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Diagnosis of Multi-drug Resistant Tuberculosis Mutations Using Hain Line Probe Assay and GeneXpert: A Study Done in Zimbabwe

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Authors' contributions

This work was carried out in collaboration between all authors. Author SDMR designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors SDMR, DZ, DK and MR managed the literature searches and analyses of the study. Author SDMR performed the laboratory analysis and authors DZ and MR managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: Tuberculosis (TB) is a global public health problem and one of the leading causes of death. Worldwide, 31% of all estimated new TB cases are from Africa. Zimbabwe is one of the 22 high TB burden countries. Multi-drug resistant TB (MDR-TB) poses challenges in TB control, hence the need for rapid laboratory diagnosis of MDR-TB for optimal treatment and reducing spread. The study aim was to investigate genetic mutations associated with MDR-TB isolates from various Harare clinics using the GeneXpert MTB/RIF® by Cepheid and

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Genotype MTBDRplus, to improve the diagnosis and management of MDR-TB.
Methods: Samples from adults aged 16 years and older, recruited from several polyclinics in the southern suburbs of Harare were used for our study. All laboratory tests prior to this study had been carried out at Biomedical research and training institute's level three bio-safety TB laboratory from January 2008-August 2012. Ethical approval was sought from BRTI Institutional review board. A total of 69 (37 MDR-TB and 32 non MDR-TB) archived isolates processed on Genotype MTBDRplus (Hains) and corresponding 39 sputum were processed on the GeneXpert. Mutations on rpoB, katG and inhA genes were observed. The gold standard was culture. Diagnostic accuracy of both methods and their level of agreement were calculated.
Results: Of the 37/69 isolates screened by culture for MDR-TB, 88.4% were confirmed by MTBDR® plus line probe assay (Hains). Within the 39 isolates tested using the Xpert MTB/RIF (GeneXpert) assay 12 were true MDR-TB. Over 8 single nucleotide polymorphisms were observed on the three genes conferring Rifampicin and Isoniazide drug resistance. The Hains and GeneXpert had an almost perfect agreement with a kappa value of 0.82.
Conclusion: Genetic markers can be used in the diagnosis of MDR-TB, to complement phenotypic methods such as culture. Using the commercial methods, Hains and GeneXpert, 88.4-94.2% of drug resistance maybe detected. Furthermore, we recommend sequencing so as to identify novel mutations and to design a kit that is custom made for the population.

Keywords: Tuberculosis; MDR-TB; molecular diagnosis; Zimbabwe; GeneXpert; hains.

1. INTRODUCTION

Tuberculosis (TB) is a global public health problem [1], and also one of the leading causes of death [2]. Worldwide, the disease takes a life every 20 seconds. Up to 30% of all estimated new TB cases are from Africa [3]. Gloomily, 90 % of TB cases and deaths occur in the 22 TB high burden countries, with Zimbabwe included [4]. The high incidence of TB is excerbated by multi drug resistant TB (MDR-TB).

MDR-TB is defined as disease caused by *Mycobacterium tuberculosis* resistant to at least the two most vital first-line anti-TB drugs, rifampicin (RIF) and isoniazid (INH). Poor diagnosis and therapeutic management of MDR-TB can lead to the development of extensive drug resistance (XDR-TB) [5]. The defination of XDR-TB is MDR-TB also resistant to second line regimen of TB treatment such as fluoroquinolones and at least one injectible drug such as aminoglycosides and cyclic peptides [6]. Globally, MDR-TB affects over half a million patients[5]. Prevalence of MDR/XDR-TB in South Africa is on the rise, estimated at 8% [7,8] which has implications on Zimbabwe due to the frequent movement of people between these two neighbouring countries. Exact numbers of MDR-TB in Zimbabwe are not yet quite known, a survey is being carried out. In 2012, a study was carried out in Harare, Zimbabwe to estimate the prevalence of MDR-TB. Of the 84 specimens processed, 20 (24%) were consistent with MDR TB [9]. A bigger survey at national level was recommended. Drug resistant TB pose huge challenges in controlling this infection, thus the need for timely and accurate laboratory diagnosis of MDR-TB for optimizing treatment and preventing spread may not be over-emphasised [10].

Drug susceptibility testing on culture media is the gold starndard method widely used for MDR-TB diagnosis in Zimbabwe. Despite having high specificity, the biggest down fall with culture is the long turnaround time of up to 8 weeks [11]. The clinical implications of the long turn around time are increased transmission, delayed therapy and increased mortality. Consequently, introducing molecular diagnosis methods is crucial so as to construct an algorithm for MDR-TB diagnosis, which would lead to rapid detection as well as accurate prescription of TB therapeutic drugs.

Several genetic markers have been used in designing molecular based laboratory diagnostic tools for MDR-TB [12]. These include; RNA polymerase beta subunit gene (rpoB), 1148 amino acids long, encoding RNA polymerase enzyme with the mutation bubble at positions 507 to 533 [13], illustrated in Fig. 1. An 81 base pair (bp) region of this gene results in over 95% of mutations associated with RIF resistance [14,15]

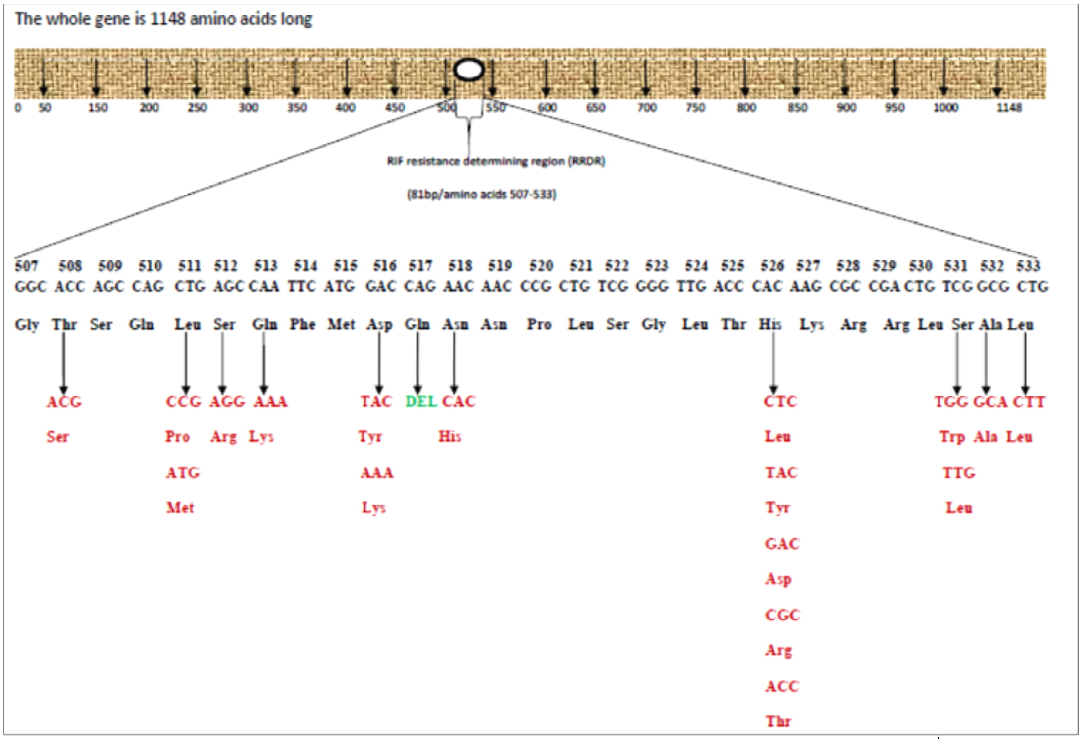

Fig. 1. The structure of rpoB gene and the 81bp RIF resistance determining region (RRDR)

katG gene encoding a catalase peroxidase [7] and inhA (isoniazid A) gene both confer INH resistance [13]. All the three genes have been used to design several commercial kits.

GeneXpert MTB/RIF® by Cepheid and Genotype MTBDRplus®(Hains) by Hain lifesciences are the two molecular diagnostic tools that have recently been introduced to Zimbabwe. The GeneXpert makes use of mutations on rpoB gene and gives results in 2 hours [16]. The GeneXpert is fully automated and uses hemi nested real time PCR to simultaneously detect both MTB and RIF resistance with the latter being used as a surrogate marker for MDR-TB [11]. Hains is based on the principle of reverse hybridization where oligonucleotide probes are immobilized on nitrocellulose strips, giving results in under 3 hours. One probe is specific for *Mycobacterium tuberculosis* complex [17]. Eight probes complementing amino acids 509-533 of rpoB gene (detecting mutations such as S531L), one for katG and two for inhA gene [18-20].

Understanding the existing mutations that are common to a particular population assists in the choice and optimization of the most appropriate diagnostic method. Data from our study will also add to molecular epidermiology used for geographic mapping and phylogenetic grouping. The aim was to investigate the genetic mutations associated with MDR-TB isolates from various Harare clinics using the GeneXpert MTB/RIF® by Cepheid and Genotype MTBDRplus with the intention to improve the diagnosis and management of MDR-TB in Zimbabwe. Delayed TB therapy exposes patients to additional anti-TB drug resistance. Prompt diagnosis of TB simultaneously with MDR-TB will faciliate timely administering of the most appropriate treatment, as a result this is cost effective and reducing further transmission.

2. MATERIALS AND METHODS

A cross sectional study of all TB isolates that had been collected from adult patients whom were at least 16 years old, reporting at several polyclinics in Harare from January 2008 to August 2012. All 69 available (37 MDR-TB and 32 non MDR-TB) archived isolates from participants screened for *Mycobacterium tuberculosis* using culture were used. Ethical approval was sought and granted by Biomedical research and training institute-Institutional review board (BRTI-IRB).

2.1 Laboratory Methods

2.1.1 Culture on Lowenstein-Jensen (LJ) media

Isolates frozen in Tryptic *Soy Broth* with 10% *Glycerol* (TSB/glycerol) and frozen sputum were retrieved from the -70°C freezer in the BRTI level 3 bio-safety laboratory. After thawing, the colony suspension was homogenized by gentle mixing then cultured on solid LJ agar. All specimens were incubated at 37°C and checked weekly for up to eight weeks. When the media surface was covered with white colonies of *Mycobacterium tuberculosis,* they were scrapped off and re-suspended in a cryotube with 500µl of distilled water. The suspension was then heat killed for 1 hour in a water bath at 80°C. The heat killed samples could now be used outside a level 3 bio-safety laboratory.

2.1.2 MTBDR plus (Line probe assay)/Hains

Hains was performed at Aibst laboratory. DNA extraction was performed using an ultra sonicator. Five hundred microlitres of processed sediment was used to perform the Genotype MTBDR plus version 2.0 assay, according to the manufacturer's instructions. Mutations on rpoB, katG and inhA genes were then observed (Fig. 1 below illustrates possible mutations on rpoB gene).

2.1.3 GeneXpert MTB/RIF (GeneXpert)

Sputum was thawed and processed in the BRTI-TB laboratory. The GeneXpert MTB/RIFsample reagent was added in a volume of 2:1 with the sputum and incubated for 15 minutes at room temperature as per manufacturer's instructions. The sample was mixed gently at 5-minute intervals during the incubation period. After the 15 minute incubation, 2 ml of the processed sample was transferred to the GeneXpert MTB/RIF cartridge using a sterile pipette provided with the kit. After loading the cartridge onto the 4-modular GeneXpert machine, the sample underwent automated washing, extraction, amplification and detection.

2.2 Data Analysis

Data was analyzed using STATA Version 11.0. Proportions of MDR TB detected using GeneXpert and Hains with culture as the gold standard were presented and so were the proportions of genes conferring drug resistance. Sensitivity, specificity, positive predictive values and negative predictive values were calculated for culture, Hains and GeneXpert. To analyze the level of agreement of Hains and GeneXpert, the kappa test was performed. Hains and culture utilized all 69 isolates, whereas GeneXpert could only be performed on 39 isolates. STATA selected the 39 samples for any calculations of the methods that included GeneXpert.

3. RESULTS

All 69 isolates were tested on Hains and 39 of these were also tested on GeneXpert. The re-cultured isolates had reproducible results.

3.1 Mutations Detected by Hains

Of the 69 isolates tested on Hains, there were 29 MDR-TB and 4 Rifampicin (RIF) mono-resistant. The most frequent mutation that conferred RIF resistance was on amino acids 530 to 533, specifically Serine to Leucine on position 531 (S531L) with a frequency of 69.7%. rpoB wild types 1, 2 and 6, representing amino acids 505-509, 510-513 and 518-523 respectively, had no mutations. 12.1% of mutations were within codons 513-519. Specific mutations could not be determined as there were no wild type bands as well as no corresponding mutation bands that were characterized by the kit. This also applied on the 30.3% isolates which showed possible mutations within codons 516-522. H526D was observed in 6.1% of MDR-TB isolates and 9.1% had no specific codon but showed possible mutations within codons 526-529.

Isoniazide (INH) resistance was mostly (42.4%) due to katG mutation 1, represented by amino acid S315T. A few isolates had INH resistance conferred by mutations on inhA, as C15T and T8A. Table 1 illustrates the proportions of mutations on katG, inhA and rpoB genes. Fig. 2 shows the oligonucleotide bands represented on Hains.

3.2 Drug Resistance Detected by GeneXpert

Of the 39 samples processed on the GeneXpert, there were 12 true MDR-TB and 27 true sensitive isolates, using culture as the gold standard. There is no detail on the actual probe that failed to hybridize.

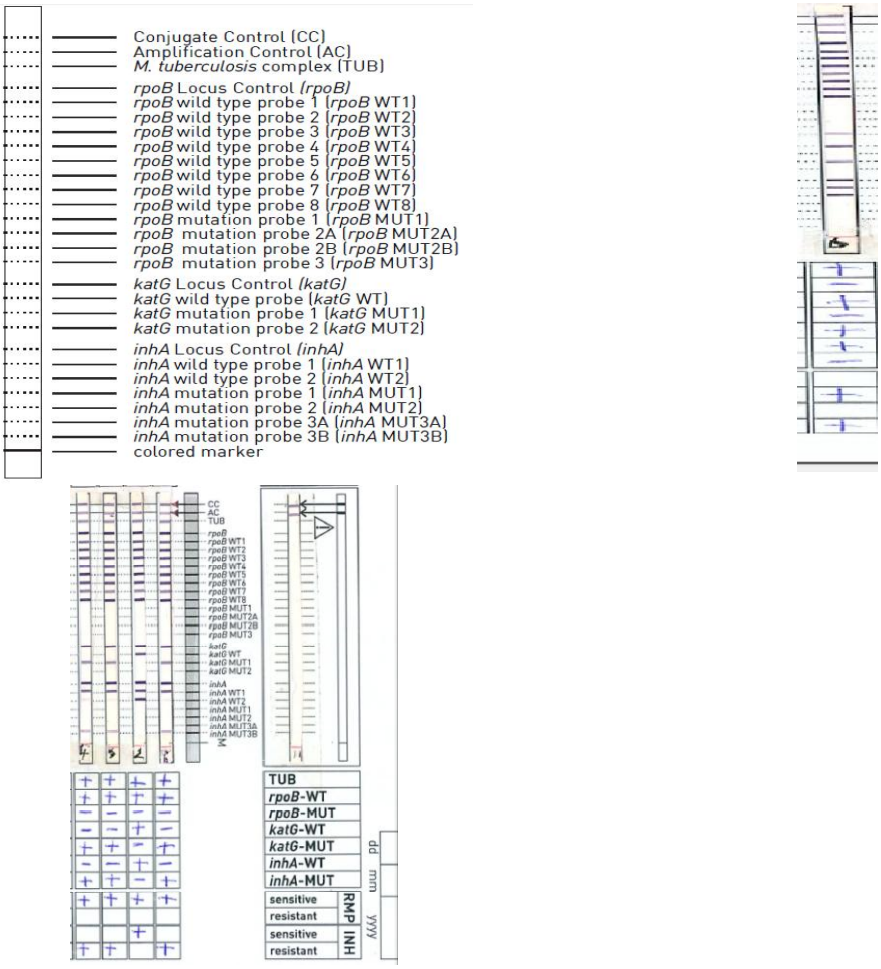

Fig. 2. Picture on left: Oligonucleotide probes represented on the Hains (Hain Lifescience, 2010). Pictures on right: Examples of *MTBDR plus (Line probe assay) / Hains.* First strip; *Mycobacterium tuberculosis* (MTB), MDR-TB, resistant to rifampicin (RIF)-rpoB S531L mutation, resistant to isoniazid (INH), katG S315T mutation and inhA no mutations. Second strip lane 1,3 and 4; MTB, susceptible to RIF, resistant to INH, katG S315T and inhA T8A mutations

3.3 Diagnostic Accuracy of GeneXpert and Hains

Only 39 samples out of 69 had sufficient volume to be processed on the GeneXpert. 12/39 (30.8%) were Rifampicin resistant and 27/39 (69.2%) were Rifampicin sensitive. The GeneXpert had 60.0% sensitivity, 87.5% specificity, 75.0% positive predictive value and 77.8% negative predictive value. The Hains had 70.3% sensitivity, 90.6% specificity, 89.7% positive predictive value and 72.6% negative predictive value. Table 2 below illustrates sensitivity, specificity and predictive values of Hains and GeneXpert.

3.4 Level of Agreement of Hains and GeneXpert

Hains and GeneXpert were able to detect 72.8% of MDR-TB and 5.7% RIF mono-resistance. Using RIF resistance as a surrogate marker for MDR-TB, molecular methods generally missed 21.4% of MDR-TB (illustrated in Fig. 3 below). Kappa test was performed on the 39 samples processed on GeneXpert and the Hains. The two methods had a 92.3% level of agreement. The kappa value was 0.82, which is almost perfect agreement.

Table 1. Frequencies of rpoB, katG and inhA genes' [wild type (WT) and mutation (MUT)] bands on Hain line probe assay

Gene fragments	% (n=69)
rpoB WT1	100
rpoB WT2`	100
rpoB WT3	94.2
rpoB WT4	89.9
rpoB WT5	95.6
rpoB WT6	100
rpoB WT7	92.8
rpoB WT8	75.4
rpoB MUT 1	0
rpoB MUT 2A	0
rpoB MUT 2B	2.9
rpoB MUT 3	33.3
katG WT	59.4
katG MUT 1	42.4
katG MUT 2	0
inhA WT 1	97.1
inhA WT 2	94.2
inhA MUT 1	2.9
inhA MUT 2	0
inhA MUT 3A	0
inhA MUT 3B	5.8

4. DISCUSSION

This was a small study carried out in one province; however it is still one of the first to report on mutations associated with MDR-TB in Zimbabwe. With Zimbabwe being one of the 22 high TB burden countries; this information is very useful for the National TB programmes in the process of reducing the incidence of MDR-TB. The results show that mutations common in the study population are similar to those detected in different geographical regions.

With such data, it is clear that commercially designed molecular diagnostic tools, with a short turnaround time, can be used so as to reduce the incidence and transmission of MDR-TB. The molecular methods (Hains and GeneXpert give results within 2-3 hours as opposed to culture with a turnaround time of 8 weeks.

Table 2. Diagnostic accuracy of GeneXpert and Hains, using culture as the gold standard

Values in %	GeneXpert	Hains
Sensitivity	60.0	70.3
Specificity	87.5	90.6
Positive predictive value	75.0	89.7
Negative predictive value	77.8	72.6

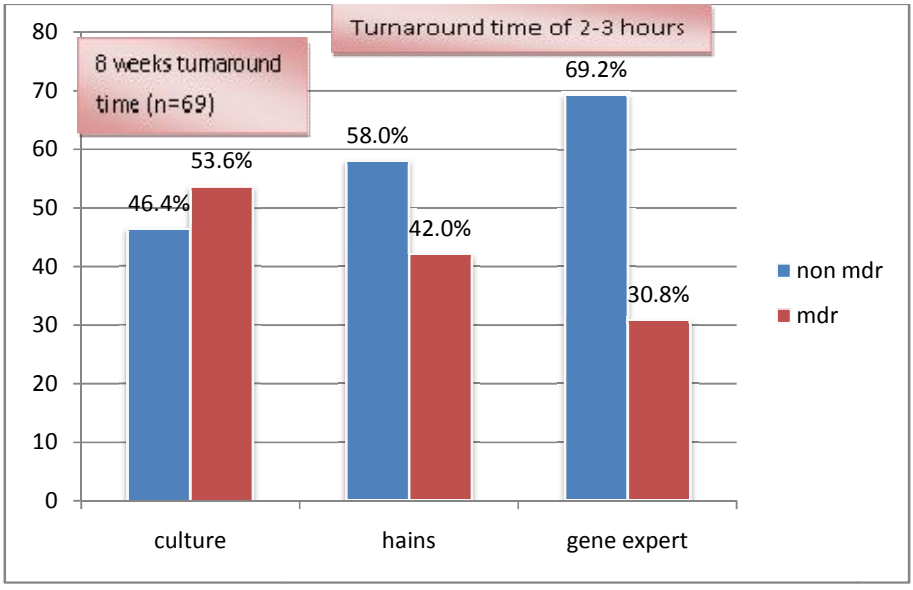

Fig. 3. Frequencies of non-MDR and MDR TB detected by Hains and GeneXpert with culture as the gold standard. Only the 39 which were consistently run on culture, Hains and GeneXpert were used for calculations

4.1 Mutations Detected by Hains

Despite the very low turnaround time of molecular methods (genotypic based), culture still detected the most MDR TB, since it is a phenotypic based method. Hains was able to detect MDR-TB in 29/69 (42.0%) of which culture had detected 37/69 (53.8%). The 5.8% which was missed by genetic methods agrees with published literature [8] demonstrated a difference of 5%, in the proportion of MDR-TB detected by culture and Hains, with the former detecting higher amounts. Within the 58% non-MDR isolates detected by Hains, there were 5.8% INH mono-resistant, 5.8% RIFmono-resistant and 46.4% sensitive isolates. The 11.6% that was missed by Hains molecular diagnostics in detecting MDR-TB could be due to mutations that would have not been characterized by the kit. If RIFmono-resistance is included and used as a surrogate marker for MDR-TB, then only 5.8% would have been missed in diagnosis. Therefore, 94.2% would have been saved if molecular methods are introduced in the diagnosis of TB and drug resistance, together with culture. Largely, prompt diagnosis of MDR-TB and appropriate treatment can then be administered, consequently reducing transmission and the mortality rate.

The mutations that were observed in the 3 genes; rpoB, katG and inhA were in agreement with recent literature [5]. Our study has shown that in Harare, Zimbabwe the most frequent mutations of rpoB are on codon S531L (69.7%). Previous studies support that rpoB mutations mostly occur on positions 531, 526 and 516. In Uganda [5], 20 isolates were observed and 70% had mutations on Ser531Leu, 10% on Ser531Trp whilst codons 513, 516 and 526 had frequencies of mutation at 5%, 5% and 10% respectively. On average 50% of MDR-TB isolates in Iran, Hondurus and Belarus had mutations on S531L. Codon 526 was the next frequent with mutations in these countries [5]. This also matches with data from Uganda, China, Belarus, Iran and Hondurus [5]. Hains has defined mutations, therefore sequencing is recommended to rule out any possible novel mutations.

In our study INH resistance was due to mutations on kat G and inhA. All katG mutations were due to S315T. Only 2 isolates had mutations on inhA that were due to C15T. For katG, 30-60% of MDR-TB isolates from Romania, China, Belarus and Hondurus had mutations on S315T. Uganda had this mutant codon in 75% of the isolates. inhA has the most frequent mutant codon C15T, though with low occurrences averaging 10%.

4.2 Drug Resistance Detected by GeneXpert

The 12 MDR-TB detected by GeneXpert had also been detected by culture as well as the 27 non-MDR-TB. Sample size was a challenge faced with this method; only 39 samples had sufficient sputum.

4.3 Diagnostic Accuracy of GeneXpert and Hains

The statistics in our study are not comparable with those of previous publications. In South Africa, for Hains, sensitivity, specificity, and positive and negative predictive values were 98.9, 99.4, 97.9, and 99.7%, respectively, for detection of rifampicin resistance; 94.2, 99.7, 99.1, and 97.9%, respectively, for detection of isoniazid resistance; and 98.8, 100, 100, and 99.7%,respectively for detection of multidrug resistance compared with conventional results. This study had much lower values, implicated on the small sample size.

4.4 Level of agreement of Hains and GeneXpert

Our study had 89.2% isolates with mutations on rpoB. This is quite comparable to studies done in China. Jun Yue and colleagues studied mutations on rpoB from isolates collected from different parts of China. DNA sequencing was used and 90.3% of the MDR-TB isolates had mutations on rpoB. One report [5] detected 94% of mutations on rpoB, 85% of isolates from Romania and Iran showed mutations on rpoB.

The samples that were INH resistant had mutations on katG and inhA at frequencies of 73.0% and 16.2% respectively. INH resistance has been widely studied worldwide using different molecular techniques such as restriction fragment length polymorphism (RFLP), microarray technology, multiplex PCR and single strand confirmation polymorphisms (SSCP). In Kwazulu natal South Africa, INH resistance was implicated on 77/79 (97.4%) and 19/79 (24.1%), for mutations on katG and inhA respectively, of TB isolates confirmed by culture. A third gene conferring INH resistance was observed, ahpC, mutations were found in 10/79 (12.1%) isolates. Our study had a relatively lower frequency of

katG mutations in comparison with other studies, whilst the frequencies of inhA mutations were slightly higher as compared to a South African study [1].

5. CONCLUSION

Molecular methods can be used in the diagnosis of drug resistance in Tuberculosis patients; however we suggest the inclusion of Hains and GeneXpert in diagnosis procedures to reduce TB mortality and occurrence of MDR-TB. The most frequent mutations are similar to documented data from other geographic regions. The turnaround time of culture opens doors for increased TB mortality and development of drug resistance as the patients are put on first line treatment and are changed from this regimen when the response to therapy is poor. Instead of this 'trial and error' method, patients can be screened for drug resistance using molecular methods, such as Hains and geneXpert, while they wait for their culture results. Further studies based on rpoB, katG and inhA sequencing are important so as to detect any novel mutations. Since this was a small study, a much bigger study covering the whole country is recommended.

CONSENT

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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