ORIGINAL ARTICLE

Comparison of Phenotypic Drug Susceptibility Testing and GeneXpert for the Detection of *Mycobacterium tuberculosis* against Rifampicin Susceptibility Assay in the South Punjab

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ABSTRACT
Background: This study aims to compare performance of conventional phenotypic Löwenstein–Jensen (LJ) -drug susceptibility testing (DST) and GeneXpert for the detection of *Mycobacterium tuberculosis* against Rifampicin (RIF) susceptibility assay from smear positive TB patients in the South Punjab region.
Methods: A total of4809 sputum samples were collected from 16 districts of South Punjab consecutively of newly enrolled and already diagnosed pulmonary TB patients. RIF susceptibility was performed by Phenotypic drug susceptibility testing in parallel with GeneXpert at Provincial TB Reference Laboratory (PRL) in collaboration with Pathology Department, Nishtar Medical College (NMC), Multan.
Results: The final analysis was performed among 4544 (96.68%) cases from enrolled 4809 cases which include 3805 (83.74%) new and 739 (16.26%) on treatment patients. Among them, 4222 (92.91%) cases were susceptible (RIF) while 322 (7.09%) were resistant to Rifampicin. The valid RIF susceptibility testing result was available from substantially more cases with GeneXpert 4400 (96.83%)than with phenotypic DST 4273 (94.04%). Discordant 40 results included 21(0.51%) MTB+RIF 'S' on phenotypic LJ-DST and 'R' on GeneXpert and 19(0.46%) MTB+RIF 'R' on phenotypic LJ-DST and 'S' on GeneXpert. This difference (0.97%) was less significant.
Conclusions: This study concluded that the GeneXpert assay had 98.9% specificity in detecting susceptibility to Rifampicin in TB patients. The use of this assay is a feasible option for treating and controlling the tuberculosis in this regional settings.

Keywords*: Mycobacterium tuberculosis*, Phenotypic LJ-DST, Rifampicin, GeneXpert.

INTRODUCTION
Among the top causes of death due to an infectious agent, *Mycobacterium tuberculosis* causing tuberculosis is second only to HIV/AIDS[1,2]. Annually, approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* and 8 to 10 million infected person progress to active tuberculosis[3]. It was estimated by WHO in 2014 that 9.6 million people became ill with active TB and 1.5million deaths occurred due to this disease[4]. *Mycobacterium tuberculosis* resistant to at least Isoniazid (INH) and Rifampin (RIF), the two most effective drugs used to treat the disease was labeled as Multidrug-resistant tuberculosis (MDR-TB)[5]. MDR- TB was transmitted primarily to healthy people indicated in many studies, despite a belief that drug resistant MTB have lowered virulence and transmissibility[6,7].

Early detection of MDR-TB patients eligible for drug susceptibility testing (DST), diagnosis and initiation of treatment are crucial to prevent disease transmission and will help to reduce high morbidity and mortality due to this disease[8]. A new test GeneXpert MTB/RIF assay detects the presence of MTB and its susceptibility to rifampicin[9] which is important first-line drug simultaneously. The MDR-TB detection by GeneXpert is recommended for screening the treated MDR-TB cases and the patients at risk like, contacts, failures and relapses as compare to new TB cases[10]. Approximately 490,000 MDR-TB cases occur worldwide every year mentioned in a recent WHO report, corresponding to approximately 4.8% of the world's TB cases[11,12]. Recent reports showing 7% of MDR isolates as extensively drug resistant TB (XDR-TB) has amplified the importance of addressing MDR-TB[13]. MDR-TB prevalence is increasing both in developed and developing

countries throughout the world[14]. The highest percentage of MDR-TB cases are found in India, China, The Russian Federation, South Africa and Bangladesh[15].

Improved diagnosis of tuberculosis is a global priority for tuberculosis control. A fast, cartridge-based GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) using PCR principle is an important new test in detection of *Mycobacterium tuberculosis* with immediate rifampicin resistance[16-18] consistently better sensitivity than sputum smear microscopy. GeneXpert MTB/RIF, showed excellent performance in a multicentre study[19] undertaken in reference laboratories.

Since 2012, patients newly diagnosed with TB have received 6-month short-course treatment[20], while those diagnosed with drug-resistant TB receive a longer second line regimen[21]. Inadequate treatment of MDR-TB can lead to worse patient outcomes, while increasing the risk of extensive drug resistance tuberculosis (XDR-TB)[22,23]. Diagnostic delays with MDR-TB are associated with worse clinical outcomes and increased transmissions[24].

PATIENTS AND METHODS

The study was conducted at Provincial Reference TB Laboratory (PRL) in collaboration with Pathology Department Nishtar Medical College (NMC), Multan from January 2015 to December 2015. PRL Lab was covering 16 Districts i.e.; (Bahawalpur, Bahawalnagar, Bhakhar, Chiniot, Dera Ghazi Khan, Jhang, Khanewal, Layyah, Lodhran, Multan, Muzafargarh, Pakpattan, Rajanpur, Rahim Yar Khan, Sahiwal and Vehari) in the South Punjab. We developed a latest model of TB case analysis/detection at PRL. The model was calibrated by National TB Control Program (NTP) to approximate the status of TB epidemics and resistant cases in Pakistan. In this setting each district had District Supervisor Laboratory (DSL). There were three Senior Laboratory Supervisors (SLS) from PRL who supervised all these districts on a monthly schedule visit. The patients having history of fever and cough more than one month attending registered Basic Medical Units BMUs in each district, early morning sputum samples were collected, Zeehl Neelson (ZN) staining done and examined by concerned DSL for AFB. Sputum smear microscopy was used as an initial screening test for TB diagnosis[25].

DSL collected the sputum samples from BMUs in the Public sector and examined these by ZN staining while Non-Governmental Organizations (NGOs) had their own structure of Laboratories in their own setup. These NGOs Medical Centers were registered in PRL as Public Private Mix (PPM), collected the cases and performed ZN staining. The samples which were AFB positive, labeled as Diagnostic Smear Positive Cases (DSPC) and transported by courier as early as possible. Samples obtained in the laboratory were in a dry ice cold box and were processed on the same day or kept at $+4^0$C in refrigerator, until their processing was done. All those cases, on treatment of 1-1.5months, follow-up sputum smears were taken and examined by ZN stain and AFB positive samples were sent to PRL for Culture and Sensitivity by phenotypic LJ-DST/GeneXpert as per recommendations by the clinician. This was labeled as Follow-up Smear Positive Cases (FSPC).

Data collection: Data of the patients from all the districts were collected quarterly. The reference range set by NTP in Pakistan as with other Asian countries and WHO for detection of TB by Diagnostic Smear Positive Rate (DSPR) as 10-20% and Follow-up Smear Positive Rate (FSPR) as 5-10%.

Phenotypic Drug Susceptibility Testing: All the sputum samples positive for AFB were processed by the conventional *N*-acetyl-L cysteine-sodium hydroxide method[26]. Decontamination of sputum samples were done with equal volumes of *N*-acetyl-L-cysteine and sodium hydroxide (2%) for 20 min and then were centrifuged at 3,000×*g* for 30 min. Finally, each sample was washed with 10 ml phosphate-buffered saline (PBS) (pH 6.8), and the sediment was re-suspended in 1.5 ml of PBS. This suspension was used to grow mycobacteria in Lowenstein-Jensen (LJ) solid medium[27]. Culture and Sensitivity by phenotypic LJ-DST were performed on all MTB isolates to RIF susceptibility at the PRL and Pathology Department, NMC Multan.

Procedure for GeneXpert testing: GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is a semi-automated and semi-quantitative nested real-time PCR (RT-PCR) assay which was marketed recently for the detection of *M. tuberculosis* and its resistance to rifampin (RIF) directly from clinical specimens[27,28]. Initial screening of sputum with GeneXpert MTB/RIF have profound effect on detection of cases and appropriate antituberculous treatment[29]. Cartridges according to the manufacturer's

recommendations were used in GeneXpert testing on sputum samples. In order to kill mycobacterium in sputum sample and to liquefy it, sample reagent (containing isopropanol and NaOH) for GeneXpert assay was added in a 2:1 ratio to the tubes. The mixture was shaken and allowed to sit for 10 min. It was shaken again and allowed to sit for another 5 min. In the GeneXpert assay cartridge, 2 ml was pipetted and then it was placed into the GeneXpert instrument for PCR testing. All other steps of measurement and analysis were conducted automatically inside instrument and reported by the GeneXpert software[30].

RESULTS

Table 1 shows that a total of 4809smear positive PTB cases were initially enrolled however, 109 cases were excluded due to history noncompliance or loss of samples or leakage of sampling containers. A total of 4700 eligible patients including 3927 (83.55%) new and 773 (16.45%) on treatment cases were put to the test for phenotypic LJ-DST or GeneXpert analysis. Out of 4700 cases, 124 were negative on phenotypic LJ-DST/GeneXpert and 32 cases showed no result with RIF on phenotypic LJ-DST/GeneXpert.

Table 2 shows that out of total 4544 (96.68%) analyzed cases, 3805 (83.74%) were new and 739 (16.26%) were on treatment. Among these, 4222 (92.91%) cases were susceptible to RIF including 3622 (95.19%) new and 600 (81.19%) on treatment. The total TB patients resistant to Rifampicin were 322 (7.09%) including 183 (4.81%) from new and 139 (18.81%) from on treatment cases.

Table 3 shows that male were 168 (52.17%) and 154 (47.83%) were female among total TB patients with RIF resistance.

Table 4 shows that out of total 4544analyzed cases in the PRL and Pathology Department Nishtar Medical College Multan, 4129 (90.87%) were performed on combined phenotypic LJ-DST and GeneXpert, while 144 (3.17%) samples on phenotypic LJ-DST and 271(5.96%) on GeneXpert were performed respectively.

Table 1: Enrolment of smear positive new and on treatment TB patients

Total cases enrolled with smear positive	Excluded cases	Eligible cases for phenotypic LJ-DST/GeneXpert		MTB Negative on phenotypic LJ-DST/GeneXpert		No results with RIF on phenotypic LJ-DST/GeneXpert	
4809	109	4700 (97.73%)		124		32	
		New cases	Treatment cases	New cases	Treatment cases	New cases	Treatment cases
		3927(83.55%)	773(16.45%)	92	32	30	02

Table 2: RIF analysis test results

Total cases for final analysis		RIF susceptible		RIF resistant	
4544 (96.68%)		**4222(92.91%)**		**322(7.09%)**	
New cases	Treatment cases	New cases	Treatment cases	New cases	Treatment cases
3805(83.74%)	739(16.26%)	3622(95.19%)	600(81.19%)	183(4.81%)	139(18.81%)

Table 3: Gender percentage of RIF resistance

Total patients with RIF resistant	Males	Females
322	168(52.17%)	154(47.83%)

Table 4: Distribution of cases performed on phenotypic LJ-DST and GeneXpert

Total cases Analyzed	Total cases on LJ- DST and GeneXpert	Total cases on LJ-DST (only)	Total cases on GeneXpert (only)
4544(96.68%)	4129(90.87%)	144(3.17%)	271(5.96%)

Table 5: Distribution of new and on treatment cases with GeneXpert, phenotypic LJ-DST and combined LJ-DST/ GeneXpert

Tests performed by	Total TB cases 4544	New TB cases 3805	Treated TB cases739
GeneXpert	271 (5.96%)	227(4.99%)	44(0.97%)
Phenotypic LJ-DST	144 (3.17%)	121(2.66%)	23(0.51%)
Combined LJ-DST/ GeneXpert	4129 (90.87%)	3457(76.08%)	672(14.79%)

Table 6: Percentages of sensitive and resistant cases by phenotypic LJ-DST and GeneXpert

Total results by phenotypic LJ-DST and GeneXpert	4129 (90.87%)
MTB+RIF 'S' on phenotypic LJ-DST and 'S' on GeneXpert	3792(91.96%)
MTB+RIF 'R' on phenotypic LJ-DST and 'R' on GeneXpert	292(7.07%)
MTB+RIF 'S' on phenotypic LJ-DST and 'R' on GeneXpert	21(0.51%)
MTB+RIF 'R' on phenotypic LJ-DST and 'S' on GeneXpert	19(0.46%)

Table 5 shows that out of total 4544 cases 227 (4.99%) new and 44 (0.97%) on treatment cases were performed by GeneXpert, 121 (2.66%) new and 23 (0.51%) on treatment cases by phenotypic LJ-DST while 3457 (76.08%) new and 672 (14.79%) on treatment cases by both GeneXpert and phenotypic LJ-DST. The valid RIF susceptibility testing results were available from substantially more cases with GeneXpert 4400 (96.83%) than with phenotypic LJ-DST 4273 (94.04%).

Table 6 shows that out of 4129 (90.87%) cases obtained by both phenotypic LJ-DST and GeneXpert, MTB with RIF sensitive were 3792 (91.96%) while RIF resistant were 292 (7.07%). Similarly MTB with RIF sensitive on phenotypic LJ-DST but resistant on GeneXpert were 21(0.51%) and MTB with RIF resistant on phenotypic LJ-DST but sensitive on GeneXpert were 19 (0.46%). Comparing the discordant 40 results of RIF-susceptibility in both, validity of false test results missed with GeneXpert and by phenotypic LJ-DST is 0.97%.

DISCUSSION

Pakistan is 4th among high burden countries for MDR-TB with estimated annual cases of 13000 among notified pulmonary TB cases. Drug resistant tuberculosis is either acquired due to poor management/inadequate drug treatment of active TB or transmission from infectious drug resistant TB patients. This may result from patient noncompliance, inappropriate drug levels, drug shortages or a number of other factors[31].

GeneXpert has been reported as having 100% sensitivity/specificity in detecting RIF resistance in a few studies[32,33].The GeneXpert assay was introduced into Pakistan in 2011 for the rapid detection of rifampicin (RIF) resistance in patients at risk of drug-resistant TB[34]. This study on MDR-TB/ RIF-susceptibility was carried out in South Punjab region of Pakistan including 16 districts using either alone conventional phenotypic DST or GeneXpert or combined testing. Study provides the data on the proportion of new and previously treated TB patients with RIF-resistant MTB cases. DST results were not available of 265(109+124+32) (5.51%) patients, either due to missing history, contamination, leakage of container, MTB negative culture or sample loss during transportation. This proportion of non-availability of result is very low as compared to that reported by other large nationwide drug resistance surveys[35,36]. This is due to provision of rapid courier services, preventing exposure to high temperature, in time processing in PRL lab.

This study shows that out of 4544 (96.68%) cases for RIF resistance, 168 (52.17%) were males and females were 154 (47.83%). In the global drug resistance survey report, the association between gender and MDR-TB was not clearly demonstrated[37]. Increasing drug resistance to commonly used drugs RIF and INH in isolates of *M tuberculosis* is a major cause of concern. Many of the research findings advocated that MDR-TB are frequently identified in patients with history of TB treatment[38,39] which is also evidenced in this study. In our study, RIF susceptible new and on treatment cases were

4222 (92.91%) and RIF-resistance cases (New and on treatment) were 322 (7.09%). The MTB resistance rate detected among the 322 cases was 183 (4.81%) in new and on treatment TB cases was 139 (18.81%), which is similar to those reported by National TB Control Program in 2014[40] in which the MDR-TB rate detected was 3.7% among new cases and 18.1% among previously treated TB cases in their survey. Similar results were also reported by other countries in the region[41]. WHO global tuberculosis report of 2014 indicating as 3.5% of new TB cases and 20.5% of previously treated cases was also correlated with our findings[42]. In this study, 4129 (90.87%) RIF-susceptibility tests were reported both on GeneXpert and LJ-DST. In addition to this 144 (3.17%) results were only by phenotypic LJ-DST and 271 (5.96%) results were only by GeneXpert. The proportion of valid GeneXpert results 4400 (96.83%) were higher than phenotypic DST 3873 (85.23%) in this study. This highlights the usefulness of molecular technologies for the surveillance of drug-resistant TB.

Collectively 40 (0.97%) discordances between GeneXpert and LJ-DST were noticed when 21(0.51%) cases were RIF sensitive on LJ-DST but resistance on GeneXpert and also 19 (0.46%) cases were RIF resistance on LJ-DST but sensitive on GeneXpert. In the case of discordant results, GeneXpert testing was repeated on culture isolates rather than on sputum samples. A recent report from Swaziland has raised questions as to the usefulness of GeneXpert[43] as 30% of RIF-resistant strains studied (although small in absolute number) were carrying an rpoB mutation not covered by the RIF susceptibility assay. Our findings indicate that technical false-resistant results on GeneXpert are very rare.

CONCLUSIONS

Our study shows that drug resistant-TB is a serious issue of concern in the study area. Among drug resistant TB cases MDR-TB is most serious and alarming. The detection by use of phenotypic LJ-DST and GeneXpert substantially reduced the loss of eligible patients. Their combined use perform equally well in both new and on treatment cases and highly reliable RIF- resistance detection. Chemotherapy evaluation can be done by drug resistance. Updated knowledge on the prevalence of MDR provides useful guidelines about standard drugs regimens for tuberculosis patients recommended by WHO.

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