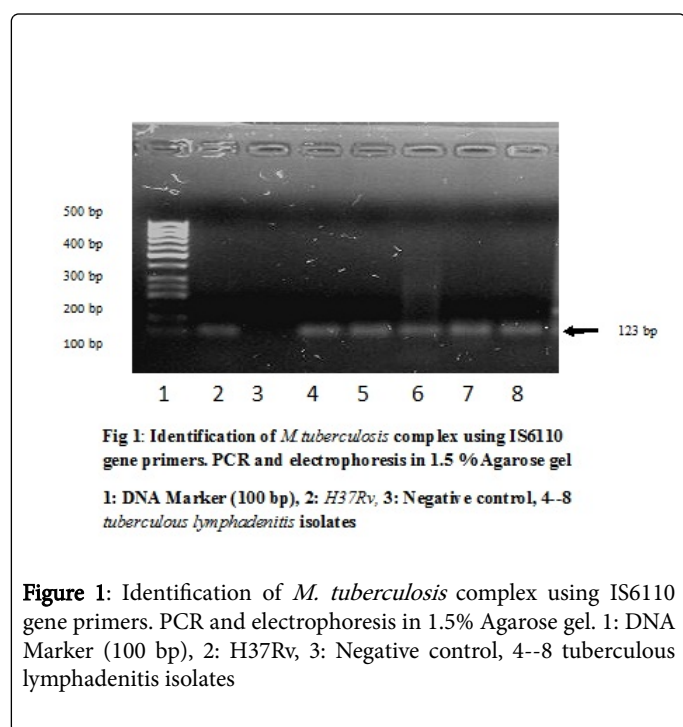
FNAC cytomorphological patterns were as follows: 8.0% of cases (6/75) were necrotizing, granulomatous 4.0% (3/75), while the necrotizing granulomatous was in 28.0% (21/75). Secondary metastasis and reactive patterns were seen as 25/75 (33.3%) and 20/75 (26.7%), respectively (Table 2).

Cytomorphological Patterns:					
Of Lymphadenopathy	M:F	Mean age	Mean ESR mm	Mean TwBCs	Mean Mantoux mm
PCR positive (n=30)	1:5	26.9 ± 11.2	87 ± 31	5.3 ± 1.6	22 ± 6.0
Necrotizing (n=6)	1:5	23 ± 8.6	69 ± 24.9	6.8 ± 2.3	19.2 ± 5.5

Granulomatous Tb (n=3)	1:2	43.7 ± 15.1	113 ± 25.1	4.6 ± 0.7	36.3 ± 7.6
Necro/Granul. Tb (21)	3:4	25.1 ± 9.2	69.3 ± 32.4	4.6 ± 0.6	21.4 ± 6.0
PCR negative (n=45)	2:1	34.6 ± 21.6	77 ± 30	5.9 ± 3.2	13 ± 10.8
Reactive (n=20)	2:1	45 ± 24.6	76 ± 35.1	5.3 ± 1.7	5.3 ± 9.2
Malignancy (n=25)	1:0	26.8 ± 25.5	56.7 ± 23.1	4.3 ± 0.6	8.5 ± 6.6

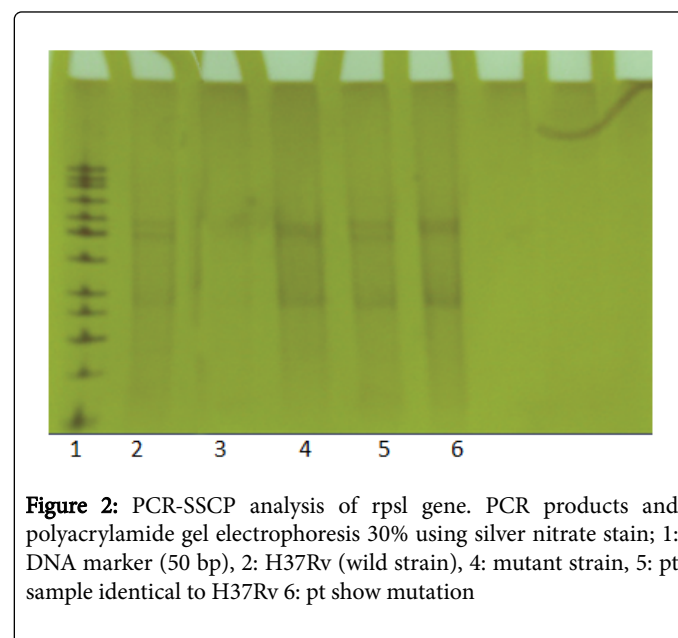
**Table 2:** Baseline characteristics, cytomorphological patterns and circulating mycobacterial DNA of the study patients

Fine Needle Aspiration Cytology (FNAC) has proved very valuable in the diagnosis of tuberculous Lymphadenitis where mycobacterial infections are endemic and when cytomorphological patterns proved to extremely concordant with microbiological and molecular present of mycobacteria. However, it has several limitations, especially in the absence of demonstrable AFB, but FNAC provides at least material for PCR in an easy and moderately invasive manner [12-14]. Recently PCR-based techniques were applied in the diagnosis of tuberculous lymphadenitis [14,15]. All cases of lymphadenitis patients (75 cases) were subjected for PCR test using IS6110 gene primer so thirty cases (n=30/75; 40%) with cytomorphological patterns of granulomatous, necrotizing and necrotizing/granulomatous showed DNA band patterns similar to *M. tuberculosis* pattern (H37RV) with DNA fragment size was 123 bp (Figure 1). The remaining of FNA samples did not give reactive with IS6110 gene primer. We used the IS6110 gene primer based on the previous findings in Sudan, the majority of tuberculous lymphadenitis is caused by *M. tuberculosis* a small percentage is caused by bovine mycobacteria. This has not changed for over thirty years [16].



The PCR-SSCP technique has been extensively used to search for gene mutations which are a whole mark for the development of resistance in microorganisms with or without the pressure of antibiotics. Recently, PCR-SSCP is used to detect missense mutations associated with antibiotics resistance in *M. tuberculosis* [17-20].

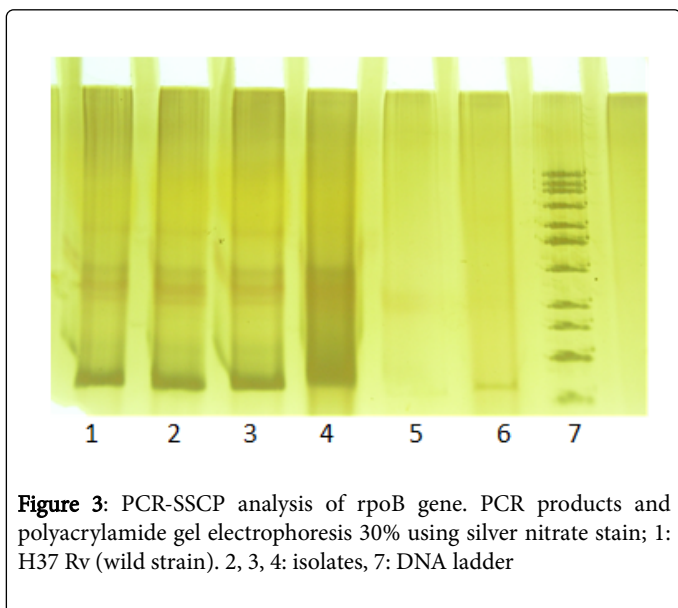
All thirty isolates of tuberculous lymphadenitis have been subjected for PCR targeted rpsl 43 and rpo B 531 that are associated with drug resistant to streptomycin and rifampicin respectively. When denatured PCR products were loaded in non-denaturing acrylamide gel, 27/30 (90%) of tuberculous lymphadenitis isolates gave similar pattern to H37RV (wild type) while 3/30 isolates (10%) gave a pattern different to H37RV (wild type) pattern, but similar to the mutant strain *M. tuberculosis* that is resistant to streptomycin (Figure 2). The rate of molecular resistance could through some information on the extent of the spread of resistance to anti-tuberculous drug and this will indicate the degree of danger in which the community faces in term of MDR and XMDR. That reported by results of this study, reports that, ten percent of the *M. tuberculosis* isolates from patients of tuberculous lymphadenitis had DNA patterns similar to streptomycin-resistant standard isolates. This finding is very different from the results that were reported by Sharaf-eldin et al., where they reported Streptomycin resistance of 30% gene mutations identified in 5/50 cases in rpsl 43 and 10/50 cases in rrs 513. The authors selected their samples from patients with pulmonary tuberculosis and probable MDR (failure to respond to anti-tuberculous drugs). The Gene mutation associated with streptomycin are identified from two genes rpsl 43 and rrs 513 and that probably may justify our result from that previous study [21]. The correlation between molecular pattern and clinical resistance to streptomycin could not elucidate from our study, because streptomycin was not given to our patients.



When denatured PCR products were loaded in non-denaturing acrylamide gel (30/30; 100%) of tuberculous isolates gave similar DNA band patterns that are similar to that of the H37RV (wild type) (Figure 3).

All our study isolates showed DNA patterns that are similar to standard rifampicin- sensitive isolates. Total rifampicin molecular sensitivity is completely to the treatment response concordant clinical

pattern in the study patients i.e. all patients responded to an anti-tuberculous combination containing rifampicin.



## Conclusion

Patterns of DNA resistance in patients with tuberculous lymphadenitis are considerable at the level of ten percent. If this proved concordant with microbiological resistance then the use of streptomycin in anti-tuberculous regimes should be critically reviewed. Rifampicin proves to be extremely effective as one of the most important first line anti-tuberculosis drugs in this country.

## Recommendations

We recommended that Detection of mutation conferring drug resistance to Isoniazid [INH] is needed to detect MDR and XMRD between *M. tuberculosis* isolates among patients with tuberculous lymphadenitis in Sudan. PCR-SSCP will be more useful when use sequencing technique to confirm missense mutation conferring drug resistance to first and second-line TB drugs.

## References

1. World Health Organization (2012) Global tuberculosis report.
2. World Health Organization (2013) Multidrug-resistant tuberculosis (MDR-TB).
3. World Health Organization (2010) Multidrug and extensively drug-resistant TB (M/XDR-TB) Global Report on Surveillance and Response
4. Hopewell PC, Pai M, Maher D, Uplekar M, Raviglione MC (2006) International standards for tuberculosis care. Lancet Infectious Diseases 6: 710-725.
5. Xiaodong C, Jianfang Z, Liu Y, Xiuli X, Jianyun L, et al. (2007) A new Multi-PCR-SSCP assay for simultaneous detection of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*. Journal of Microbiological Methods 70: 301-305.
6. Rüscher-Gerdes S, Pfyffer GE, Casal M, Chadwick M, Siddiqi S (2006) Multicenter laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of *Mycobacterium tuberculosis* to classical second-line drugs and newer antimicrobials. Journal of Clinical Microbiology 44: 688-692.
7. Hillemann D, Rüscher-Gerdes S, Richter E (2006) Application of the Genotype MTBDR assay directly on sputum specimens. Int J Tuberc Lung Dis 10: 1057-1059.
8. Hillemann D, Rüscher-Gerdes S, Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol 45: 2635-2640.
9. Yassin MA, Olobo JO, Kidane D, Negesse Y, Shimeles E, et al. (2003) Diagnosis of tuberculous lymphadenitis in Butajira, rural Ethiopia. Scand J Infect Dis 35: 240-243.
10. Das DK, Pant CS, Chachra KL, Gupta AK (1992) Fine needle aspiration cytology diagnosis of tuberculous thyroiditis. A report of eight cases. Acta Cytol 36: 517-522.
11. Lee H, Cho SN, Bang HE, Lee JH, Bae GH, et al. (1998) Molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* isolated from Korea by polymerase chain reaction-single strand conformation polymorphism sequence analysis. The Int J Tuberc Lung Dis 2: 585-589.
12. Gupta SK, Chugh TD, Sheikh ZA, al-Rubah NA (1993) Cytodiagnosis of tuberculous lymphadenitis .A correlative study with microbiologic examination. Int Academy Gynecol Cytol 37: 329-332.
13. Ghoshal AG , Roy PP (2000) Diagnosis of tuberculosis. J Indian Med Assoc 98: 115-118.
14. Jain A, Verma RK, Tiwari V, Goel MM (2005) Dot-ELISA vs. PCR of fine needle aspirates of tuberculous lymphadenitis: a prospective study in India. Acta Cytology 49: 17-21.
15. Baek CH, Kim SI, Ko YH, Chu KC (2000) Polymerase chain reaction detection of *Mycobacterium tuberculosis* from fine-needle aspirate for the diagnosis of cervical tuberculous lymphadenitis. Laryngoscope 110: 30-34.
16. Aljafari AS, Khalil G, Elsidig EIA, Ibrahim ME (2004) Diagnosis of Tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. Cytopathology 15: 1-5.
17. Telenti A, Imboden P, Marchesi F, Schmidheini T, Bodmer T (1993) Antimicrob Agents Chemotherapy automated detection of rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. Biology direct 37: 2054-2058.
18. Telenti A, Honoré N, Bernasconi C, March J, Ortega A, et al. (1997) Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. J Clin Microbiol 35:719-723.
19. Lodha R, Kabra SK (2004) Newer diagnostic modalities for tuberculosis. Indian J Pediatr 71: 221-227.
20. Kim BJ, Kim SY, Park BH, Lyu MA, Park IK, et al. (1997) Mutations in the rpoB gene of *Mycobacterium tuberculosis* that interfere with PCR-single-strand conformation polymorphism analysis for rifampin susceptibility testing. J Clin Microbiol 35: 492-494.
21. Ghada SS, Saeed NS, Hamid ME, Jordaan AM, Van der Spuy GD, et al. (2002) Molecular analysis of Clinical isolates of *Mycobacterium tuberculosis* collected from patients with persistent diseases in Khartoum region. J Infect 44: 244-251.