Original Article

Drug susceptibility testing using molecular techniques can enhance tuberculosis diagnosis

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Abstract

Background: Sputum samples were collected from tuberculosis patients in a high tuberculosis incidence area in the Western Cape, South Africa. The aim of this study was to evaluate the performance and time to diagnosis of a genotypic drug susceptibility testing method.

Methodology: During June 2000 and November 2003, a total of 1,540 samples were sent for drug susceptibility testing (DST) to the national health laboratory services, and of those, a phenotypic DST result was obtained for 1,373 samples whereas a genotypic DST result was obtained for 1,301 of 1,540 samples. Performance-based calculations were done on 1,244 samples for which both a phenotypic and genotypic DST result was available.

Results: The reproducibility of the genotypic and phenotypic DST methods was 97% and 95%, respectively. The sensitivity and specificity of the genotypic DST method was 68% and 99% for Isoniazid and 87% and 99% for Rifampicin, respectively. Smear gradation was found to influence the performance of the genotypic DST method. The genotypic DST method gave accurate DST results for 75% of the samples within 20 days (range, 15-25), whereas the phenotypic DST results were only available for 75% of the samples after 38 days (range, 26-115) (p<0.001).

Conclusion: This study showed that the genotypic DST could improve tuberculosis control by rapid diagnosis of drug resistant tuberculosis. This finding may have important implications for the control of drug resistant tuberculosis as it may reduce the chance for further transmission events.

Key Words: drug resistant phenotype, drug resistant genotype, probe method, time to positivity, smear gradation, rapid diagnosis.

Introduction

In resource poor countries, the World Health Organization (WHO) guidelines recommend the diagnosis of tuberculosis (TB) by smear microscopy in all new TB cases and smear microscopy, culture and drug susceptibility testing (DST) in re-treatment cases [1]. If a new case fails to convert after 2 to 3 months of first line therapy, culture and DST is requested. Routine phenotypic DST methods are culture based and are initially done to detect isoniazid (INH) and rifampicin (RIF) resistance. If resistance to INH and RIF is found, DST for ethambutol (EMB) is requested. DST usually takes between 3 to 6 weeks, resulting in long diagnostic delays. These delays are further exacerbated in new cases with primary drug resistance, given that DST is only initiated after 2 to 3 months of first line therapy. Such long delays and the administration of inappropriate therapy during the delay period may lead to the further acquisition of drug resistance, as well as the dissemination of drug resistant strains through transmission. Thus, to improve the outcome and prevent transmission of drug resistant TB, robust and effective alternative diagnostic tests are required that will enable the identification of drug resistant TB (DR-TB) within a few days after sputum collection.

Resistance to first line anti-TB drugs develops through the sequential accumulation of mutations in genes targeted by the different drugs. To date, 11 genes have been linked to resistance to the first

line anti-TB drugs [2,3]: *katG, inhA ,inhA* promoter, *ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance *embB* for EMB resistance, *pncA* for pyrazinimide (PZA) resistance and *rpsL* and *rrs* for streptomycin (STR) resistance [4,5]. Mutations in specific codons can therefore be used to rapidly detect drug resistance, since drug susceptible samples lack the corresponding gene mutation. In the last few years this concept has led to considerable progress in the development of screening tools for the detection of DR-TB. The first paper on mutation detection for drug resistant TB was published in 1993 [6] and since then, numerous markers and molecular methods have been described to detect drug resistant gene mutations. These methods include polymerase chain reaction (PCR) or other nucleic acid amplification methods followed by DNA sequencing, probe methods, PCR-restriction fragment length polymorphism (PCR-RFLP), single–strand conformation polymorphism (SSCP), heteroduplex analysis (HA), molecular beacons and ARMS-PCR [2,6-11]. Application of these molecular tools proved to be rapid and effective in low burden settings.

The aim of this study was to compare the performance (sensitivity, specificity and reproducibility) and time to diagnosis of a molecular genotypic DST method to a phenotypic DST method in order to detect INH and RIF resistance in a high incidence TB community as fast and accurate detection of INH and especially RIF resistance can aid in the diagnosis and treatment of MDR-TB patients.

Materials and Methods
Study Setting
TB cases from the Boland, Overberg, South Cape and Karoo region were treated according to the National Tuberculosis programme (NTP) of South Africa. Sputum samples were collected from suspect TB cases attending 9 primary health care clinics and a TB referral hospital during the period June 2000 and November 2003 and sent to the National Health Laboratory services (NHLS) for TB diagnosis by smear microscopy. Sputum from re-treatment and new cases who failed to convert after 2 months of intensive phase anti-TB treatment was subjected to culture and DST.

Phenotypic DST
Sputum samples were decontaminated with a mixture of sodium hydroxide, sodium citrate and N-acetyl-L-cysteine after which sodium phosphate buffer was added [12]. At this point each sample was divided into two equal parts. Part 1 was sent to the Division of Molecular Biology and Human Genetics, University of Stellenbosch, for genotypic DST. Part 2 was used at NHLS for routine microscopy, culture and phenotypic DST analysis. Cultures were grown in BACTEC 12B medium (Becton Dickinson, Maryland, USA) with PANTA, and growth of Mycobacteria was confirmed by Ziehl-Neelsen (ZN) staining. All ZN positive samples were then subjected to a niacin test to confirm the presence of *Mycobacterium tuberculosis* complex. If the culture was ZN negative after the 35 days of incubation, a final result of negative was reported. DST testing was only done on niacin positive cultures using the proportion method on Middlebrook slopes containing critical concentrations of 0.2 µg for isoniazid (INH) or 1 µg for rifampicin (RIF). Resistance was defined as 1% or more bacterial growth in comparison with a control in which the tested drug was absent.

Genotypic DST
Decontaminated sputum samples (part 1) were collected (usually 20-25 samples) weekly from the NHLS for genotypic DST. The remaining sample (approximately 500µl) was inoculated into 1 ml of BACTEC 12B media, which contained 0.1 ml PANTA–plus (Becton Dickinson, Maryland, USA), and incubated in a 50 ml Falcon tube (Greiner Bio-One, Germany) at 37℃ for 5 days. A negative control containing water was inoculated after every 5th sputum sample in a batch to control for possible cross-contamination. After 5 days of culture, the bacteria were pelleted by centrifugation at 10,000 x g for 20 minutes and the supernatant was aspirated into a final volume of 150 µl. A crude DNA template was prepared by boiling each sample at 100℃ for 20 minutes. These crude DNA templates were used to PCR amplify chromosomal domains containing mutations associated with INH (*katG315, inhA-15* promoter) and RIF (*rpoB531, rpoB526, rpoB516*) resistance as described previously [7,13]. PCR amplification of each batch was performed in 4 separate rooms to minimize amplicon

contamination. A water control, DNA from genotypically characterized drug resistant and susceptible controls, and the negative control prepared during short-term culture were incorporated with each PCR amplification reaction [13]. Amplicons generated were visualized after electrophoretic fractionation in 1.5 % agarose gel in 1 x TBE buffer and staining with ethidium bromide. Ten microliters of the PCR amplified product was then denatured by the addition of 190 μL of denaturing buffer containing 0.4 N NaOH and 25 mM EDTA and then spotted onto a Hybond-N+ membrane using a dot-blot apparatus (Bio-Rad). Hybridization was done using ^{32}P labelled oligonucleotide probes which were directed towards mutations in drug resistant genes most frequently found in the local isolates and in other parts of the world as described previously [7,13]. Results were scored based on discrimination between genotypically well-characterized controls on the blot. DNA sequencing was done on selected PCR amplified products using the ABI PRISM DNA model 3130xl sequencer (Applied Biosystems, Foster City,CA 94404, USA).

Data Analysis

The statistical program Statistica 7.1 (stasoft, Inc (2005) (www.statsoft.com)) was used to calculate the performance (sensitivity and specificity) of the molecular method at a confidence interval of 95%. Reproducibility of the phenotyping and the probe method was calculated by comparing follow-up DST results from the same patient, while the accuracy of the genotypic DST method was compared to the results obtained by DNA sequencing of the *rpoB* gene. The time for reporting DST results was calculated by subtracting the date the phenotypic or genotypic DST result was available from the date the sputum samples were received. The time for reporting DST for samples that had lost viability during phenotypic DST was calculated from the first time that specific isolates were received for DST and reported lost viability until a second or third sample was requested and the DST results were available. The time for reporting genotypic DST results was calculated from the day a specific batch of samples was received at the University of Stellenbosch until the genotypic results were available.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of the Faculty of Health Science, Stellenbosch University, for ongoing molecular studies on drug resistance in local communities (project numbers 2000/C061 and 2002/C118).

Results

Between June 2000 and November 2003, 3,038 sputum samples were collected and subjected to culture from patients residing in the study setting (Figure 1). DST was requested and performed on 1,540 of these cultures, of which 1,373 (89 %) cultures gave a DST result. The remaining 167 cultures were either contaminated (n=123), lost viability (n=43) or were found to be Non-Tuberculosis Mycobacteria (NTM) (n=1). Phenotypic INH and RIF resistance was identified in 279 (20 %) and 177 (13 %) of the cultures, respectively. Resistance to both INH and RIF (MDR-TB) was identified in 165 (12 %) of the cultures. The reproducibility of the routine phenotypic DST method was 95% (kappa value 0.8) when the DST results of sequential follow-up samples were compared.

Figure 1. Flow diagram showing phenotypic and genotypic DST of sputum samples.
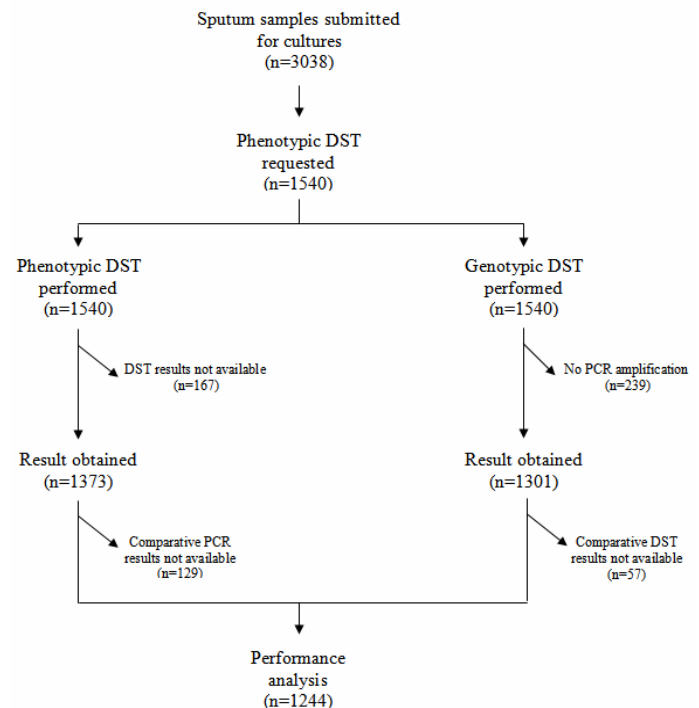
Genotyping was done on all the samples submitted for culture and DST testing in order to determine the efficiency of mutation detection by the genotyping method. A definitive genotypic DST result was obtained for 1,301 of the 1,540 samples. The remaining 239 failed to produce a product after PCR amplification. Genotypic DST of the 1,301 samples showed that 188 (14%) of the samples were INH resistant, 155 (12%) were RIF resistant and 117 (9%) of the samples were MDR-TB. The reproducibility of the genotyping DST method was 97% (kappa value 0.9) when sequential follow-up samples were compared. Genotypic DST had an accuracy of 97% (kappa value 0.9) when compared to DNA sequencing of the rpoB gene.

Phenotypic and genotypic DST results were available for 1,244 of the samples and the performance was calculated on this set of samples (Table 1). Results of performance calculations comparing the genotyping DST method to the gold standard phenotypic DST method are tabulated in Table 2.

Table 1. Comparative analysis of phenotypic and genotypic DST.

	n=1244	
	Phenotype	**Genotype**
INH^S	1000 (80 %)	1065 (86 %)
INH^R	244 (20 %)	28 *inhA* prom (2 %)
		151 *katG* (12 %)
RIF^S	1095 (88 %)	1102 (89 %)
RIF^R	149 (12 %)	142 (11 %)
MDR	141 (11 %)	108 (9 %)

Table 1 only reflects the number of cases where results were available for both phenotypic and genotypic DST. S-susceptible; R-resistant.

To determine whether smear gradation strongly influenced the predictive value of the genotyping method, PCR amplification ability was compared to the sample bacterial load. A positive correlation between the bacterial load and amplification was observed, as 6% (37/311) of the high (smear 2+ and 3+) and 12% (51/881) of the low (smear 1+) bacterial load samples were not amplifiable. Smear results were not available for 181 sputum samples.

To determine whether the genotypic DST method could shorten the interval for diagnosing drug resistance, the time to a positive DST result, between the phenotypic and genotypic methods, was compared. Genotype DST results were available for 75% of the samples within 20 days (range, 15-25), whereas 75% of the phenotype result were only available after 38 days (range, 26-115) (Figure 2). This difference was statistically significant according to the Wilcoxon matched pair test (p<0.001).

Table 2. Performance calculation of genotypic DST method to phenotypic DST methods.

	INH (%)	RIF (%)	MDR (%)
Sensitivity	68	87	72
Specificity	99	99	99
Positive predictive value	92	92	94
Negative predictive value	93	98	97

INH-isoniazid; RIF-rifampicin; MDR-multi drug resistant.

Thirty-four (20%) of the samples that failed to give a phenotypic DST result were PCR amplifiable. These samples either lost viability or were contaminated and subsequent samples were requested for DST. In these cases the mean delay was 133 days (range 50-1403 days) before a phenotypic DST result was available. In contrast, the genotypic DST results were available within 27 days (range, 18 to 30).

Figure 2. Time to a positive DST result using either phenotypic or genotypic DST. The mean diagnostic delay for the phenotypic method was 38 days (range 26 to 115), while the mean diagnostic delay for the genotypic method was 20 days (range 15 to 25).
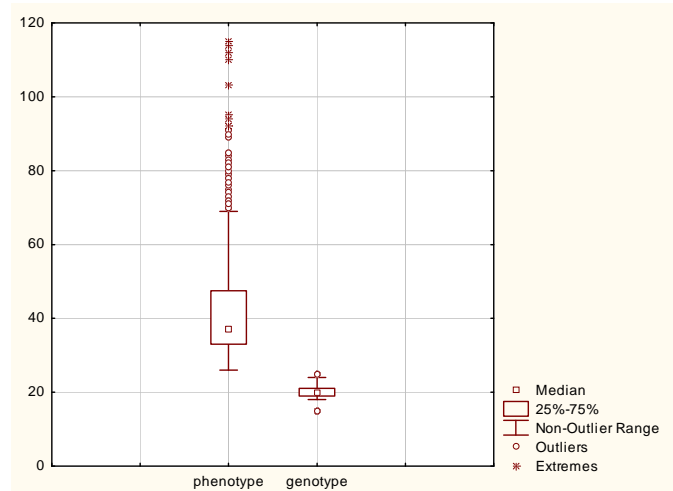
Discussion

It is well known that DST for some drugs is difficult due to technical reasons and these results are therefore not always accurate [14,15]. In addition, it may take up to 6 weeks to get a phenotypic DST result and during this time many transmission events may take place. Alternative methods need to be evaluated to improve the speed of diagnosis of DR-TB and especially MDR-TB. Molecular techniques have been applied widely to detect mutations associated with specific drugs to overcome these problems.

In this study, we concentrated on evaluating the performance (sensitivity and specificity) of a genotypic method to detect drug resistance in a high TB incidence area. In this study we have concentrated on identifying the most frequent mutations associated with resistance to INH and RIF [6,11]. We found that our genotypic DST method performed favourably to the culture method for INH and RIF. However, it was noted that the sensitivity for INH was low since many of the resistance carrying gene mutations could not be identified. This observation is not unique since the molecular mechanisms for INH resistance are not fully understood and 25 to 30% of phenotypic INH resistance associated mutations are still unaccounted for [16]. An important finding was that 75% of the genotypic results from all samples tested were available in a significantly shorter time interval in comparison to conventional culture based phenotyping methods. Such a time saving may have important implications for the control of DR-TB as it may reduce the chance for further transmission events of DR-TB. Furthermore, the genotypic DST method was able to detect drug resistance in samples which lost viability, circumventing the need to request follow-up sputum and thus reducing the overall cost and resources as it will not be necessary to track patients in rural areas.

Amplification of drug resistant genes directly from sputum samples without the need for culture remains a major problem. For many genotypic methods PCR analysis directly from sputum samples with very low bacterial load is extremely difficult. This may be the most important reason that molecular testing has not yet been introduced for routine testing on a large scale. In this study we found that the short-term mini culture method can overcome this problem and give consistent results.

In addition, we found that the implementation of DNA extracted protocols using the NucliSENS magnetic extraction kit (Biomérieux, Netherlands) as well as the use of Hot start Taq polymerase (Qiagen) significantly improve PCR amplification ability of marginally positive sputum samples. We are aware of the fact that there are currently two commercial methods available to detect drug resistance TB, the Commercial strip assay INNO-LiPA Rif (Innogenetics,Ghent,Belgium) and the Genotype MTBR assay (Hain Life Science GmBH, Hehren,Germany) that is currently under evaluation in South Africa [17-20]. However, although both these tests perform very well (data not shown), we have found that bacterial load still remains an important factor in PCR amplification ability and subsequent analysis of marginally positive sputum samples.

In the local community, drug resistance is largely due to transmission of a previously drug resistant strain [21]. If any genotypic method could rapidly (despite low sensitivity) detect the majority of drug resistant strains in a high incidence community, the use of such a technique will be an advantage for the control of DR-TB. Therefore, is it urgent that molecular methods are developed and evaluated with the aim of rapid detection of MDR- and XDR-TB.

The following recommendations can be made to efficiently identify drug resistant genotypes in a high incidence TB community: i) molecular methods can aid in the rapid detection of RIF resistance which can be used as a marker for MDR-TB; ii) implementation of any new test must be done in close collaboration with clinicians and the control program; iii) a positive genotype result can be regarded as true resistance; iv) discrepant results between genotype and phenotype testing should be subjected to DNA sequence analysis. We conclude that this study showed that a molecular method to rapidly detect drug resistance can add considerable value to the control of DR-TB in a high incidence area.

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Conflict of interest: No conflict of interest is declared.