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Diagnostic Accuracy of IS6110 Insertion Gene, *Hsp*65, and Xpert MTB/RIF for Rapid Diagnosis of Pulmonary Tuberculosis

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Abstract

Purpose: To evaluate diagnostic accuracy of *IS*6110 insertion genes, *hsp*65, and Xpert MTB/RIF for rapid diagnosis of pulmonary tuberculosis. **Methods:** Sixty patients, medically reported HIV negative, clinically suspected of having pulmonary tuberculosis, were included in this study, and consented before enrolment. Sputum samples were gathered once, and tested by smear for Acid Fast Bacilli (AFB). Cultured in the Loewenstein-Jensen (LJ) medium for *M. tuberculosis* growth, *M. tuberculosis* DNA was detected by conventional PCR targeting *IS*6110, and *hsp*65 genes using specific primers, and automated nested real-time PCR targeting *rpoB* gene. Sensitivity, specificity and diagnostic accuracy were calculated for each method compared to culture. **Results:** Compared with culture as reference method, smear, *IS*6110, *hsp*65, and Xpert MTB/RIF had sensitivity 77.14%, 100%, 100%, and 100%, specificity 92%, 96%, 96%, and 96.97%, and diagnostic accuracy 83.33%, 98.33%, 98.33% and 98.21% respectively. Molecular diagnostic methods had the highest diagnostic accuracy, whereas smear had the lowest. No statistical significance, (p value > 0.05) was detected between the patients' demographic data and the presence or absence of TB infection. **Conclusion:** The diagnostic accuracy that we got from the molecular methods, confirmed the diagnostic value of molecular detection of *M. tuberculosis* in pulmonary cases, supporting the application of automated and conventional PCR in rapid analysis. Smear could be more efficient when used for treatment monitoring. Combination between one-molecular techniques with smear as a routine method could be valid for rapid diagnosis of TB.

Keywords

Rapid Diagnosis, Pulmonary TB, Molecular Tests

1. Introduction

Tuberculosis (TB) is known to be a worldwide health issue. It has been recorded to influence more than 12 million individuals every year, and the vast majority of the cases are connected with the Human Immune deficiency Virus (HIV) as TB/HIV co-infection, which builds mortality rate [1]. Tuberculosis is an airborne ailment brought by the bacteria known as *Mycobacterium tuberculosis* [1].

In recent years, there is an increased TB incidence in Saudi Arabia. Several predisposing factors such as the high economic living in Saudi Arabia, attract many workers from affected areas, and also the frequent visit of pilgrims to the holy cities [2] [3] [4].

The detection of TB bacteria in sputum smear (Acid-fast bacilli smear) still remains one of the good choices of diagnosis especially in rural areas, and low-income countries. Though, a few nations use sputum smear for treatment observing instead of analytic purpose. Culture method (the current reference standard) is used in countries with good laboratory infrastructure. Recently, the frequent use of molecular methods to diagnose tuberculosis has raised hopes for the early detection of the disease [1].

Diverse gene targets like *IS*6110, 16S *rDNA*, *rpoB*, *recA*, *and hsp*65, which are used to distinguish *M. tuberculosis* gene in sputum tests, has demonstrated variability in affectability and specificity. *IS*6110 is a standout amongst the most well-known genes effectively used to recognize and distinguish *M. tuberculosis* DNA in clinical specimens by PCR [5] [6] [7] [8]. The gene demonstrated variability in affectability and specificity as indicated by the kind of tests, of the TB restriction in the body, and nearness or nonappearance of TB/HIV co-infection [6]-[12]. Likewise, the quality encoding 65-kDa heat stun protein (*hsp*65) has been accounted as a valuable focus for mycobacterium discovery by PCR- based methods [13].

A new rapid technology such as Xpert MTB/RIF detects DNA of *M. tuberculosis* and rifampicin resistance gene by Real Time-PCR [14] [15]. It is an automated Nucleic Acid Amplification Tests (NAAT), which isolates and detects *M. tuberculosis* in raw materials or sediment of sputum specimens, simultaneously. It detects most of the relevant rifampicin resistance and mutant genes. Besides, Xpert MTB/RIF required a very little technical training, and results were obtained within 90 minutes from raw sputum samples [16].

In our study, we evaluated the conventional PCR targeting *IS*6110, and *hsp*65 (*TB*11/12) genes, and automated nested real-time PCR targeting *rpoB* gene to recognize *M. tuberculosis* DNA among suspected TB patients.

2. Materials & Methods

2.1. Ethical Approval

This study was ethically approved by the Jazan University standing committee for biomedical research ethics (SCBRE) with approval Ref No. 2198/60.

2.2. Patients

Sixty patients who are reported HIV negative, clinically suspected of having pulmonary tuberculosis, attended to the chest diseases hospital Laboratory at the Jazan area south-west Saudi Arabia, were included in this study, were as patients whom started treatment were excluded from this study. All included patients consented before enrollment in the study.

2.3. Samples Collection and Preparation

Sixty sputum tests were gathered, and prepared by standard research facility methods. For cleaning and processing, we took after the method portrayed by (Kent and Kubica, 1985) [17]. Briefly about 3 ml of sputum sample was added to an equal volume of BBL Myco Prep (cat#240862) containing NALC-NaoH with PBS in 15 ml falcon tube (BD), kept for 15 minutes for digestion, and then PBS was added till 15 ml, centrifuged at 3000/rpm 4°C, for 15 minutes, and the supernatant was discharged and 3 ml PBS was added to the pelted sample for DNA extraction and detection assays. Smears and cultures were performed before sample deactivation. The sample deactivation as described by Doig *et al.* 2002 [18], was done for the sample before transferring to a molecular biology lab for PCR.

2.4. Ziehl Neelsen (ZN) Staining Method

Sputum tests were researched for the nearness of acid-fast bacilli (AFB) by Ziehl Neelsen staining method, smear arrangement and staining was done by maker's guideline of Crescent Ref MS412, and Lot number 32706F.

2.5. Culture

About 0.5 ml of all sputum samples was cultured in the Loewenstein-Jensen (LJ) medium. Liquid culture was not used in this study because it was not available at the time when we did the study. A negative culture was affirmed following 8 weeks of incubation, and a positive culture was affirmed by the typical growth development of *M. tuberculosis* trailed by AFB smear and Real-time PCR discovery. The positive control strain of Mycobacterium species was generously provided by the laboratories of the microbiology department of the faculty of medicine King Khalid University.

2.6. DNA Extraction

DNA was extracted from sputum, and a positive culture By Promega DNA extraction kit according to the manufacturer's instruction with minor modifications, as lyses step sputum samples and purified culture was treated with a 20ul freshly prepared lysosyme solution(Cat#L6876, Sigma, USA) at concentration 10 mg/ml. The extracted DNA was kept in −20°C till used.

2.7. Amplification of *IS*6110 Gene by PCR

A 20 μL of PCR reaction was prepared by adding 1 μL of 10x dNTPs (1.25 μM each of dTTP, dGTP, dCTP, and dATP), 1 μL of each primer (0.5 μM), 4 μL of

10min PCR buffer, 2 μL of MgCl$_2$ (50 μM), 5.8 μL of sterile distilled water, 0.2 μL of *Taq* polymerase of (5 Unit/μL), and 5 μL DNA sample. DNA was amplified by PCR using the following cycle conditions: 94˚C for 5 minutes, then 38 cycles: (94˚C for 1 minute, 68˚C for 1 minute). The primers of *IS*6110 genes were (F, 5'-CCT GCG AGC GTA GGC GTC GG-3'; R, 5' CTC GTC CAG CGC CGC TTC GG), which amplified 123 bp fragment as described by [19] [20]. The presence of a single band equivalent to 123 bp (**Figure 1**) was taken as positive results, while the same band produced by the positive control, and the negative control offered no response.

2.8. Amplification of *hsp*65 by PCR

PCR master mix for *hsp*65 (*TB*11/12) gene was prepared by the same method for *IS*6110 genes with cycle conditions as follows: 95˚C for 4 minutes, followed by 30 cycles: (95˚C for 30 seconds, 56˚C for 1 minute, 72˚C for 40 seconds), and a final extension of 72˚C for 7 minutes. The primers of *hsp*65 (*TB*11/12) *insertion gene* were TB11 (5'-ACC AAC GAT GGT GTG TCC AT), and TB12 (5'-CTT GTC GAA CCG CAT ACC CT), which amplified a 440 bp fragment as described by [21]. The presence of a single band equivalent to 440 bp (**Figure 2**) was taken as positive results while the same band produced by the positive control, and the negative control offered no response.
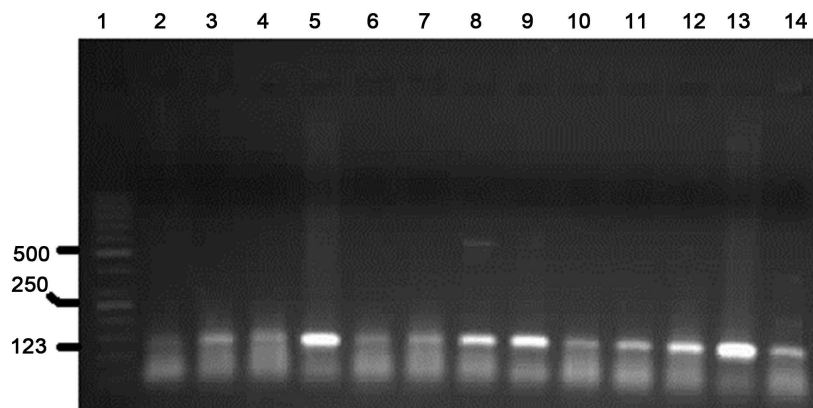

Figure 1. Sputum samples tested by *IS*6110 gene, lanes1 a 50 pb Mwt marker, Lane2 negative sample, lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 positive samples produced 123 pb.
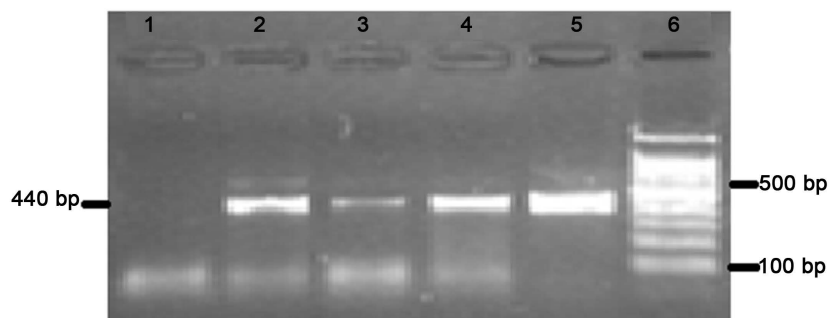

Figure 2. Sputum samples tested by *hsp*65 (TB11/12) gene, lanes1 negative sample, lanes 2, 3, 4, and 5 positive samples produced 440 pb, lane 6 a 100 pb marker.

2.9. Electrophoresis of Amplified Products

Amplified products were visualized on 1.8% agarose gels stained with flouro-safe dye (0.5 mg/L), and examined under UV light.

2.10. Xpert MTB/RIF Test

This test was performed on a robotized Cephoid Gene Xpert Instrument framework (Ref GXMTB/RIF-US-10), quickly, around 0.5 ml of concentrated sputum silt was added to the Gene Xpert cartridge, that holds the Real Time-PCR and PCR reagents and host every one of the procedures. The outcomes were investigated by utilizing Gene Xpert Software adaptation 4.3. The identification of *M. tuberculosis* (MTB) was accounted for when no less than two tests in *Ct* values inside the substantial extent, and MTB not recognized was accounted for when there was only one or no positive analysis.

2.11. Statistical Analysis

Data analysis was directed utilizing (SPSS 20.0 for Windows, SPSS inc. Chicago, IL, USA). The results of the different samples were analyzed and statistically matched. Since the investigation was led utilizing known culture-positive and-negative samples, the greater part of the samples were thought to be true positives (Tps) or true negatives (Tns). The four analyses results were named Tp, false negative (Fn), Tn, or false positive (Fp). The sensitivity and specificity were computed in like manner. Graphic statistics centered on frequency distribution and percentages and Chi-square test was also conducted. A p-value < 0.05 was reflected as statistically significant.

3. Results

Sixty TB suspected patients were included in this study, 43 (71.7%) were male, and 17 (23.3%) were female, 60% were Saudi nationality, and 40% were Non Saudi nationality, ages were ranged between 10 - 70 years old, with the majority range between 25 - 44 years (56.7%), followed by 10 - 24 years (26.7%), and 45 - 70 years (16.7%) as shown in Table 1. Our results showed no statistical significance, (p value > 0.05) was detected between the age, sex, and nationality with the presence or absence of TB infection (Table 2).

Out of 60 patients specimens 35 (58.3%) were positive for MTB, and 25 (41.7%) were negative for MTB culture (Table 1, Figure 4). Positive *M. tuberculosis* cultures were affirmed by Real-Time PCR amplification curve, and it created *Ct* values ranged between 11.5 and 25.5 (Figure 3).

Smear results showed that 29 (48.3%) were AFB positive, and 31 (51.7%) were non AFB (Table 1, Figure 4). Contrasted with culture as reference method, smear had sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and analytical exactness of 77.14% (95% as a confident interval (CI) 60.98, 87.93[1]), 92% (95%(CI) 75.03, 97.78[1]), 93.1% (95% (CI) 78.04, 98.09[1]), 74.19% (95% (CI) 56.75, 86.3[1]), 83.33% (95% (CI) 71.97, 90.69[1]), respectively as shown in (Table 3, Figure 5).
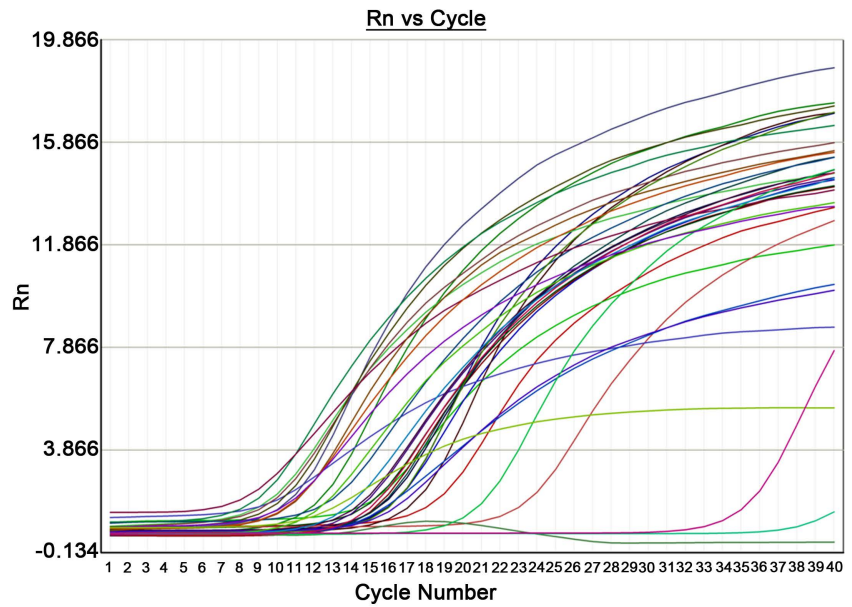
Figure 3. Amplification plot of isolated *M. tuberculosis* DNA by *IS*6110 gene, *Ct* values ranged between 11.5 - 25.5 (Positive control Ct 25.5, Negative control Ct 37.3).

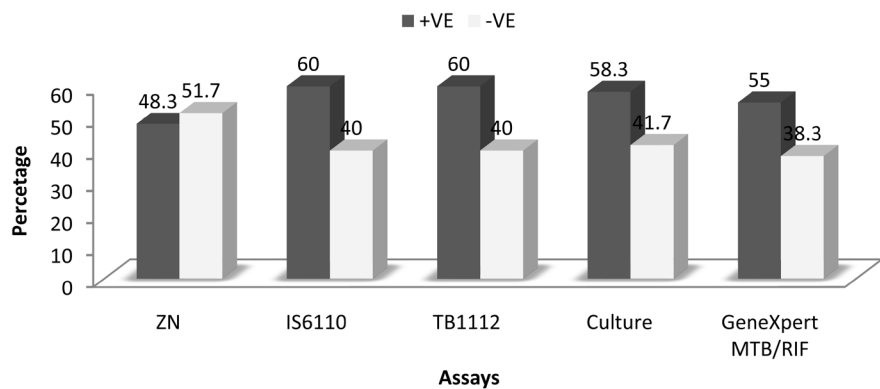**Figure 4.** Results of ZN smear, *IS*6110, *TB*11/12, GeneXpert MTB/RIF assays and culture method.
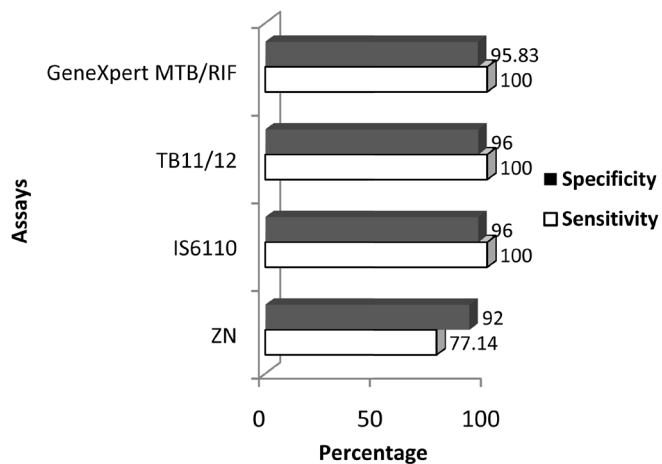

Figure 5. Sensitivity and specificity, of ZN smear, *IS*6110, *TB*11/12 and GeneXpert MTB/RIF assays using culture method as reference.

Table 1. Characteristics of the 60 patients, specimens analyzed and culture results.

Characteristics	N	%
Age groups		
10 - 24 years	16	26.7
25 - 44 years	34	56.7
45 - 70 years	10	16.7
Gender		
Male	43	71.7
Female	17	28.3
Nationality		
Saudi	36	60.0
Non-Saudi	24	40.0
ZN smear		
+VE	29	48.3
−VE	31	51.7
IS6110		
+VE	36	60.0
−VE	24	40.0
TB11/12		
+VE	36	60.0
−VE	24	40.0
Culture		
+VE	35	58.3
−VE	25	41.7
Xpert MTB/RIF		
+VE	33	55.0
−VE	23	38.3
ND	4	6.7

+VE(Positive), −VE(Negative), ND(Not done).

Table 2. Comparison of demographic data of the patients and positive results of ZN smear, *IS*6110, *TB*11/12 and Xpert MTB/RIF assays with the culture method as reference.

Characteristics	NO. and% of positive samples				
	ZN smear	IS6110	TB11/12	Xpert MTB/RIF	Culture
Age groups					
10 - 24 years	9 (56.2)	10 (62.5)	10 (62.5)	9 (60.0)	10(62.5)
25 - 44 years	16 (47.1)	21 (61.8)	21 (61.8)	19 (61.3)	21 (61.8)
45 - 70 years	4 (40.0)	5 (50.0)	5 (50.0)	5 (50.0)	4 (40.0)
Gender					
Male	22 (51.2)	27 (62.8)	27 (62.8)	24 (61.5)	27 (62.8)
Female	7 (41.2)	9 (52.9)	9 (52.9)	9 (52.9)	8 (47.1)
Nationality					
Saudi	15 (41.7)	20 (55.6)	20 (55.6)	18 (54.5)	19 (52.8)
Non- Saudi	14 (58.3)	16 (66.7)	16 (66.7)	15 (65.2)	16 (66.7)
Total	29 (48.3)	36 (60.0)	36 (60.0)	33 (58.9)	35 (58.3)

No statistical significance was detected between the specified assay and the culture.

Table 3. Sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy of the ZN smear, *IS*6110, *TB*11/12 and Xpert MTB/RIF assay with the culture method as reference.

Specimen type	Sensitivity	Specificity	PPV	NPV	Diagnostic Accuracy
	No. (%), 95% confidence interval (CI)				
ZN smear	77.14% (60.98, 87.93[1])	92% (75.03, 97.78[1])	93.1% (78.04, 98.09[1])	74.19% (56.75, 86.3[1])	83.33% (71.97, 90.69[1])
*IS*6110	100% (90.11, 100[1])	96% (80.46, 99.29[1])	97.22% (85.83, 99.51[1])	100% (86.2, 100[1])	98.33% (91.14, 99.71[1])
*TB*11/12	100% (90.11, 100[1])	96% (80.46, 99.29[1])	97.22% (85.83, 99.51[1])	100% (86.2, 100[1])	98.33% (91.14, 99.71[1])
Xpert MTB/RIF	100% (89.28, 100[1])	96.97% (79.76, 99.26[1])	96.97% (84.68, 99.46[1])	100% (85.69, 100[1])	98.21% (90.55, 99.68[1])

In the current study, both of *IS*6110 and *Hsp*65 (*TB*11/12) gave similarity in results 36 (60%) were positive for *M. tuberculosis* DNA, and 24 (40%) were negative (Table 1, Figure 5). Compared with culture as reference method, both *IS6110* and *Hsp*65 (*TB*11/12) genes produced same sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of 100% (95% (CI) 90.11, 100[1]), 96% (95% (CI) 80.46, 99.29[1]), 97.22% (95% (CI) 85.83, 99.51[1]), 100% 995% (CI) 86.2, 100[1]), 98.33% (95% (CI) 91.14, 99.71[1]), respectively as shown in (Table 3, Figure 5).

In this study Xpert MTB/RIF showed that 33 (55%) out of 56 tested samples were positive for MTB, 23 (38.3%) were negative, and 4 (6.7%) were not done (ND) for the machine factor, as shown in (Table 1, Figure 4). Compared with culture as reference method, Xpert MTB/RIF gave sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of 100% (95% (CI) 89.28, 100[1]), 96,97% (95% (CI) 79.76, 99.26[1]), 96.97% (95% (CI) 84.68, 99.46[1]), 100% (95% (CI) 85.69, 100[1]), 98.21% (95% (CI) 90.55, 99.68[1]), respectively as shown in (Table 3, Figure 5).

4. Discussion

The early diagnosis of TB is very important to start early treatment and to establish preventive measures of the disease control [1]. In this study, the diagnostic accuracy of AFB smear, conventional and automated nested PCR was evaluated compared with culture as a gold standard method.

In this study one patient specimen gave negative result with culture method and positive with molecular methods utilized as a part of this study, this may be due to either by the non-viable bacilli existent in the sample or the small quantity of the bacteria.

When compared with other molecular tests smear produced the lowest sensitivity, which indicated that smear is still not the suitable method for diagnosing TB, although it is a quick and cheap method for diagnosis. On the other hand,

smear could be more efficient when used for treatment monitoring as described before [1].

Our findings confirm the diagnostic value of *IS*6110 and *hsp*5 genes in rapid detection of pulmonary TB, the same findings were mentioned by Ritis *et al.* (2000) [22] when he used *IS*6110 gene, although the sources of the samples were different, and of Maccente *et al.* (2013) [23], when she evaluated the *hsp*65 gene for rapid diagnosis of TB in a high-burden country. Besides the highest diagnostic accuracy of this method, it has many advantages. Firstly, the method is fast, the results can be obtained within one to two working days. Secondly, the method is cheap, it cost about 7 USD. One patient gave a positive result in this method and a negative result by culture, although this sample statistically was calculated as false positive (FP), it could be the occurrence of non-viable bacilli in this sample.

The Automated Xpert MTB/RIF produced a high diagnostic accuracy for pulmonary TB in sputum samples, our finding was in strong agreement with a study done by Vinod *et al.* (2014) [24], although he used samples from Tuberculous meningitis. In our study, one patient sample was positive with Xpert MTP/RIF and negative by culture, the test can detect *M. tuberculosis* DNA from either nonviable or non-intact bacilli [25], taking into account also the culture limitation. Many advantages of the Xpert MTB/RIF are, first, it is fast, the result can be obtained within 90 minutes, second, positive result reflect the true detection of *M. tuberculosis* rather than *nontuberculous mycobacteria* [25], third detection of Rifampicin resistant bacteria. Besides all these advantages, the test is expensive, one test cost about 18 USD, which is not suitable for low-income countries.

5. Conclusion

Our results and the diagnostic accuracy that we got from the molecular methods, confirm the diagnostic value of molecular detection of *M. tuberculosis* in pulmonary cases, supporting the application of automated and conventional PCR in rapid analysis. Combination between one-molecular techniques with smear as a routine method could be valid for rapid diagnosis of TB.

6. Limitations

Culture result itself can be influenced by numerous elements including sample blunders and other mechanical causes can likewise influence culture sensitivity, which demonstrates our study restrictions.

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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