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LABORATORY STUDY

MTBDRplus for the rapid diagnosis of ocular tuberculosis and screening of drug resistance

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Abstract

Purpose **Timely diagnosis of intraocular tuberculosis (IOTB) along with detection of drug resistance can save many eyes from visual impairment. With the growing incidence of IOTB and rising drug resistance, a reliable diagnostic platform for simultaneous detection of the agent and mutated gene is urgently needed. The MTBDRplus assay was evaluated directly on vitreous fluid samples for the same.**

Patients and methods **In a prospective study, The MTBDRplus assay was performed on 127 vitreous fluid samples (77 'study group' comprising cases of presumed ocular tuberculosis and 50 'control group' cases of disease controls ($n = 25$) and non-uveitic controls ($n = 25$)). All samples positive by MTBDRplus assay were subjected to gene sequencing to confirm the mutations for rifampicin and isoniazid resistance.**

Results **The MTBDRplus assay produced a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 36.36%, 100%, 100%, and 50.50%, respectively, for the detection of IOTB. Among the 28 cases from study group that were positive by MTBDRplus assay, rifampicin resistance was reported in six and isoniazid resistance in two cases. On sequencing of *rpoB* and *katG* gene, one case of false rifampicin-resistant by MTBDRplus was found. The other resistant isolates showed concordant mutations between MTBDRplus assay and sequencing.**

Conclusion **The MTBDRplus assay is an effective tool for the rapid diagnosis of IOTB along with detection of drug resistance, thereby improving the outcome in IOTB.**

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Introduction

Intraocular tuberculosis (IOTB) is one of the myriad presentations of *Mycobacterium tuberculosis*, a bacillus known for extrapulmonary spread. Eyes bear the dual brunt of the disease either directly via hematogenous spread or indirectly via hypersensitivity reaction from infection at some other site,[1] making IOTB a not so uncommon disease entity especially in endemic countries. The incidence of IOTB varies among different studies. It is not only rampant in high-endemic areas constituting 10.5% of all cases of uveitis,[2] but also in low-endemic areas where it ranges between 1 and 7%,[3,4] and is on a rise.[5] In the absence of a uniform case definition of IOTB,[6] these figures may as well be gross underestimation. With as high as 75% of infected eyes succumbing to moderate to severe visual impairment within a span of 6 months by IOTB,[7] a quick and correct diagnosis is imperative. The growing percentage of multidrug resistant tuberculosis highlights the importance of testing for resistance genes in patient population of IOTB as well.[8]

Diagnosis of IOTB is a major challenge. Clinical simulation by other etiologies, both infectious and non-infectious, and immune response to tubercle antigens at distant sites contribute to diagnostic confusion. Overlapping clinical manifestations and limited help from ocular angiography and ultrasound,[6] leave the ophthalmologist with the guarded definition of 'presumed ocular tuberculosis'.[9] This definition is based on the supportive circumstantial evidences like immune response to tubercle antigen (tuberculin skin test, γ-interferon assays), chest radiography and response to anti-tubercular therapy with other causes of uveitis excluded.[1] This scheme of diagnosis, too, suffers from several loopholes.[9] Although demonstration of the tubercle bacilli is the gold

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standard for diagnosis, it is rarely possible to isolate it by culture or microscopy. The inability to collect sufficient sample (fluid or biopsy) from the intricate eye structures and the paucibacillary nature of disease preclude isolation of organism. While polymerase chain reaction, targeting the most commonly used genes has many limitations for the diagnosis of paucibacillary tuberculosis including a low sensitivity,[10,11] multiplex PCR[12], and LAMP assay[13] have not yet been widely available commercially. MTBDRplus line probe assay is a commercially available diagnostic technique that is currently recommended for smear positive pulmonary specimen and from culture.[14] To the best of our knowledge, there are only two studies[15,16] addressing the role of MTBDRplus assay, on 11 and five cases, respectively, for the diagnosis of IOTB and detection of resistance simultaneously. We, therefore, evaluated the diagnostic potential of MTBDRplus assay on substantial number of vitreous fluid samples to add to the existing literature in this field.

Materials and methods

Study design

The study was approved by the Institute Ethics committee. In a prospective study, 127 vitreous fluid (VF) samples collected from eyes undergoing pars plana vitrectomy (PPV) between January 2012 and June 2015 and processed in the mycobacteriology laboratory of our institute were included in the study. They were divided into the following groups: IOTB group ($n=77$)—patients of presumed ocular tuberculosis diagnosed on the basis of previously described criteria,[17] control group I ($n=25$)—patients with a non-tubercular intraocular inflammation, and control group II ($n=25$)—patients with a non-inflammatory vitreoretinal disorder, as described in a prior study.[12] All the samples were subjected to MTBDRplus assay for the presence of *M. tuberculosis* and detection of resistance to rifampicin and isoniazid. All samples giving positive result by MTBDRplus assay were subjected to gene sequencing for confirmation of mutations.

Sample collection

The undiluted vitreous fluid was collected from the affected eye by a standard 23-gauge PPV. In this way, ~2 ml of VF was collected for each eye.

Sample processing and DNA extraction

The samples were processed inside level-II biosafety cabinets in accordance with standard bacteriological procedures to be followed for *M. tuberculosis*. All 127 samples were coded, properly labeled and then randomly distributed before carrying out any test on them so as to blind the investigator about their original grouping. For each case, 500 µl–1 ml of VF was aliquoted for DNA extraction for the MTBDRplus assay. The rest of the sample was stored at −20 °C for future reference. The DNA was extracted by commercially available Qiagen Mini DNA extraction kit as per manufacturer's instructions.

MTBDRplus assay

All the coded samples were subjected to analysis by the Genotype MTBDRplus V 2.0 (Hain Lifescience, GmbH, Nehren, Germany). The three crucial preanalytical steps of preparing the master mix, adding the DNA template and running the amplification PCR were carried out in sharply delineated rooms designated for each of these steps. A volume of 45 µl of the master mix per sample was prepared in the contamination-free room and 5 µl of the DNA template was added to it in a separate area. The amplification consisted of a 2-step multiplex PCR using biotinylated primers that underwent reverse hybridization with their specific probes. The reagents provided by the manufacturer as amplification mixes A and B were optimized and the preinstalled hybridization protocol was employed using Twin Cubator (Hain Life Sciences, GmbH, Nehren, Germany). Positive banding pattern against wild-type probes indicate absence of mutation while absence of banding pattern against wild-type probes and/or presence of any mutation band indicate resistance to the respective drug.

Sequencing

All MTBDRplus positive VF samples were subjected to *rpo*B and *kat*G sequencing using Big-Dye 3.1 Terminator (Applied Biosystems, Foster City, California, USA) and ABI 3130 sequencer (Applied Biosystems). H37Rv was used as the reference sequence.

The results of MTBDRplus assay for the detection of drug resistance were evaluated taking gene sequencing as the gold standard.

Statistical analysis

The sensitivity, specificity, positive predictive values, and negative predictive values were calculated using standard formulae.

Table 1 Performance of MTBDRplus assay for the diagnosis of IOTB

Assay	*Test results*	*IOTB group* (n = *77*)	*Control group* (n = *50*)	*Sensitivity*	*Specificity*	*PPV*	*NPV*
MTBDRplus	Positive	28	0	36.36%	100%	100%	50.50%
	Negative	49	50				

Table 2 Results of MTBDRplus assay vs gene sequencing for the diagnosis of IOTB

Group	*N*	*MTBDRplus assay*						*Sequencing*					
		MTBDRplus +ve	*RIF S*	*RIF R*	*INH S*	*INH R*	*MDR (RIF +INH)*	*rpoB GS S*	*rpoB GS R*	*katG GS S*	*katG GS R*	*inhA GS S*	*inhA GS R*
IOTB	77	28 (36.36%)	22	6	26	2	2	23	5	26	2	28	0
Control group I	20	—	—	—	—	—	—	—	—	—	—	—	—
Control group II	20	—	—	—	—	—	—	—	—	—	—	—	—

Results

MTBDRplus assay for detection of M. tuberculosis

Among the IOTB group, MTBDRplus assay detected the presence of *M. tuberculosis* in 28/77 (36.36%) cases. All samples from the two control groups tested negative for *M. tuberculosis*. The test strips gave valid results for all the samples as all the six control bands were optimal in every case. The overall sensitivity, specificity, positive predictive value, and negative predictive value for MTBDRplus in detecting *M. tuberculosis* form patients of IOTB was 36.36%, 100%, 100%, and 50.50%, respectively (Table 1).

MTBDRplus assay for detection of drug resistance

Among the 28 cases that showed presence of *M. tuberculosis* by MTBDRplus assay, rifampicin resistance was detected in six cases and isoniazid resistance in two cases (Table 2). There were two cases in which mutations to both rifampicin and isoniazid were present; these were labeled as multidrug resistant tuberculosis (MDR-TB). Out of the remaining samples, 22/28 samples were rifampicin susceptible and 26/28 samples were isoniazid susceptible. The total time taken for the MTBDRplus assay, from sample preparation to reading of results was ~24 h.

Molecular pattern of gene mutations in MTBDRplus assay

Among the six rifampicin-resistant cases, the mutation bands corresponded to codon 531 (rpoB MUT3) in four cases, and to codon 526 (rpoB MUT2A) and codon 516 (rpoB MUT1) in one case each. The two isoniazid-resistant cases had mutation at codon 315 (katG MUT1).

Comparison of drug resistance detected by MTBDRplus assay with gene sequencing

On *rpoB* gene sequencing of the 28 samples, there were only five cases in which mutation for rifampicin was detected, unlike six cases in MTBDRplus assay. Thus, MTBDRplus assay reported one case of 'false' rifampicin resistance as the *rpoB* gene sequencing of the isolate did not reveal any mutation in the *rpoB* gene. Other than this one isolate, the other five samples had concordant results between MTBDRplus assay and sequencing for the precise site of mutation. The results of isoniazid resistance were concordant with gene sequencing as both cases reporting katG (high-level) mutation by MTBDRplus assay also had mutated codon at the 315 position of the *katG* gene on sequencing (Table 3). Taking gene sequencing as the gold standard, the sensitivity, specificity, PPV and NPV of MTBDRplus assay was 95.65, 100, 100, and 83.33%, respectively, for detection of rifampicin resistance. The values were 100% for detection of isoniazid resistance.

Discussion

In the present study, *M. tuberculosis* was detected in 28 out of 77 (36.36%) patients of IOTB. This is in accordance with previous studies wherein MTBDRplus assay reported a sensitivity of 33% for detecting *M. tuberculosis* from cerebrospinal fluid (CSF) samples.[18] Since the sterile body fluids are paucibacillary in nature, a reduced sensitivity of detection, when performing the assay directly from the sample, is not surprising. Another study has reported *M. tuberculosis* detection of 55% when CSF sample was analyzed from 31 'confirmed' cases of tubercular meningitis.[19] Another possible reason for the relatively low sensitivity of the assay could be the criteria used for diagnosis of presumed IOTB. Since a favorable response

Table 3 Correlation between mutations detected by MTBDRplus assay and gene sequencing

Case	*MTBDRplus assay*		*Sequencing*	
	Rif	*Inh*	*rpoB*	*katG*
1	MTB+ RR c531	MTB+ IR c315	531 (TCG → TTG)	315 (AGC → ACC)
2	MTB+ RR c531	MTB+ IR c315	531 (TCG → TTG)	315 (AGC → ACC)
3	MTB+ RR c531	MTB+ IS	531 (TCG → TTG)	No mutation
4	MTB+ RR c526	MTB+ IS	526 (CAC → TAC)	No mutation
5	MTB+ RR c516	MTB+ IS	516 (GAC → TAC)	No mutation
6	MTB+ RR c531	MTB+ IS	No mutation	No mutation

to anti-tubercular therapy is a part of the defining criteria,[17] it is likely that the assay could not detect *M. tuberculosis* in patients undergoing therapy.

For the detection of drug resistance, the sensitivity of MTBDRplus assay in the present study was 95.65% for rifampicin and 100% for isoniazid. Maurya *et al*,[20] on evaluating MTBDRplus assay on both PTB ($n=423$) and EPTB ($n=127$) isolates have reported a sensitivity of 98 and 98.4%, respectively, for detecting rifampicin and isoniazid resistance. This difference in sensitivities could be attributed to following three contrasting factors between the two studies. While the present study is dedicated to VF samples only, Maurya *et al* may have included a variety of EPTB samples including pus and tissue samples which have relatively higher mycobacterial loads. Second, while we have taken gene sequencing as the standard of comparison, Maurya *et al* have used the phenotypic drug susceptibility testing (DST) by 1% proportion method. Third, keeping in view the paucibacillary nature of IOTB, the VF samples were subjected directly to the MTBDRplus assay in the present study, without first culturing them. This in contrast to Maurya *et al* who have subjected only culture-positive isolates (both PTB and EPTB) to MTBDRplus assay.

Other previous studies analyzing MTBDRplus assay for detecting rifampicin and isoniazid resistance have reported a sensitivity ranging from 92.8 to 93.3%, respectively, on 51 non-ocular EPTB samples;[21] 83.3 and 85.7%, respectively, on 60 bone and joint tuberculosis (BJTB) samples;[22] to 80 and 93%, respectively, on 89 cerebrospinal fluid (CSF) samples.[19] These differences could have appeared due to choice of reference standard and the version of the machine[23] used. Further, unlike our study, none of these studies have confirmed their results by carrying out gene sequencing for the offending drug. Two other studies,[15,24] comparing results of MTBDRplus assay version 2.0 with sequencing have reported a sensitivity of 96.7 and 100%, respectively, for detecting rifampicin resistance and 100% each for detecting isoniazid resistance. The present study differs from these two by the fact that while the former was carried out on 115 culture-positive undefined (PTB or EPTB) samples, the latter was conducted on mere eleven VF samples from cases of IOTB.

The MTBDRplus assay showed rifampicin resistance in 6/28 (21.42%) cases and isoniazid resistance in 2/28 (7.14%) cases. The mutations were detected at codon 531 ($n=4$), 526 ($n=1$), and 516 ($n=1$) in the *rpoB* gene; and codon 315 ($n=2$) of the *katG* gene. The findings are similar to results obtained in previous studies[16,20,21,25] as these are the most commonly encountered mutations contributing to drug resistance for rifampicin and isoniazid, respectively.

In the present study, one case of 'false' rifampicin resistance, i.e., resistant on MTBDRplus assay and no known mutation found on *rpoB* gene sequencing, was observed. On one and a half year of follow-up, this particular patient improved clinically without receiving MDR treatment. Similar observation has been made by prior researchers who have documented at least one case of false rifampicin resistance by MTBDRplus assay in comparison with rpoB sequencing,[24] DST,[20,21] or CRS.[18] Such cases of false rifampicin resistance could have arisen due to cross-contamination at any step of the assay thus emphasizing the importance of separate working areas for each step. Interestingly, another commercially available molecular platform, the GeneXpert MTB/RIF assay, is also known to report 'false' rifampicin resistance[26,27]despite being a semi-automated closed system with minimal chances of cross contamination. Prior studies have also documented cases of false rifampicin-susceptibility by MTBDRplus assay, i.e., these cases were wrongly labeled rifampicin susceptible by MTBDRplus assay whereas they were resistant either by sequencing ($n=2$)[24] or DST ($n=1$ each).[20,22] Discordant results for isoniazid resistance, as reported earlier,[20–22] could arise due to loss of DNA during manual processing (unlike GeneXpert), presence of inhibitors, or the phenomenon of 'heteroresistance'. Comparing the performance of MTBDRplus assay with gene sequencing is more prudent than with DST or CRS as it not only defines the exact loci of mutation in the respective gene but also confirms the dubious cases that are reported as 'intermediate' (absence of both the wild-type and the corresponding mutation band) by MTBDRplus assay.

Although no such 'intermediate' results were obtained in the current study, prior studies have advised caution while interpreting results of MTBDRplus assay by confirming with sequencing.[28]

The MTBDRplus assay has several advantages over other methods of DST. It is relatively simpler, faster and economical than conventional methods of DST. While phenotypic DST is not only cumbersome, it requires at least 4–6 weeks of incubation and the results may not always be reliable. The automated liquid culture systems like MGIT have no-doubt decreased the incubation time required but they are limited by their high costs in resource-limited countries with high TB-endemicity.[29] Among the commercial platforms, the GeneXpert has an edge over MTBDRplus assay in terms of its automation which minimizes chances of manual error and contamination but it is limited by the fact that only rifampicin resistance can be detected by GeneXpert in contrast to both rifampicin- and isoniazid resistance detected by MTBDRplus assay. This advantage of MTBDRplus over GeneXpert helps in correct identification of drug resistance thus preventing over-estimation of MDR-TB in areas of mono-rifampicin resistance endemicity. On one hand GeneXpert is more rapid (TAT 3 h) as compared to MTBDRplus assay (TAT 24 h), on the other hand the discordant results for rifampicin resistance are reported more with GeneXpert than MTBDRplus assay.[30] The cost per isolate for GeneXpert and MTBDRplus assay is $10 and $22, respectively.

Infectious uveitis, especially IOTB, is notorious for its non-specific clinical presentation and serious sequelae. The situation is further complexed by delayed diagnosis and poor response in settings where drug resistance is common. A recent study advocates that whenever possible, culture and/or molecular testing should be conducted on ocular fluids for the simultaneous detection of the agent or its DNA and to delineate the mutant gene by sequencing.[8]

Conclusion

Although its rate of detection of *M. tuberculosis* from IOTB samples is relatively lower than other molecular assays, its major potential lies in the substantial reduction of turn-around time as compared to conventional methods of DST and in reliably labeling the case as MDR-TB after analyzing gene mutations for both rifampicin and isoniazid. The MTBDRplus assay is thus an important diagnostic modality for timely detection and therapeutic management of cases of IOTB.

Summary

What was known before

- Diagnosis of intra-ocular tuberculosis (IOTB) is a major challenge.
- Conventional microscopy and culture are too insensitive.
- Molecular techniques not widely available commercially.
- MTBDRplus conducted first time on substantial number of vitreous fluid samples.

What this study adds

- MTBDRplus assay is a rapid test for the screening of IOTB and simultaneous detection of rifampicin and isoniazid resistance.
- Substantial reduction of turn-around time.
- Reliable labeling the case as MDR-TB after analyzing gene mutations for both rifampicin and isoniazid.

Conflict of interest

The authors declare no conflict of interest.

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