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Detection of Multidrug-Resistant Tuberculosis Using PCR Compared to the Conventional Proportional Method

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Objective: To evaluate the PCR technique for the rapid defection of Multidrug-Resistant (MDR) *Mycobacterium tuberculosis* compared to the conventional proportional drug sensitivity testing.

Design: Cross sectional laboratory based study.

Setting: Alshaab Teaching Hospital, Abu-Angah Hospital and the National Health Laboratory, Sudan.

Method: One hundred thirty tuberculosis suspected individuals of both sexes and of different ages were included in the study. Sputum samples were cultured on Lowenstein-Jensen (LJ) medium. Resistant strains were tested for the presence of mutations conferring resistance using molecular techniques to amplify 315 base pair (bp) rifampicin (RIF) and 146 bp isoniazid (INH), as markers for MDR among *Mycobacterium tuberculosis.*

Result: One hundred nineteen (91.5%) showed *Mycobacterium tuberculosis*-like colonies, 65 of which were randomly subjected to PCR and examined for the presence of IS6110 insertion sequences. Fifty-six (86.2%) were confirmed members of the *Mycobacterium tuberculosis*. The result of antibiotics susceptibility testing revealed that 32/56 (57.1%) of the strains were resistant to RIF, 36/56 (64.3%) to INH and 30/56 (53.6%) were resistant to both drugs (MDR). The conventional method showed 21/56 (37.5%) were resistant to RIF, 32/56 (57.1%) to INH and 16/56 (28.6%) were resistant to both drugs (MDR).

Conclusion: Not all resistant strains detected by conventional were detected by PCR method; 14 (25%) were missed for RIF, 9 (16.1%) for INH and 4 (7.1%) for both. This represents a significant lack of sensitivity of the PCR technique, which could be due to the presence of other types of mutations that needs other primers and PCR protocol.

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The most effective anti-TB drugs are isoniazid (INH) and rifampicin (RIF). Resistant Mycobacteria to at least one of these drugs are the cause of Multidrug-resistant tuberculosis (MDR-TB). This type of resistance is highly problematic due to limited sources of drugs as well as the high toxicity, low efficacy and high cost of second-line tuberculosis drugs[1].

In 2008, an estimated 390,000 - 510,000 cases of MDR-TB emerged globally. Among TB cases, 3.6% are estimated to have MDR-TB. Twenty-two of 48 African countries reported first-line TB drug resistance, the estimated number of MDR-TB cases (primary and acquired) in 2008 was 69,000 (53,000 - 110,000)[2].

Since the early study by Cavanagh, data on drug resistant TB are lacking in the Sudan[3]. Tuberculosis control program in Khartoum 2006 showed that there was no system to detect the prevalence of MDR-TB or HIV among the TB cases[4].

Although the rate of drug resistance is continuously increasing, only around 7% of estimated cases are detected. The control of drug resistant disease is difficult especially in high burden countries due to poor laboratory services and the slow nature of conventional drug susceptibility testing[5].

Therefore, implementation of rapid molecular methods for detecting drug-resistant TB may be a viable alternative to culture-based DST. Recently the WHO recommended the use of molecular techniques such as Line probe assay (LPA) for rapid screening of MDR-TB in low and middle-income settings[6].

The role of mycobacterial catalase-peroxidase gene (katG) was determined by cloning and sequencing of this gene. Mutations in this gene were found in 42 - 58% of isoniazid-resistant clinical isolates, confirming the effect of KatG enzyme in INH activity[7].

Other mutations were detected in Ser315Thr, about 40% of isoniazid-resistant strains[8]. This mutation was found to result in the production of a catalase enzyme that retains about 50% of isoniazid catalase-peroxidase activity, which is sufficient for the ability of the organism to evade the action of host active radicals[9].

Spontaneous mutations (deletions/substitutions/insertions) were found to occur in the 81-bp hotspot region of the rpoβ gene which encodes DNA-dependent RNA polymerase (the target for rifampicin binding); this result into replacement of the aromatic with non-aromatic amino acids in the target RNA polymerase enzyme, which consequently leads to poor bonding between rifampicin and the RNA polymerase[10,11].

The aim of this study is to evaluate the PCR technique for the rapid defection of MDR *Mycobacterium tuberculosis* in comparison to the conventional proportional drug sensitivity testing.

METHOD

Randomized prospective cross sectional study was performed. Smear-positive sputum was collected from 130 patients with persistent tuberculosis. Informed consent was obtained from each patient. The study was approved by the Federal Ministry of Health and the ethical committee of the Scientific Research Council of Sudan University of Science and Technology.

Sputum samples were screened for acid-fast bacilli (AFBs) using Ziehl-Neelsen (ZN) smear microscopy. Handling and processing of specimens were performed in biosafety cabinet (BSL 3). Sputum was processed with the N-acetyl-L-cysteine-sodium hydroxide (NaOH) method (NaOH final concentration, 1.5%) and cultured in Lowenstein-Jensen (LJ) slants. Isolation and conventional identification of *Mycobacterium tuberculosis* were performed following standard method[12]. Drug susceptibility testing was performed by LJ proportion method[13].

Sixty-five *Mycobacterium tuberculosis* isolates were randomly selected from the positive cultures and were confirmed as *Mycobacterium tuberculosis* by detecting species-specific IS6110 (123 bp) using primers and method previously described[14]. Resistant strains detected by conventional methods were tested for the presence of mutations conferring resistance to isoniazid (INH) and rifampicin (RIF) using PCR method.

SPSS for windows version 11.0 was used for data analysis.

RESULT

One hundred thirty sputum specimens from patients were examined, 82 males and 48 females, age range was 12 to 67 years, 119 (91.5%) showed *Mycobacterium tuberculosis*-like colonies, 65 were randomly subjected to PCR and examined for the presence of IS6110 insertion sequences. PCR revealed that 56 (86.2%) were *Mycobacterium tuberculosis* and 9 (13.8%) were MOTTs, see table 1 and figure 1.

Table 1: Susceptibility Testing of IS6110 Positive *Mycobacterium Tuberculosis* for Rifampicin and Isoniazid by Proportion Method Compared to Multiplex PCR Method

	LJ proportion method		PCR method	
	RIF	INH	RIF	INH
	Number (%)		Number (%)	
Resistant	32 (57.1%)	36 (64.3%)	21 (37.5%)	32 (57.1%)
Sensitive	23 (41.1%)	19 (33.9%)	35 (62.5%)	24 (42.9%)
ND	1 (1.8%)	1 (1.8%)	-	-
Total	56	56	56	56

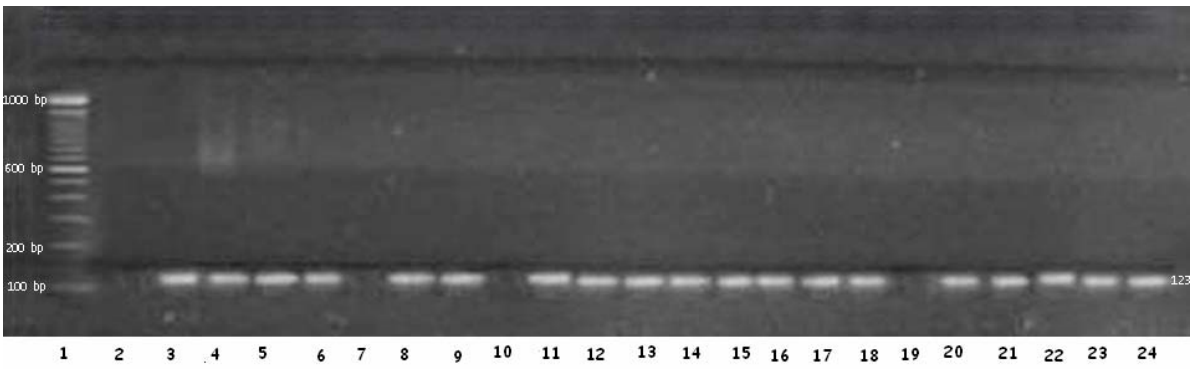

Figure 1: PCR Amplified IS6110 Sequences, Lane 1, DNA Marker; Lane 2, Negative Control; Lane 3, Reference *Mycobacterium Tuberculosis* Strain H37v (Positive Control)

Antibiotics susceptibility testing revealed that 32/56 (57.1%) of the strains were resistant to RIF using the conventional proportion method compared to 21/56 (37.5%) using PCR method. Resistant for INH were 36/56 (64.3%) using the conventional method compared to 32/56 (57.1%) using PCR method; 51.8% of the strains were found resistant for both drugs (MDR) using the conventional method compared to 28.6% for both drugs (MDR) using the PCR method, see figures 2 and 3.

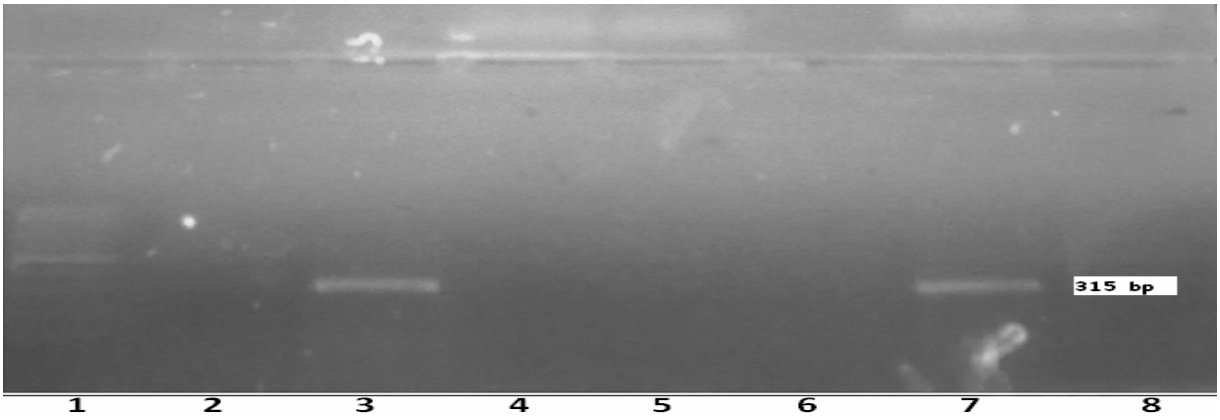

Figure 2: The Amplicon of Rifampicin Resistant *Mycobacterium Tuberculosis*; Lane 1, DNA Marker; Lane 2, Negative Control; Lane 3, Positive Control; Lane 7; RIF Resistant Strain

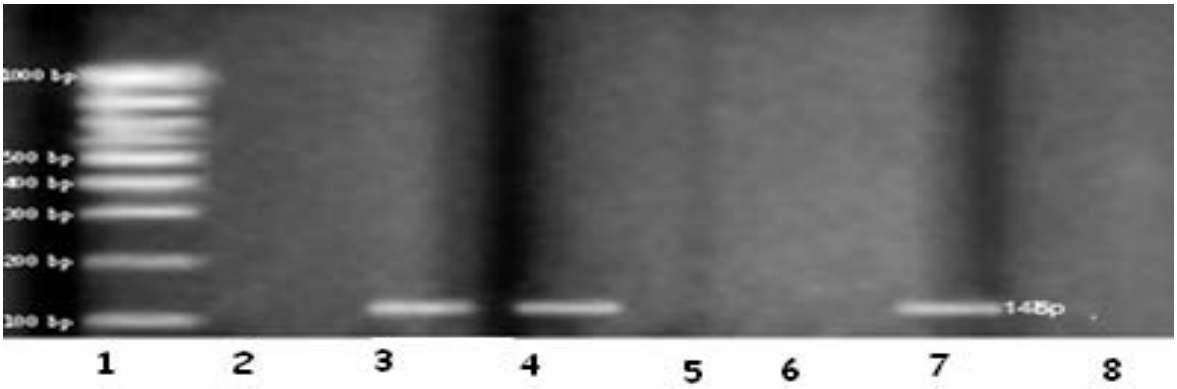

Figure 3: The Amplicon of Isoniazid Resistant *Mycobacterium Tuberculosis;* Lane 1, DNA Marker; Lane 2, Negative Control; Lane 3, Positive Control; Lanes 4 And 7, INH Resistant Strains

DISCUSSION

The result of antibiotics susceptibility testing revealed that there were obvious differences between the conventional and PCR method, this may be due to the presence of resistant mutations, which could not be detected using current primers.

It was noticed that the majority of the isolates, which showed resistance to the two drugs on LJ medium proportion method showed resistance on the PCR to the two drugs or at least to one of them, mostly isoniazid; therefore, resistance to Isoniazid can be considered as a marker for multidrug resistance[15]. However, Sharma et al considered resistance to rifampicin as a marker for MDR TB[16].

In this study, a rising resistance rates of MDR among TB patients in Sudan compared with some African countries were revealed. In Ethiopia 14% of the isolates were found MDR, in Kenya 33.3%, in South Africa 60.2% and in Saudi Arabia 11% resistance to isoniazid and 9.7% to rifampicin was recorded[17-20]. In the present study, a rate of 21.5% was detected, which could be compared to Sharaf el Din et al who applied PCR based dot-blot method and found that 12% of strains were resistant to INH (*katG* gene), 8% were resistant to RIF (*rpoB* gene), 30% to STM (*rpsl* gene) and 4% to EMB (*embB* gene) but no record of MDR was investigated by these authors in patients with persistent TB[21].

Similarly, a novel PCR-based reverse hybridization method Genotype MTBDR assay (Hain Lifescience GmbH, Nehren, Germany) was evaluated for rapid detection of rifampicin (RIF) and isoniazid (INH) resistance in Turkish *Mycobacterium tuberculosis* isolates revealed that RIF resistance

was correctly identified in 95.1% of the isolates and in 73% of INH-resistant isolates. However, the recommendation of the author is to confirm the result with phenotypic methods[22].

The GenoType® MTBDR*plus* assay has been validated as a rapid and reliable first-line diagnostic test on AFB-positive sputum or MTB isolates for INH resistance, RIF resistance and MDR-TB in Bangkok, it was found reliable, but its impact on treatment outcome, feasibility and the cost associated with widespread implementation needs to be evaluated[23].

The presence of some isolates in this study, which are sensitive in the proportional method and appeared as resistant in the PCR to one of the two drugs or both of them could not be attributed to contamination, rather it could be due to mutations, which are not necessarily strongly related to resistance. Moreover, since the target region of the genes for RIF and INH resistance are not mentioned by the manufacturer, it is difficult to explain this part of the result.

CONCLUSION

Classical DST is still a gold standard method for diagnosis of MDR TB in developing countries. However, substitution of the classical DST with the rapid and sensitive PCR needs to be carefully evaluated as the protocol used in this study failed to detect all types of mutations present. Other PCR protocols targeting all known mutations in RIF and INH genes could be of more useful.

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REFERENCES

1. Migliori GB, Lange C, Girardi E, et al. Extensively Drug-resistant Tuberculosis Is Worse than Multidrug-Resistant Tuberculosis: Different Methodology and Settings, Same Results. Clin Infect Dis 2008; 46(6): 958-9.
2. World Health Organization (WHO). Multidrug and Extensively Drug-resistant TB (M/X DR-TB): 2010 Global Report on Surveillance and Response. http://whqlibdoc.who.int/publications /2010/9789241599191eng.pdf. Accessed 17.2.2011.
3. Cavanagh P. The Sensitivity to Streptomycin, PAS and Isoniazid of Strains of *Myco. Tuberculosis* Isolated from Patients in Khartoum and Wad Medani. Tubercle 1965; 46(3): 250-5.
4. Ahmed Suleiman MM, Aro AR, Sodemann M. Evaluation of Tuberculosis Control Programme in Khartoum State for the Year 2006. Scand J Public Health 2009; 37(1): 101-8.

5. World Health Organization (WHO). Use of liquid TB Culture and Drug Susceptibility Testing (DST) in Low and Medium Income Settings. Summary Report of the Expert Group Meeting on the Use of liquid Culture Media. http://www.who.int/tb/laboratory/ use_of_liquid_tb_culture_ summary_report.pdf. Accessed 25.1.2011.
6. World Health Organization (WHO). Molecular line Probe Assays for Rapid Screening of Patients at Risk of Multidrug-Resistant Tuberculosis (MDR-TB): Policy Statement. Geneva, World Health Organization, 2008. http://www.who.int/tb/laboratory/1papolicy. pdf. Accessed 12.2.2011.
7. Somoskovi A, Parsons LM, Salfinger M. The Molecular Basis of Resistance to Isoniazid, Rifampin, and Pyrazinamide in *Mycobacterium Tuberculosis*. Respir Res 2001; 2(3): 164-8.
8. Marttila HJ, Soini H, Eerola E, et al. A Ser315Thr Substitution in KatG is Predominant in Genetically Heterogeneous Multidrug-resistant *Mycobacterium Tuberculosis* Isolates Originating from the St. Petersburg Area in Russia. Antimicrob Agents Chemother 1998; 42(9): 2443-5.
9. Rouse DA, DeVito JA, Li Z, et al. Site-directed Mutagenesis of the katG Gene of *Mycobacterium Tuberculosis*: Effects on Catalase-peroxidase Activities and Isoniazid Resistance. Mol Microbiol 1996; 22(3): 583-92.
10. Goble M, Iseman MD, Madsen LA, et al. Treatment of 171 Patients with Pulmonary Tuberculosis Resistant to Isoniazid and Rifampin. N Engl J Med 1993; 328(8): 527-32.
11. Telenti A, Imboden P, Marchesi F, et al. Detection of Rifampicin-Resistance Mutations in *Mycobacterium Tuberculosis*. Lancet 1993; 341(8846): 647-50.
12. Public Health Mycobacteriology, A Guide for a Level III laboratory. US Department of Health and Human Services, Public Health Service, Centers for Disease Control. USA: Atlanta, 1985: 159-184.
13. National Committee for Clinical Laboratory Standards (NCCLS). Susceptibility Testing of *Mycobacteria, Nocardiae,* and Other Aerobic *Actinomycetes;* Approved Standard; NCCLS document M24-A [ISBN 1-56238-500-3]. NCCLS, 940 Pennsylvania, USA: 2003. http://www.iso90.ir/phocadownload/csli/M24-A.pdf Accessed 17.3.2011.
14. Eisenach KD, Cave MD, Bates JH, et al. Polymerase Chain Reaction Amplification of a Repetitive DNA Sequence Specific for *Mycobacterium Tuberculosis*. J Infect Dis 1989; 161(5): 977-81.
15. Vijdea R, Stegger M, Sosnovskaja A, et al. Multidrug-resistant Tuberculosis: Rapid Detection of Resistance to Rifampin and High or Low Levels of Isoniazid in Clinical Specimens and Isolates. Eur J Clin Microbiol Infect Dis 2008; 27(11): 1079-86.
16. Sharma SK, Mohan A. Multidrug-resistant Tuberculosis. Indian J Med Res 2004; 120(4): 354-76.
17. Bruchfeld J, Aderaye G, Palme IB, et al. Molecular Epidemiology and Drug Resistance of *Mycobacterium Tuberculosis* Isolates from Ethiopian Pulmonary Tuberculosis Patients with and without Human Immunodeficiency Virus Infection. J Clin Microbiol 2002; 40(5): 1636-43.
18. Githui WA, Jordaan AM, Juma ES, et al. Identification of MDR-TB Beijing/W and Other *Mycobacterium Tuberculosis* Genotypes in Nairobi, Kenya. Int J Tuberc Lung Dis 2004; 8(3): 352-60.
19. Calver AD, Falmer AA, Murray M, et al. Emergence of Increased Resistance and Extensively Drug-Resistant Tuberculosis Despite Treatment Adherence, South Africa. Emerg Infect Dis 2010; 16(2): 264-71.
20. Alrajhi AA, Al-Barrak AM. *Mycobacterium Tuberculosis* Susceptibility in Saudi Arabia. Saudi Med J 2002; 23(10): 1227-31.
21. Sharaf-Eldin GS, Saeed NS, Hamid ME, et al. Molecular Analysis of Clinical Isolates of *Mycobacterium Tuberculosis* Collected from Patients with Persistent Disease in the Khartoum Region of Sudan. J Infect 2002; 44(4): 244-51.
22. Cavusoglu C, Turhan A, Akinci P, et al. Evaluation of the Genotype MTBDR Assay for Rapid Detection of Rifampin and Isoniazid Resistance in *Mycobacterium Tuberculosis* Isolates. J of Clin Microbiol 2006; 44(7): 2338-42.

23. Anek-Vorapong R, Sinthuwattanawibool C, Podewils LJ, et al. Validation of the GenoType® MTBDR *Plus* Assay for Detection of MDR-TB in a Public Health Laboratory in Thailand. BMC Infect Dis 2010; 10: 123.