

Detection of Multidrug-Resistant Tuberculosis in Sudan using PCR Method in Comparison to the Conventional Proportional Method

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Objective: To evaluate the usefulness and application of PCR based technique for the rapid detection of Multidrug-Resistant (MDR) *Mycobacterium tuberculosis* in comparison to the conventional proportional drug sensitivity testing.

Design: Prospective cross sectional laboratory based study.

Setting: Different TB centers in Khartoum State, Sudan.

Method: One hundred and thirty tuberculosis patients of both sexes and different ages were included in this 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 *M. tuberculosis*.

Results: 56/65 (86%) of isolated *M. tuberculosis* was confirmed as members of the *M. tuberculosis* complex using PCR amplified IS6110. 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 while 30/56 (51.8%) of the strains were found resistant to both drugs (MDR) using the conventional method compared to 21/56 (37.5%) resistant to RIF, 32/56 (57.1%) to INH while 16/56 (28.6%) were resistant to both drugs (MDR) when using the PCR method.

Conclusion: Not all resistant strains detected by conventional method were detected by PCR method, 14 (25%) were missed for RIF, 9 (17.9%) 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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INTRODUCTION

The most effective anti-TB drugs are isoniazid (INH) and rifampicin (RIF). Mycobacteria that are resistant 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 drugs¹.

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 anti-TB drug resistance, the estimated number of MDR-TB cases (primary and acquired) in 2008 in this region was 69 000 (53 000–110 000)².

Since the early study by Cavanagh, data on drug resistant TB are lacking in the Sudan³. Tuberculosis control programme in Khartoum State for the year 2006, as evaluated by Suleiman et al, showed that there was no system to detect the prevalence of MDR-TB among the TB cases⁴.

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, such a delay that results in an adverse effect⁵

Therefore, implementation of rapid molecular methods for detecting drug-resistant TB may be a valuable 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 settings⁶.

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 activity⁷.

Other mutations were detected in Ser315Thr in around 40% of isoniazid-resistant strains⁸

This mutation was found to result in the production of a catalase enzyme that retains around 50% of isoniazid catalase-peroxidase activity which is sufficient for the ability of the organism to evade the action of host active radicals⁹

It is proposed that over-expression of one or more of certain other genes including those which encode for the mycolic acid-synthesis intracellular proteins: fatty-acid enoyl-acyl carrier protein reductase (*InhA*), acyl carrier protein (*AcpM*) and a β -ketoacyl-ACP synthase (*KasA*)¹⁰ may be the reason for isoniazid resistance in these strains. Moreover, mutations in the promoter region of a gene that encodes an alkyl hydroperoxidase reductase (*ahpC*) have been found in approximately 10% of isoniazid-resistant isolates, but mutations in *katG* were also found in these isolates¹¹.

Spontaneous mutations (deletions/substitutions/insertions) were found to occur in the 81-bp hotspot region of the *rpoB* gene which encodes DNA-dependent RNA polymerase (the target for rifampicin binding). This results 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 and activity of the enzyme (transcription), is preserved, thus explaining resistance to rifampicin in bacteria¹¹.

The objective of this study was to evaluate the usefulness and application of a PCR based technique for the rapid detection of MDR *Mycobacterium tuberculosis* in comparison to the conventional proportional drug sensitivity testing method.

METHOD

A prospective cross sectional study was carried out. Smear-positive sputum was collected from 130 patients (62 males and 48 females, age range from 12-67 years) with persistent tuberculosis who were randomly selected from patients attending Alshaab Teaching Hospital, Abu-Angah Hospital and the National Health Laboratory (Khartoum). Sputum samples (spot or morning) were collected per patient in 50 ml sterile conical centrifuge tubes, smear positive patients were enrolled after being given their informed consent. The ethical approval for this study was obtained from the Federal Ministry of Health and from 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 methodology¹². Drug susceptibility testing was performed by LJ proportion method¹³.

Sixty five *M. tuberculosis* isolates were randomly selected from the positive cultures and were confirmed as *M. tuberculosis* by detecting species-specific IS6110 (123 bp) using primers and method previously described¹⁴. Resistant strains detected by conventional methods were tested for the presence of mutations conferring resistance to isoniazid (INH) and rifampicin (RIF) by PCR methods using PCR kits from Genekam Biotechnology, Germany, (Ref K112)

following the instructions of the manufacturer.

The PCR program was set according to manufacturer and the PCR product was visualized on 1% agarose gel electrophoresis stained with 0.5 mg/ml ethidium bromide and run at 100 V for 30 min.

Statistical package for social science (SPSS) for windows version 11.0 was used for data analysis.

RESULT

Results of drugs susceptibility testing for rifampicin and isoniazid by LJ proportion and PCR methods are shown in Table 1. Out of the 130 sputum specimens, 119 (91.5%) showed *M. tuberculosis*-like colonies, 65 of which were randomly subjected to PCR and examined for the presence of IS6110 insertion sequences (Figure 1). Depending on PCR results, 56 (86.2%) samples of these were *M. tuberculosis* and 9 (13.8%) were MOTTs.

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 (Figure 2); whereas those resistant for INH were 36/56 (64.3%) using the conventional method compared to 32/56 (57.1%) using PCR method (Figure 3); 51.8% of the strains were found resistant for both drugs (MDR) using the conventional method compared to 28.6% for both drugs (MDR) when using the PCR method.

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

	LJ proportion	PCR method
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	method			
	RIF	INH	RIF	INH
Resistant: No.	32	36	21	32
(%)	(57.1 %)	(64.3 %)	(37.5 %)	(57.1 %)
Sensitive : No.	23	19	35	24
(%)	(41.1 %)	(33.9 %)	(62.5 %)	(42.9 %)
ND: No.	1	1	-	-
(%)	(1.8%)	(1.8%)		
Total	56	56	56	56

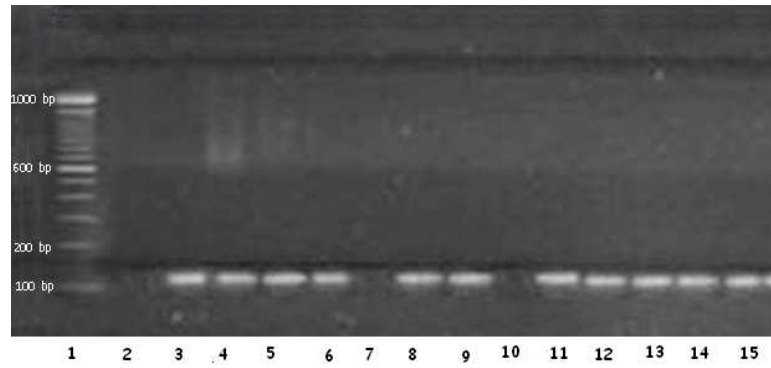


Figure 1: PCR Amplified IS6110 Sequences, Lane 1, DNA marker; lane 2, negative control; lane 3, reference *M. tuberculosis* strain H37v (positive control).

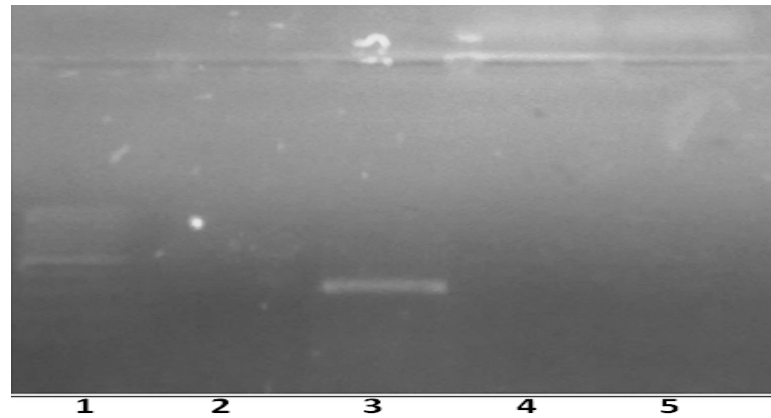


Figure 2: The amplicon of rifampicin resistant *M. tuberculosis*; Lane 1, DNA marker; lane 2, negative control; lane 3, positive control; Lane 7; RIF resistant strain.

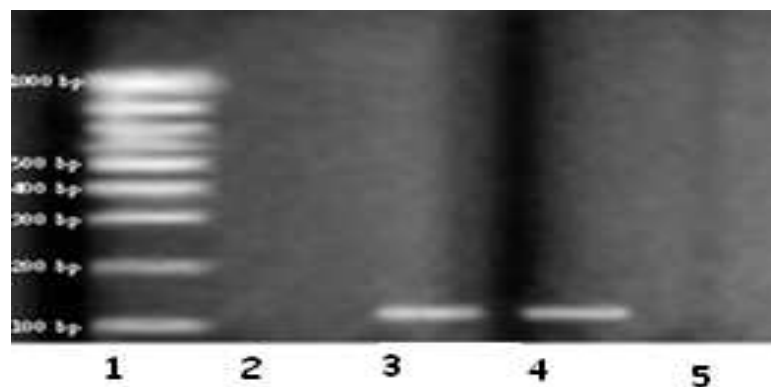


Figure 3: The amplicon of isoniazid resistant *M. tuberculosis*; Lane 1, DNA marker; lane 2, negative control; lane 3, positive control; lanes 4 and 7, INH resistant strains.

DISCUSSION

The result of antibiotics susceptibility testing revealed that there were obvious differences between the conventional and PCR methods, this may be due to the existence of resistant mutations which could not be detected using current primers and company protocol (Genekam).

Depending on the results obtained in this study, it was noticed that the majority of the isolates which showed resistance to the two drugs on LJ medium proportion method showed on the PCR resistance to the two drugs or at least one of them, mostly isoniazid, so resistance to Isoniazid can be considered as a marker for multidrug resistance¹⁵. However, Sharma and Mohan (2004) considered resistance to rifampicin as a marker for MDR TB¹⁶.

Results of the present report demonstrated a rising resistance rates of MDR among TB patients in Sudan compared with some African countries. For instance, in Ethiopia 14.0% of isolates were found MDR; 33.3% of drug-resistant TB were found MDR in Kenya; 60.2% of patients with drug-resistant TB in South Africa were classified as MDR and in Saudi Arabia 11% resistance to isoniazid and 9.7% to rifampicin was recorded¹⁷⁻²⁰. These rates are comparable to 21.5% detected in the present study and can be compared to those recorded by Sharaf el Din et al who applied a 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 TB²¹.

The increased rates of MDR can be attributed to different reasons which may include limited and less sensitive diagnostic tools, the absence of well trained personnel, abuse and interruption of treatment and socioeconomic status.

Similarly, a novel PCR-based reverse hybridization method Genotype MTBDR assay (Hain Lifescience GmbH, Nehren, Germany) was evaluated for rapid detection of rifampin (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 was that the test results should always be confirmed with phenotypic methods²².

The GenoType® MTBDR^{plus} assay has also 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, Thailand and was found reliable but its impact on treatment outcome and the feasibility and cost associated with widespread implementation was found in need to be evaluated²³.

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 can not be attributed to contamination as the negative control in each reaction is always negative, rather, it can 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 results.

CONCLUSION

The study concluded that the classical DST is still a gold standard method for diagnosis of MDR TB in developing countries like Sudan. 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 prevalent. Other PCR protocols targeting all known mutations in RIF and INH genes could be of more usefulness.

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