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Discordant line probe genotypic testing vs culture-based drug susceptibility phenotypic testing in TB endemic KwaZulu-Natal: Impact on bedside clinical decision making

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ABSTRACT

The recommendations for *Mycobacterium tuberculosis* drug susceptibility testing include both phenotypic and genotypic methods. This concurrent use of differing testing platforms has created an emerging challenge of discordant results, creating a diagnostic dilemma for the laboratorians as well as attending clinicians. We undertook a retrospective study to determine the prevalence of discordant results between the MTBDR*plus* line probe assay and solid culture-based drug susceptibility testing for rifampicin and isoniazid. The analysis was conducted for the period January 2013 and December 2015 at the Inkosi Albert Luthuli Central Hospital. Rifampicin and isoniazid resistance testing data were "paired" on 8273 isolates for culture-based drug susceptibility testing and line probe assay. The latter method showed high sensitivity and specificity of 93% and 95% respectively for isoniazid testing. For rifampicin testing, sensitivity and specificity were 95% and 75%. Overall, discordance was 14.6% for rifampicin and 7.2% for isoniazid. This report is not intended to determine superiority of one method over another. It is merely to show that discordance does exist between different methods of testing. Given the burden of HIV and Tuberculosis in Sub-Saharan Africa, these findings have clinical significance and huge public health implications. Clinicians should understand the limitations of phenotypic testing methods.

1. Introduction

Rapid, accurate and cost-effective methods to detect multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) infections are urgently required to ensure that affected patients are identified early, treated appropriately, become non-infectious rapidly and have favorable clinical outcomes [1]. Inadequate or delayed detection of MDR-TB contribute to suboptimal treatment and poor clinical outcomes, perpetuating onward transmission of MDR-TB strains within communities [2]. Furthermore, recently published findings show that person-to-person transmission of MDR-TB strains, rather than inadequate treatment of MDR-TB, was the underlying driver of extensively drug-resistant tuberculosis (XDR-TB) transmission in TB-endemic KwaZulu-Natal, South Africa [3].

World Health Organisation TB guidelines, adopted by most countries globally, have long advocated microbiologic confirmation of *Mycobacterium tuberculosis* (*Mtb*) infections for entry of patients into the TB programme [4-6].These guidelines have been updated and recommend genotypic testing platforms such as the MTBDR*plus* (Hain Lifesciences) and GeneXpert MTB/RIF assays (Cepheid), both shown to shorten the time to diagnosis, for the diagnosis of TB and MDR-TB in developing and high-burden countries[7,8].The current recommendations for TB drug susceptibility include both phenotypic and genotypic methods of detection [2]. Conventional phenotypic methods are culture based and dependent on *Mtb* growth. The gold-standard of diagnosis, solid culture-based drug susceptibility testing (DST), provides results in 4–6 weeks, whereas liquid culture-based DST takes approximately 2 weeks [9].As per WHO recommendations, many laboratories use

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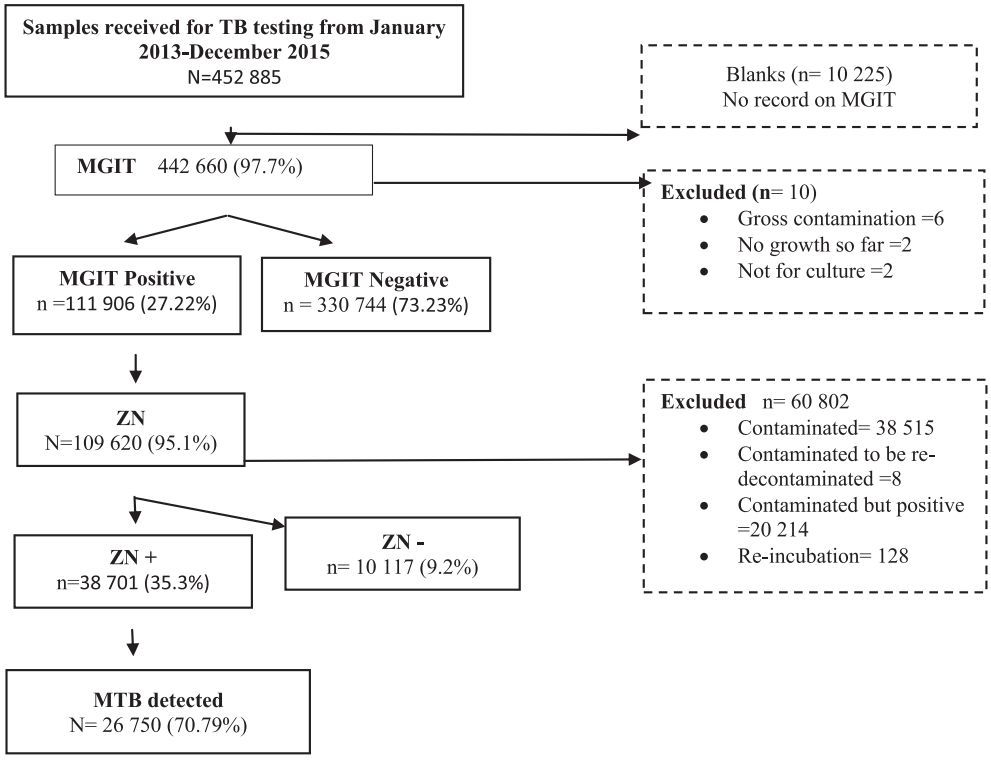

Fig. 1. Laboratory results of samples received for MTB testing from January 2013-December 2015.

additional phenotypic methods to confirm the molecular drug susceptibility results and to also test susceptibility to second and third line anti-TB drugs [10-12]. This concurrent dual use of testing platforms has created an emergence of discordant results for rifampicin (RIF) and isoniazid (INH) testing on the same patient sample produced from different platforms, resulting in a diagnostic dilemma for attending clinicians [13].

Discordance between laboratory tests invariably occur. Root causes of discordant results range from human or laboratory errors pre- and post-analysis and limitations of assays, to co-infection with different *Mtb* strains and heteroresistance. In TB-endemic settings, the extent of mixed strain infections is not known, and the process of isolating single colonies is tedious and time consuming [14,15]. The laboratory plays a major role in TB patient care and most medical decisions are based on laboratory test results. The sensitivity and specificity of a test frequently differ with the prevalence of a disease and many factors contribute to this [16]. The sensitivity of TB detection tests is decreased in smear negative specimens and certain specimen types.

Discordant INH resistance may be of little significance, as clinical management currently remains unchanged irrespective of the presence or absence of INH mono-resistance. Conversely, discordant RIF resistance results do have clinical consequences as the interpretation of such results and anti-TB regimen choice is left in the hands of the attending clinician. This has implications for both adverse patient outcomes and for programmatic reporting, with either under or over-reporting of actual numbers of either drug susceptible or MDR-TB cases. Moreover, given the length and toxicity associated with MDR-TB treatment, attending clinicians face difficult decisions on whether to initiate MDR-TB treatment based on the molecular result or stop MDR-TB treatment based on a DST result that becomes available few weeks later. Patients diagnosed with MDR-TB are admitted into an MDR-TB facility based on a molecular result and experience close contact and prolonged exposure to other MDR-TB patients, thereby making the decision to withdraw MDR-TB treatment in such instances problematic. In addition, discordant results may lead to multiple repeat testing to exclude laboratory or human error, incurring more costs to the health service and further delays in instituting appropriate treatment. In developing countries such as South Africa, this is not cost effective and also time consuming for the laboratory.

Empiric evidence describing the extent to which discordance in results between MTBDR*plus* (LPA) and solid culture-based DST for RIF and INH exists is lacking. Understanding the extent to which discordance exists in TB endemic settings will help guide development of appropriate laboratory diagnostic algorithms, clinical decision making, and an approach to recording and reporting of infections. We therefore undertook a retrospective study to determine the prevalence of discordant results between the MTBDRplus (LPA) and solid culture-based DST for RIF and INH. We report briefly on our findings.

2. Methods

A retrospective analysis of all discordant results for RIF and INH between the MTBDR*plus* (LPA), and solid culture-based DST using the 1% agar proportion method from Mycobacterium Growth Indicator Tube (MGIT) positive cultures was conducted between January 2013 and December 2015 at the Inkosi Albert Luthuli Central Hospital, the only TB Reference laboratory (TB culture) for the province of KwaZulu-Natal.

All samples underwent liquid culture testing using the Becton Dickinson BACTEC MGIT™ 960 instrument. All MGIT tubes were retained in the instrument to allow for a 42-day incubation period before they were removed. MGIT tubes were also removed if the instrument alerted a positive MGIT. For any MGIT that was deemed positive by the instrument, Ziehl-Neelsen (ZN) staining was performed. This allowed the laboratory technologist to determine the presence of acid-fast bacilli (AFB) via microscopy and cording characteristics. For all MGITS with AFBs seen, further MPT antigen testing was conducted. As part of the routine laboratory workflow, all *Mtb* positive isolates were subjected to line probe assay (LPA) testing (Hain Lifescience GmbH, Nehren, Germany) for anti-TB drug susceptibility, as per standard operating procedures. Criteria to conduct additional solid culture-based DST testing using the 1% agar proportion from MGIT positive cultures were

based on internal policies and was only conducted on LPA samples that demonstrated resistant results, LPA samples that demonstrated inconclusive results or requests for solid culture-based DST from attending clinicians.

We identified all samples that were sent for both MTBDR*plus* (LPA) testing and solid culture-based DST testing using the 1% agar proportion from positive MGITs. We then selected all samples that had results for both INH and RIF from both methods. These results were paired into DST: LPA (INH) and DST: LPA (RIF) respectively [Fig. 1]. From each of the DST: LPA; INH and RIF pairs, we identified concordant and discordant pairs, with the latter used for determining the level of discordance [Fig. 1]. All discordant isolates retrieved had repeat LPAs performed from the initial and sub-cultured samples. All LPA results were quality controlled.

3. Results

In a high throughput laboratory processing almost half a million samples over a 2-year period, about 111 906 ((25.3%); 95% CI: 25.2–25.3%) of the 442 650 MGITs were positive for *Mtb*. Of the 111 906 positive MGITs, 60 802 (5%) samples were contaminated and underwent decontamination while others were re-incubated. The remaining 109 620 (95%) were processed by the ZN staining method, of which, AFBs were observed in 38 701 ((35, 3%); 95% CI: 35.0–35.6%). Of the 38 701 samples, *Mtb* complex was detected in 26 750 ((69%); 95% CI: 97.1%-97.5%) [Fig. 1], all of which were processed by the LPA method [Fig. 2]. Of these, 7030 (26.3%); 95%CI:25.8–26.8%) were INH resistant whilst 19 608 ((73.3%);95%CI:72.8–73.8%) were susceptible, with the remaining 112 ((0.4%); 95%CI:0.4–0.5%) having no INH result. For RIF testing, about 7 124 ((26.6%); 95% CI: 26.1–27.2%) of the samples demonstrated resistance while 18 892 ((70.6%); 95% CI: 70.8–71.2%) were susceptible with the remaining 734 ((2.7%;95% CI: 2.6–2.9%) having inconclusive results. This yielded 575 (21.5%) MDR-TB, 1 041 (5.0%) RIF mono-resistant, 1132(4.2%) of INH mono-resistant strains and 17 728 (66.3%) susceptible strains. The remaining 3% were strains that were inconclusive for either INH or RIF.

Only 31% (8 275) of the 26 750 isolates were processed by the solid culture-based DST method of which 80.4% (6 651) were RIF resistant and 19.6% (1 622) were susceptible to RIF. For INH testing, 6 818 (82.4%) were resistant whilst 1 455 (17.6%) were INH susceptible. This yielded about 5 870 (21.9%) MDR, 780 (2.9%) RIF mono-resistant, 945 (3.5%) INH mono-resistant and 675 (2.5%) susceptible TB strains.

The number of solid culture-based DSTs performed limited the "pairing" of DST and LPA to only 8 273 pairs. From the 8 273 pairs, solid culture-based DST testing demonstrated that RIF was resistant in 6 651 ((80%); 95% CI: 79.5–81.2%) and susceptible in 1 622 ((20%); 95% CI: 18.7–20(0.5%) of these isolates. For INH, there were 6 818 ((82.3%); 95%CI: 81.5–83.2%) resistant and 1 455 ((17.5%; 95% CI: 16.8–18.4%) susceptible TB strains resulting in 71% (5 870) of MDR-TB, 8% (675) of DS-TB and 9% (780) of RIF mono-resistant and 945 (11%) INH susceptible strains. The remaining 1% had a missing result for either INH or RIF.

From the 8 273 pairs, LPA DST testing demonstrated that INH had 1 367 (16.5%) SS (Susceptible Susceptible)and 6 311(76.3%) RR (Resistant Resistant) concordant pairs, and 69 (1.0%) SR (Susceptible Resistant), 446 (5.3%) RS (Resistant Susceptible), and 80 (1.0%) RZ and SZ discordant pairs. For RIF testing, there were 923 (11.2%) SS and 6 135 (74.1%) RR concordant pairs, and 309 (3.7%) SR, 353 (4.3%) RS, and 553 (6.7%) RZ and SZ discordant pairs (Tables 1 and 2).

Using the RS and SR pairs, overall discordance was 7.2% (595/8 273) and 14.6% (1 215/ 8 273 for INH and RIF respectively. The LPA method showed high sensitivity and specificity, 93% and 95%

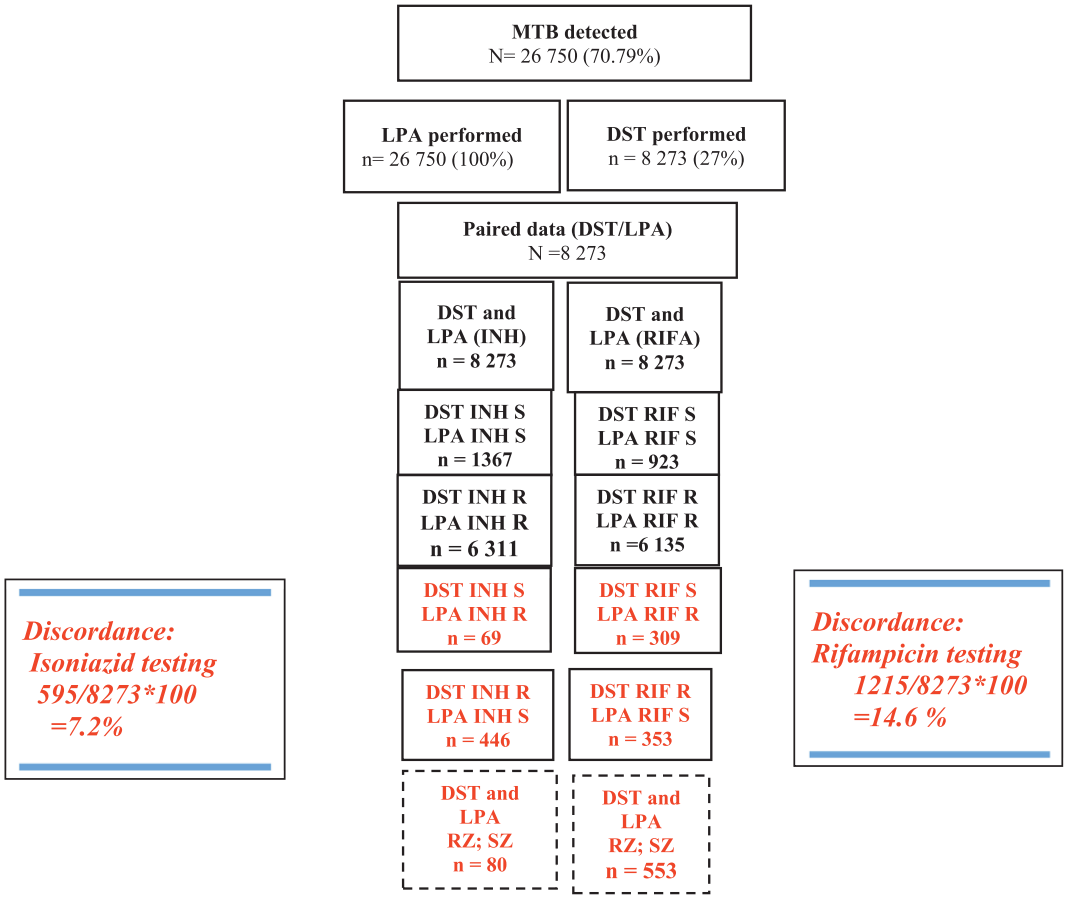

Fig. 2. Discordance between the LPA and the DST methods.

Table 1
Sensitivity and specificity of LPA in RIF testing.

		Solid culture-based DST			
		R	S	Z	Total
LPA	R	**6135**	**309**	**0**	6444
	S	**353**	**923**	**0**	1276
	Z	163	390	0	553
	Total	6651	1622	0	8273
	Total	**6488**	**1232**	**0**	**7720**
	Specificity	74,91883			
	Sensitivity	94,55919			
	PV +	95,20484			
	PV −	72,33542			

Table 2
Sensitivity and specificity of LPA in INH testing

		Solid culture-based DST			
		R	S	Z	Total
LPA	R	**6311**	**69**	**0**	6380
	S	**446**	**1367**	**0**	1813
	Z	61	19	0	80
	Total	6818	1455	0	8273
	Total	**6757**	**1436**	**0**	**8193**
	Specificity	95,19499			
	Sensitivity	93,39944			
	PV +	98,9185			
	PV-	75,39989			

R = Resistant, S = Susceptible, Z = Inconclusive.

respectively for INH testing, Predictive Value Positive (PVP) and predictive Value Negative (PVN) were 98% and 72%, respectively. For RIF testing, sensitivity and specificity were 95% and 75% with PVP and PVN of 95% and 72% respectively (Tables 1 and 2).

4. Discussion

Discordance in RIF susceptibility results was observed in 14.6% of isolates. Taking culture-based DST as the "gold standard", if all clinicians initiated treatment based on the initial molecular result (LPA), then 25% of patients would have been incorrectly treated for MDR-TB and 5% of patients with MDR-TB would have been inadequately treated with susceptible anti-TB drugs. In addition, the 25% patients would have possibly been transferred to an MDR-TB facility and exposed to resistant *Mtb* strains before the culture-based DST result became available. Discordance in INH susceptibility results was observed in 7.2% of isolates. For INH testing, most isolates yielded completely concordant results, with 93% and 95% for resistance and susceptibility, respectively. In the case of a true INH susceptible result, treatment would be appropriate. However, in the case of a false INH sensitive result, concerns of inadequate treatment and transmission also arise. Given the burden of HIV and TB in Sub-Saharan Africa, these findings have clinical significance and huge public health implications. In disease endemic settings, this can lead to a host of public health challenges such as misinterpretation of disease burden, undermining of TB outcomes and incorrect planning of TB control programmes.

Molecular assays detect resistance at a genetic level and thus do not detect the eventual phenotypic expression [2,17]. In this study, the LPA utilized, targets selected resistance conferring mutations only: the *rpoB* gene that confers resistance to RIF and the *katG* and *inhA* gene that confer resistance to high level and low level INH resistance respectively [18]. Published reports have highlighted the inability of molecular assays to detect mutations outside targeted "hotspots" and resistance determining regions. In a Swaziland study, 30% of MDR-TB strains were due to the *Ile491Phe rpoB* gene, a disputed mutation outside the 81 base pair hotspot region. This mutation is not routinely detected by commercial molecular tests such the GeneXpert MTB/RIF (Cepheid) assays and the LPA [19]. An additional study demonstrated 100% sensitivity and specificity for detecting *Mtb* using the GeneXpert® MTB/RIF, with culture as the gold standard. However, the assay incorrectly assigned RIF resistance in 4/13 (31%) of cases [20]. Furthermore, in a Myanmar study, repeating the GeneXpert® MTB/RIF assay was required in order to compare the result with other available tests, particularly in results reported as "MTB detected very low, RIF resistance detected' [21]. In countries where the GeneXpert® MTB/RIF assay is used as a first-line diagnostic test for MTB and RIF resistance, this finding is of importance. In a retrospective review, the presence of discordant RIF results was common with the GeneXpert® MTB/RIF assay and was associated with probe delays and the use of probe B [22].

Another common reason for discordant results is the presence of mixed infections and heteroresistance. Mixed infections with multiple strains of *Mtb* create a challenge for DR-TB testing and treatment as attending clinicians receive results that are dependent on the level of detection of the assay utilized [23]. Furthermore, clinicians may receive conflicting results if populations of both sensitive and resistance *Mtb* strains are present in the patient's sample. Heteroresistance can arise as a result of within-host diversification following a single infection with both DR-TB and DS-TB infection which can be spontaneous or driven by antibiotic selection pressure [24]. Heteroresistance is picked up by the LPA, molecular phenotyping (MIRU-VNTR) or by genome sequencing [25,26]. However, even assaying single samples does not allow definition of the full extent of heteroresistance. This is significant in TB-endemic populations in South Africa, where TB- HIV co-infected patients have a 2-fold higher prevalence of MDR-TB and are at a higher risk of re-infection [27-29].

In our study, the LPA was compared to culture-based drug susceptibility phenotypic testing. The long-standing debate regarding the "Gold-standard" for TB DR-TB testing continues [30,31].The agar proportion method has been regarded as the "gold standard" method for several decades [32]. Although some laboratories utilize liquid-based testing for second-line drugs, the FDA has cleared this method for first-line drugs only [33-35]. This is of importance as resistance may be missed by standard, growth-based systems, especially with the use of automated liquid systems [36-38]. Van deun et al demonstrated that certain *rpoB* mutations such as *Asp516Tyr, His526Leu His526Ser, Ile572Phe, Leu511Pro* and *Leu533Pro* were associated with discordant DST results. These mutations led to "low–level" or "borderline" resistance. In this study the authors concluded that clinically relevant resistance may not be covered by the high critical concentrations, suggesting modifications of existing testing methods in order to detect resistance. Examples of modifications were prolongation of the incubation period and/or using a larger inoculum size when preparing samples for testing [39].

There is very limited data available on the clinical correlation associated with discordant genetic mutations [40,41]. Williamson et al assessed the impact of *rpoB* mutations on clinical correlation and outcomes. Treatment failure was significantly associated with the presence of *rpoB* mutations that were not detected in DST. In this study, 94 patients with INH resistant, RIF sensitive detected by MGIT DST had 4 *rpoB* mutations, *Leu511Pro/Met515Ile, His526Asn/Ala532Val, Asp516Tyr and His526Leu* [42].Three of the four patients with mutations were treatment failures and the other was unknown [42]. In a study that evaluated INH, Kim et al showed that 4.8% of discordant INH results between liquid and solid media existed, with majority of RIF sensitive *Mtb* demonstrating INH resistance in liquid medium and INH sensitivity on solid medium [43]. Results suggested that in patients with RIF sensitive *Mtb*, modifications of treatment regimens based on DST results from liquid medium could improve treatment outcomes. It was concluded that in this particular population, unfavorable outcomes may be reduced by treating for INH resistance [43].

From a laboratory perspective, the resolution of a discordant result

is challenging. Sequencing-based techniques may assist with the identification of exact single nucleotide polymorphisms that will be required to resolve a discrepancy [44-47]. In addition, a precision-based medicine approach, whereby selection of a treatment regimen is guided by characterization of different mutations shows promise. However, these approaches pose challenges in high burden, developing countries. [48] In a study that compared commercial and non-commercial phenotypic and genotypic rapid drug susceptibility tests, RIF resistant 'disputed' mutations were frequently missed by MGIT. These mutations were *L430P, D435Y, L452P, and I491F*. Furthermore, phenotypic and genotypic DSTs varied greatly when detecting occult RIF resistance and none of the methods used detected all disputed mutations without misclassifying wild-type strains [49]. Accuracy problems in the laboratory will always exist and laboratories must have protocols in place to detect errors. The training on the importance of proper capturing of patient identifying information and critical elements should be ongoing.

Laboratory results impact on patient care. In the province of KwaZulu-Natal, clinicians that review initial *Mtb* laboratory results and manage patients are usually newly qualified. This is the case for most developing countries with shortages of medical staff. Therefore, results should be easy to understand with a standardized reporting language easily interpreted by a new intern or medical doctor handling the cases. Results from genotypic and phenotypic tests for DR-TB need to be used in conjunction with one another and thus the attending doctor should have a basic understanding of the limitations of assay/platform. Educational partnerships that involve the laboratory and clinicians should be developed at all institutions. Although it is important to know the limitations of all platforms and the causes of discordance, it is equally important to look at these results from a "bedside" perspective. Tests should not be interpreted in isolation and clinical correlation with initial laboratory results remains pivotal in the appropriate management of patients. The value of good history taking, clinical judgement and expertise as well as adjunct tests such as radiology cannot be over emphasized. A study in Uganda, demonstrated that clinical judgment identified a small number of additional culture-positive cases with poor specificity, highlighting the importance of clinical judgment [49]. When rapid molecular tests are negative but clinical judgment and suspicion for MDR-TB is high, then MDR-TB treatment should continue until the phenotypic susceptibility results become available. The question of repeat testing using a different platform has also been suggested for initial laboratory results on specimens that are RIF sensitive and INH resistant [13,50]. Conversely if an initial result is positive for RIF resistance and there is no clinical suspicion of DR-B, the result should be treated with reserve [51,52]. High rates of *Mtb* transmission in high endemicity populations increase the prevalence of mixed infections, and therefore in majority of cases, the standard rule remains to treat patients with mixed populations for both DS-TB and DR-TB. Furthermore polydrug-resistant strains should be analyzed for RIF resistance as studies have demonstrated that the failure and relapse rates are almost similar in isolates with a recognized or disputed rpoB mutation [53]. Patients with disputed *rpoB* mutations should be treated for MDR-TB ± high dose rifampicin as the clinical outcome is worse with standard treatment [54,55].

5. Conclusion

Discordance between genotypic and phenotypic tests are increasingly recognized and with the advent of new diagnostic platforms, laboratories will have to deal with greater discordance between results. This study highlights the need for good communication between the laboratory and the clinician in the management of patients with a discordant result. Despite all the advances in susceptibility testing in MTB, a "perfect gold standard" is still not available. This report is not intended to determine superiority of one method over another. It is merely to show that discordance does exist between different methods of *Mtb* susceptibility testing and clinicians should understand their limitations.

5.1. Limitations

Only 31% of all isolates were processed by the conventional DST method, which limited the degree of "pairing" between the two methods and possibly influenced the degree of discordance. Almost half (35%) of the isolates that were processed by the ZN method were excluded from the analysis as most were contaminated and others were marked for re-incubation, causing further delays in diagnosis and consequently treatment initiation. Repeat samples constituted 8% of total samples that were received for processing, however, all discordant isolates had repeat LPAs from both the initial sample and the sub-cultured samples and there was no difference in the initial and final result. There were some errors that were associated with incorrect capturing of patient demographics and these include misspelt patient names and surnames as well as missing gender and the age. These errors narrowed the criteria for removing duplicated results, resulting in multiple entries of the same patient as the system could not recognize such errors. We did not further investigate the isolates to determine the nature or cause of the discordance as the purpose of this study was to establish the impact of the initial laboratory result on the clinician and patient at the bedside. It would be informative to know if any of the LPA probes were more strongly associated with discordance. Knowing that certain probes are more prone to discordance with the agar proportion method than other probes may assist the physician in determining their level of confidence in the LPA results for INH and RIF. The study could also not ascertain if the samples were from incident or prevalent cases.

Submission declaration and verification

The work described has not been published previously.

Authorship

All authors have made substantial contributions as per journal requirements.

Role of the funding source

No funding was required for this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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