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Original Article

In Silico and *In Vitro* Analyses of Previously Reported Primer Pairs for the Detection of *Mycobacterium tuberculosis* and Their Comparison With Two Novel Designed Primer Pairs

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Abstract

Background: The accurate and timely diagnosis of tuberculosis (TB) is crucial in the effective management and control of this infectious disease. Several molecular tests have been developed and are currently utilized for the diagnosis of *Mycobacterium tuberculosis* (MTB) in suspected patients. This study aimed to investigate various criteria affecting both the analytical and clinical specificity and sensitivity of primer pairs derived from published literature. The study also compared these established primer pairs with two novel primer pairs using *in silico* and *in vitro* methodologies.

Methods: All studies related to the detection of MTB using polymerase chain reaction (PCR) and quantitative PCR (qPCR) were included based on established criteria. Eventually, all reported primer pairs were analyzed using *in silico* online software tools.

Results: Among the 386 primarily retrieved articles, 98 met the eligibility criteria and were included for data extraction and analysis. Of these, 85 studies (86.7%) employed endpoint PCR, while 13 studies (13.3%) utilized q-PCR. All reported primers were thoroughly analyzed, resulting in the selection of six primers. Approximately 50% of the analyzed primer pairs targeted *IS6110* and demonstrated a clinical sensitivity exceeding 80%. The *rpoB* gene revealed that nearly 35% of the primer pairs exhibited a clinical sensitivity greater than 80%.

Conclusion: The newly designed primers, based on *in silico* analysis, represented favorable clinical sensitivity and specificity. Consequently, PCR using these primers may prove to be highly beneficial for the diagnosis and management of TB in low-resource settings.

Keywords: PCR, *Mycobacterium tuberculosis*, Primer pairs, Analytical sensitivity, Clinical sensitivity

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Introduction

Mycobacterium tuberculosis (MTB) ranks among the top 10 causes of death worldwide (1), accounting for 95% of cases and 98% of TB-related deaths in developing countries (2). An infected person can transmit the disease to 10–15 people among their family members and relatives within a year (3). Therefore, timely and accurate diagnosis is vital in preventing the transmission and spread of TB (4). Today, a wide range of tests are employed for TB diagnosis, including various molecular nucleic acid amplifications, such as polymerase chain reaction (PCR) and quantitative PCR (q-PCR) (5,6). Accurate and rapid results, provided by molecular methods, including the detection of mutations that lead to antibiotic resistance, enhance the likelihood of targeted treatment. This process not only reduces hospitalization time and costs but also minimizes the risk of transmission and spread of the disease (7,8).

The selection of the target gene and region and the efficiency of the designed primers for that target gene are vital in enhancing the analytical and clinical sensitivity and specificity of molecular detection methods (9,10). This study seeks to conduct an *in silico* analysis of primer pairs for the PCR diagnosis of MTB retrieved from published articles using online bioinformatics software and to compare the results with those of the two designed

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primer pairs using both *in silico* and *in vitro* methods. In general, the specificity, sensitivity, and other criteria for appropriately designed primers are evaluated and compared in this study.

Materials and Methods

Searching the Scientific Literature

A systematic analysis was performed using several databases, including PubMed, Scopus, ScienceDirect, and Google Scholar, employing an appropriate search strategy with relevant keywords, and all papers related to the detection of MTB by PCR and q-PCR were included in the analysis. Eligible papers were selected and analyzed following the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Figure 1).

Various characteristics of the primers, including the author, year of study, target gene, primer sequences, PCR product length, sample size, clinical specificity and sensitivity, and the positive and negative predictive values, were extracted from the final eligible papers.

Analysis of Primers

The melting temperatures (Tm) of each primer were calculated using OligoCalc software (11), according to the basic, nearest neighbor, and salt-adjusted formula. Moreover, the annealing temperature (Ta) was determined by taking the average of the nearest neighbor Tm of the forward and reverse primers, then adding 3 ˚C. Next, a comparison was made between the Ta of forward and reverse primers and that of the primer pairs.

The hairpin, hetero-dimer, and homo-dimer (self-dimer) forms of all retrieved primers were analyzed using Oligo-analyzer (12), with the threshold for each formation set at a ΔG (Gibbs free energy) of -9 kcal/mol. The ΔG value was recorded if the ΔG of the primer was less than -9; otherwise, the cases were indicated as NO in the table.

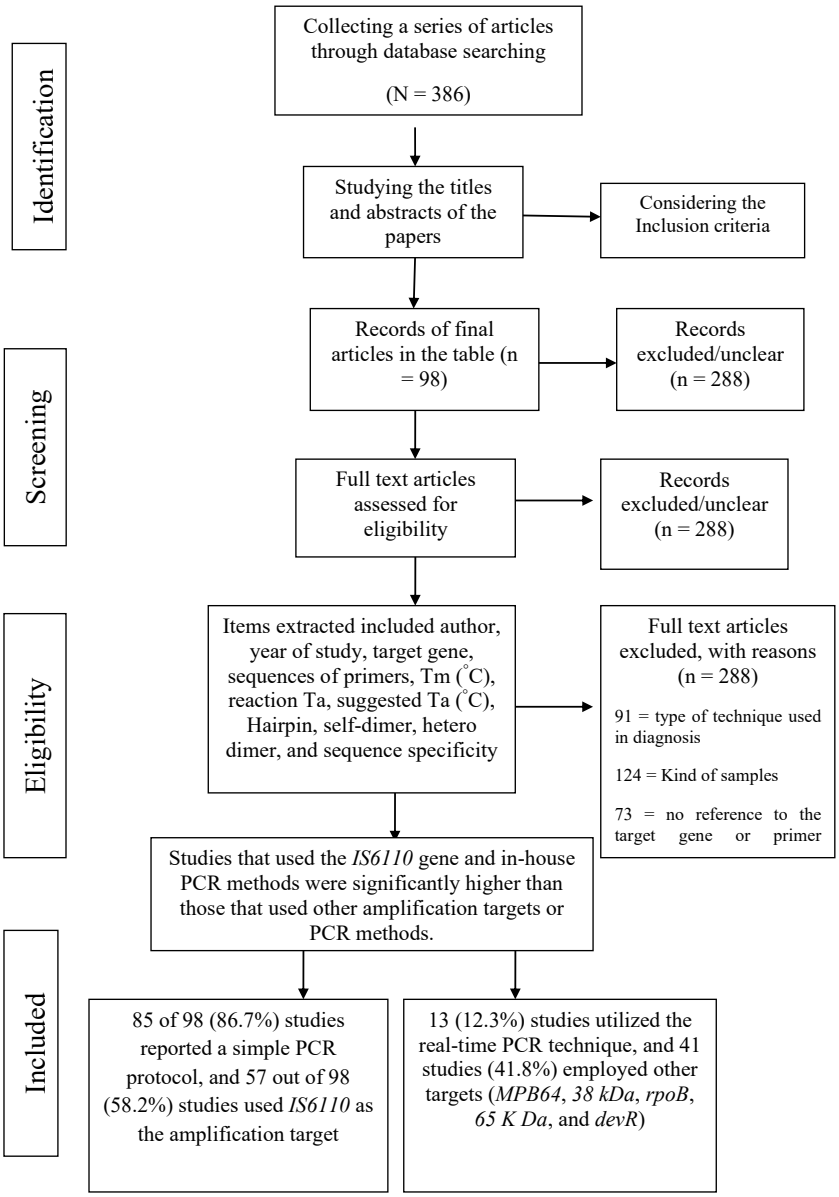

Figure 1. Analysis Procedure of Retrieved Papers. *Note*. Tm: Melting temperature; PCR: Polymerase chain reaction; Ta: Annealing temperature.

Subsequently, Primer BLAST was utilized to evaluate the specificity of primer pairs (13).

In summary, the sequences of the forward and reverse primers were analyzed against at least 100 whole genome sequences of MTB, and the results regarding non-specific products were documented. It should be noted that a product was considered nonspecific if there was even a single nucleotide mismatch at the 3' end of either primer, if there were five or more nucleotide mismatches between each primer and the product, or if nonspecific product(s) were present (6).

Sputum Collection and Detection of Mycobacterium tuberculosis

The study was conducted on patients with suspected TB who were referred to a lung disease center in Kermanshah from August 2019 to June 2020.

All collected sputum samples were decontaminated and analyzed using direct smear, GeneXpert, and culture methods, as well as PCR using all selected primer pairs, which are conventional techniques for diagnosing TB. The Ziehl-Neelsen method was employed for staining the concentrated smear. Additionally, digested and concentrated sputum samples were cultured on Lowenstein-Jensen medium.

Polymerase Chain Reaction Assay for the Detection of IS6110 and rpoB Genes

DNA was extracted from the sputum samples using the G-Spin DNA Isolation Kit according to the manufacturer's instructions. Subsequently, a Synergy HTX Multi-Mode Microplate Reader was used to measure the concentration of the extracted DNA. In addition, specific primers were utilized in a thermal cycler to amplify the *IS6110* and *rpoB* genes. A 25 µL PCR typically includes 100 ng DNA template, 0.1 µM of each primer, 1X master mix, and nuclease-free water up to 25 µL, followed by thermal cycling for the amplification of the target DNA sequence. The PCR products were visualized following electrophoresis and staining. The TB H37Rv reference strain and a PCR containing all elements, except the DNA template, were used as positive and negative controls.

Moreover, the PCR amplification was performed using all selected primer pairs in a thermal cycler (Bio-Rad, Singapore), with an initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of denaturation at 95 ˚C for 30 seconds, annealing at the appropriate temperature for 30 seconds, and extension at 72 ˚C for 1 minute, concluding with a final extension at 72 ˚C for 5 minutes. The PCR products were then analyzed by electrophoresis and visualized after staining.

Results

Scientific Literature Database Search

A total of 386 articles related to PCR and q-PCR diagnosis of MTBC were retrieved from multiple databases using selected keywords covering the period from 1990 to 2020.

Following an initial screening of titles and abstracts, 98 articles were deemed eligible for final inclusion, while 285 articles were excluded from the investigation. A summary of the characteristics of the eligible studies is presented in Table S1 (Supplementary file 1). IS6110 as a target and in-house PCR methods had the highest frequency in the studies.

Analysis of Primer Pairs

Overall, 85 out of 98 (86.7%) studies were conducted using the endpoint PCR protocol, while 13 (12.3%) studies utilized q-PCR. Furthermore, 57 out of 98 (58.2%) studies used *IS6110* as the target gene. Moreover, six (6.1%) studies employed the *MPB64*, and three (3%) studies applied the *38kDa* genes. Other genes, such as r*poB*, *65KDa*, *devR*, *sdaA*, *pncA*, *secA1*, *hsp65*, and *dnaJ*, were used as targets in 32 studies (32.7%).

The comparison of melting temperatures revealed that 61 primer pairs (62.2%) exhibited a difference in Tm, ranging from 0 ˚C to 2 ˚C. In addition, 32 primer pairs (32.7%) showed a difference between 2˚C and 5˚C, and 5 primer pairs (5.1%) had a difference of more than 5 ˚C.

The comparison between the suggested Ta (sTa) and the applied Ta indicated that 43 primer pairs (43.9%) had a difference ranging from 0 ˚C to 3 ˚C, and 19 primer pairs (19.4%) displayed a difference between 3 ˚C and 5 ˚C. Additionally, 19 primer pairs (19.4%) showed a difference between 5 ˚C and 10 ˚C, and 7 primer pairs (7.1%) represented a difference of 10˚C or more, indicating a significant discrepancy. The Ta utilized in the reaction was also not reported in 10 studies (10.1%). Most primer pairs had sTa between 60˚C and 68˚C, with 29 (29.5%) items applied at a Ta in the PCR that was lower than the Ta calculated by OligoCalc. The most commonly used primer was "Eisenach et al. 1991," which was employed in 10% of the studies (14).

The clinical sensitivity and specificity of MTB detection using the *IS6110* target were in the range of 14–100% and 35.9–100%, respectively. The positive and negative predictive values were in the range of 16.81–100% and 70.6–100%. Moreover, the corresponding values were 85.8–89.2% and 94.3–96% for efficiency and accuracy, respectively.

The results of the *in silico* analysis for all primers indicated that 6 primer pairs (6.1%) exhibited low specificity, which could lead to a higher likelihood of false positive results. Further, 58 primer pairs (59.2%) demonstrated 100% specificity in the primer BLAST analysis, demonstrating a complete match with the target in the whole genome sequence of 100 known standard MTB strains without any significant mismatch, particularly at the 3' end. Additionally, 30 primer pairs (30.6%) illustrated specificity between 80% and 99%, while 7 (7.1%) of them had specificity between 40% and 80%. The analytical specificity of 3 primer pairs (3.1%) was extremely low, implying complete non-specificity.

Table 1 presents the comparison results between the *in*

Table 1. Relationship Between *In Silico* Specificity and Differences Between sTa and aTa, Tm, and the Homo-Dimer Form of Forward and Reverse Primers, and the Hetero-Dimer Form of Primer Pairs

Homo-DimerR			Homo-DimerF			Heterodimer			Differences Between TmF-TmR				Differences Between sTa and aTa						Spe BLAST
Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	5-UP	2.1-5	0-2	Total	Not reported	10.1-UP	5.1-10	3.1-5	0-3	
7	5 (71.5%)	2 (28.5%)	7	4 (57.2%)	3 (42.8%)	7	2 (28.5%)	5 (71.5%)	7	0	3 (42.9%)	4 (57.1%)	7	0	1 (14.3%)	1 (14.3%)	0	5 (71.4%)	0-80
30	21 (70%)	9 (30%)	30	5 (16.6%)	25 (83.4%)	30	22 (73.7%)	8 (26.7%)	30	0	4 (13.7%)	26 (86.7%)	30	2 (6.6%)	0	5 (16.7%)	6 (20%)	17 (56.7%)	81-99
58	24 (41.4%)	34 (58.6%)	58	19 (32.8%)	39 (67.2%)	58	7 (12.1%)	51 (87.9%)	58	5 (8.7%)	24 (41.3%)	29 (50%)	58	8 (13.8%)	5 (8.6%)	13 (22.4%)	12 (20.7%)	20 (34.5%)	100
3	1 (33.3%)	2 (66.7%)	3	1 (33.3%)	2 (66.7%)	3	0	3 (100%)	3	0	1 (33.3%)	2 (66.7%)	3	0	1 (33.33%)	0	1 (33.33%)	1 (33.33%)	Non-spe
98	51	47	98	29	69	98	31	67	98	5	32	61	98	10	7	19	19	43	Total
$P \leq 0.05$				-			$P \leq 0.05$				$P \leq 0.05$					-			*P*-value

Note. speBLAST: Calculated specificity using Primer-BLAST; sTa: Suggested T annealing; aTa: Applied annealing temperature; TmF: Melting temperature of forward; TmR: Temperature melting of reverse; Non-spe: Non-specific.

silico specificity of primer pairs and the differences between the sTa and the aTa, Tm, as well as the homo-dimer and hetero-dimer formations of forward and reverse primer pairs.

Table 2 provides the data related to the relationship between the reported clinical specificity and the differences between the sTa and the aTa, Tm, as well as the homo-dimer and hetero-dimer formations of the forward and reverse primer pairs.

The results associated with the relationship between the clinical sensitivity reported in the articles and the differences between the sTa and the aTa, Tm, as well as the homo-dimer and hetero-dimer formations of the forward and reverse primer pairs, are summarized in Table 3.

In total, 82 primer pairs (83.7%) successfully amplified a single fragment with the expected size of the PCR product, while 16 primer pairs (16.3%) could amplify two or more product fragments of different sizes than expected, which were considered non-specific products.

Overall, 48 studies (49%) defined gold standard methods for MTB detection. Among them, 26 articles (54.1%) defined traditional bacteriological culture on Lowenstein-Jensen medium as the gold standard. In other studies, culture, the BACTEC TB system, acid-fast direct microscopy, and fluorescent staining, in combination with clinical presentation and clinical response to specific treatment, were considered the gold standard.

Specificity and sensitivity ranged from 30% to 100% and from 14% to 100%, respectively. In addition, the positive and negative predictive values ranged from 16.81% to 100% and from 42% to 100%, respectively. Additionally, efficiency and accuracy were reported to be within the range of 48–100% and 60–100%, respectively.

Results of Primer Analysis

All reported primers were analyzed, six of which were chosen based on common criteria. Approximately 50% of the primer pairs analyzed for the diagnosis of MTB targeted the *IS6110* gene and demonstrated a clinical sensitivity of over 80%. Consequently, this gene was selected as the primary target, and three pairs of primers were selected from it.

The second target gene, *rpoB*, was selected because nearly 35% of the analyzed primer pairs exhibited a clinical sensitivity greater than 80%.

The *MPB64* gene was not selected because about 20% of the related primer pairs displayed a clinical sensitivity of less than 50%.

Primer No. 12 (Table S1) was the first selected primer targeting the *rpoB* gene, reported by Nimesh et al (15). The second selected primer was primer No. 14 (Table S1), which targeted the *IS6110* gene and was reported by the mentioned researchers (15). This primer, which was designed and introduced by Eisenach et al, is more commonly used for MTB complex detection (14). Primer No. 40 (Table S1) was the third selected primer targeting the *IS6110* gene and was reported by Kim et al (25). The fourth selected primer was primer No. 55 (Table S1), which targeted the *IS6110* gene and was reported by Kox et al (16). The fifth selected primer was primer No. 81 (Table S1), which targeted the *IS6110* gene and was reported by Gouveia et al (17).

Sputum Collection and Detection of Mycobacterium tuberculosis

The sputum samples of 186 suspected TB patients referred to the Kermanshah TB Reference Laboratory were collected for analysis . The ages of the suspected TB patients ranged from 17 years to 85 years, with a mean age of 46 years. Within this population,

Table 2. Relationship Between Clinical Specificity and Differences Between sTa and aTa, Tm, and the Homo-Dimer Form of Forward and Reverse, and the Hetero-Dimer Form of Primer Pairs

Homo-DimerR			Homo-DimerF			Hetero-Dimer			Differences Between TmF and TmR				Differences Between Ta and aTa						Clinispe
Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	5-UP	2.1-5	0-2	Total	Not reported	10.1-UP	5.1-10	3.1-5	0-3	
41	22 (53.7%)	19 (46.3%)	41	9 (22%)	32 (78%)	41	15 (36.5%)	26 (63.5%)	41	1 (2.5%)	16 (39%)	24 (58.5%)	41	2 (5%)	5 (12.2%)	11 (26,8%)	6 (14.6%)	17 (41.5%)	80.1-100
7	4(57.2%)	3(42.8%)	7	2(28.5%)	5(71.5%)	7	4(57.2%)	3(42.8%)	7	0	2(28.5%)	5(71.5%)	7	2 (28.5%)	0	1 (14.3%)	0	4(57.2 %)	50.1-80
6	2 (33.4%)	4 (66.6%)	6	3 (50%)	3 (50%)	6	1 (16.6%)	5 (83.4%)	6	2 (33.3%)	2 (33.3%)	2 (33.3%)	6	0	0	0	1 (16.7%)	5 (83.3%)	0-50
44	23 (52.3%)	21 (47.7%)	44	15 (34.1%)	29 (65.9%)	44	11 (25%)	33 (75%)	44	2	12	30	44	6 (13.6%)	2 (4.5%)	7 (15.9%)	12 (27.3%)	17(38.7%)	Not reported
98	51	47	98	29	69	98	31	67	98	5	32	61	98	10	7	19	19	43	Total
-				-			-				$P \leq 0.05$					-			*P*-value

Note. Clinispe: Clinical specificity; sTa: Suggested annealing temperature; aTa: Applied annealing temperature; TmF: Melting temperature forward; TmR: Melting temperature reverse.

Table 3. Relationship Between Clinical Sensitivity and Differences Between sTa and aTa, Tm, and Homo-Dimer Form of Forward and Reverse, and Hetero-Dimer Form of Primer Pairs

Homo-DimerR			Homo-DimerF			Heterodimer			Differences Between TmF and TmR				Differences Between sTa and aTa						Clinisen
Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	Less than -9	Higher than -9	Total	5-UP	2.1-5	0-2	Total	Not reported	10.1-UP	5.1-10	3.1-5	0-3	
46	25 (54.4%)	21 (45.6%)	46	11 (23.9%)	35 (76.1%)	46	16 (34.8%)	30 (65.2%)	46	3 (6.5%)	17 (37%)	26 (56.5%)	46	4 (8.7%)	4 (8.7%)	9 (19.6%)	4 (8.7%)	25 (54.3%)	80.1-100
15	8 (53.3%)	7 (46.7%)	15	7 (46.6%)	8 (53.4%)	15	4 (26.6%)	11 (73.4%)	15	1 (6%)	6 (40%)	8 (54%)	15	2 (13.3%)	2 (13.3%)	2 (13.3%)	5 (33.4%)	4 (26.7%)	50.1-80
3	2 (66.7%)	1 (33.3%)	3	0	3 (100%)	3	1 (33.3%)	2 (66.7%)	3	0	1 (33.3%)	2 (66.7%)	3	2 (66.7%)	0	0	0	1 (33.3%)	0-50
34	16 (47%)	18 (53%)	34	11 (32.4%)	23 (67.6%)	34	10 (29.5%)	24 (70.5%)	34	1 (3%)	8 (23.5%)	25 (73.5%)	34	2 (6%)	1 (3%)	8 (23.5%)	10 (29.4%)	13 (38.1%)	Not reported
98	51	47	98	29	69	98	31	67	98	5	32	61	98	10	7	19	19	43	Total
-				-			-				-					$P \leq 0.05$			*P*-value

Note. Clinisen: Clinical sensitivity; sTa: Suggested T annealing; aTa: Applied annealing temperature; TmF: Melting temperature of forward; TmR: Melting temperature of reverse.

there were 66 female patients (35.5%) and 120 male patients (64.5%). All samples underwent digestion and decontamination, followed by the direct smear, GeneXpert, and culture and DNA extraction for PCR assays.

Among these samples, 53 (28.5%) tested positive in both the direct smear and GeneXpert tests, while 133 (71.5%) tested negative. In the culturing process, 51 samples (27.5%) tested positive, while 135 samples (72.5%) were negative (Table 4).

Polymerase Chain Reaction Assay for the Detection of IS6110 and rpoB Genes

Out of 186 DNA samples extracted from the sputum, 77 (41.4%) tested positive using the Nimesh IS6110 primer pair, whereas 109 (58.6%) tested negative (Figure 2A). Using the Kim primer pair, 81 samples (43.5%) tested positive, while 105 (56.5%) tested negative (Figure 2B). Similarly, the Kox study primer pair showed that 79 samples (42.5%) tested positive, whereas 107 (57.5%) tested negative (Figure 2C).

Additionally, the Gouvenia study primer pair indicated that 77 samples (41.4%) tested positive, while 109 samples (58.6%) tested negative (Figure 2D). The PCR method using the Nimesh *rpoB* primer pair demonstrated that 67 samples (36%) tested positive, whereas 119 (64%) tested negative (Figure 3).

In the PCR method, using the Alvandi *IS6110* primer pair, 78 (41.9%) and 108 (58.1%)

samples tested positive and negative, respectively. Additionally, using the Alvandi *rpoB* primer pair, 69 samples (37.1%) tested positive, while 117 samples (62.9%) tested negative. A summary of the diagnostic value test results is presented in Table 5.

Discussion

The rapid and accurate identification of MTB infections is essential for effective clinical management of the disease. Moreover, adequate time to prescribe the appropriate antibiotic, contact precautions, and preventive measures are effective strategies that stem from the rapid identification of MTB infections (18). Traditional diagnostic methods for MTB infections typically involve preparing a smear from suitable samples, such as sputum, and employing acid-fast staining techniques, such as a carbol fuchsin (Ziel-Nelson method) or fluorochrome dyes (auramine-rhodamine method) (19,20).

Although acid-fast staining is a cost-effective, rapid, and straightforward method that provides high specificity, it suffers from low sensitivity, which presents a diagnostic challenge (20,21). Furthermore, cultivation on specific media (e.g., Lowenstein-Jensen and Middlebrook media) is still recognized as the gold standard for the diagnosis of MTB. However, this method is constrained by its lengthy operational duration, which can extend for several weeks, and by the requirement for specialized laboratory infrastructure that is usually available only in reference centers (21).

Molecular techniques that detect MTB DNA (e.g., PCR and qPCR) have been adopted for diagnosing MTB. They offer high sensitivity and specificity, thereby enabling the rapid analysis of numerous samples (22,23).

The direct detection of MTB in infectious samples (e.g., sputum) is becoming increasingly preferred. Different studies have investigated the sensitivity and specificity of diagnosing MTB using molecular techniques that target various genes. An important factor influencing the sensitivity and specificity of these methods is the use of appropriate primers. PCR is an effective technique for diagnosing MTB due to its simplicity, lower cost, and greater accessibility (22,24).

The current study analyzed 186 sputum samples from patients suspected of having TB. The samples were initially assessed using smear microscopy, the GeneXpert method, and culture. Additionally, PCR tests were performed using various primers designed or selected based on previous studies. The selected primers were chosen through a comprehensive *in silico* screening process and were subsequently compared *in vitro*.

The average age of the suspected TB patients was 46 years, with 64.5% men and 35.5% women. Among these samples, 53 (28.5%) tested positive in both the smear and GeneXpert tests, while 133 (71.5%) were negative. In the culture results, 51 (27.5%) samples were positive, whereas 135 (72.5%) were negative.

As previously mentioned, a total of 186 sputum samples from patients suspected of having TB were analyzed in this study. Among these samples, 53 (28.5%) tested positive in both the smear and GeneXpert tests, while 133 (71.5%) were negative.

After performing PCR analysis using the selected *IS6110* primers on the 186 extracted DNA from the sputum samples, the highest positivity rate of 43.5% was obtained with primers from the study by Kim et al (25). Additionally, the primers from the studies by Kox et al (16), Nimesh et al (15), and Gouveia et al (17) identified 42.5%, 41.4%, and 41.4% of sputum samples as positive for TB, respectively. In comparison, our designed primer pair identified 41.9% of sputum samples as positive for TB. In addition, 36% of the suspected TB samples tested positive using the *rpoB* primer pair from the study by Nimesh et

Table 4. Frequency of Results Based on the Intended Tests

Methods	Results	Frequency (%)	Total
Smear	Positive	53 (28.5)	186 (100%)
	Negative	133 (71.5)	
GeneXpert	Positive	53 (28.5)	
	Negative	133 (71.5)	
Culture	Positive	51 (27.4)	
	Negative	135 (72.6)	

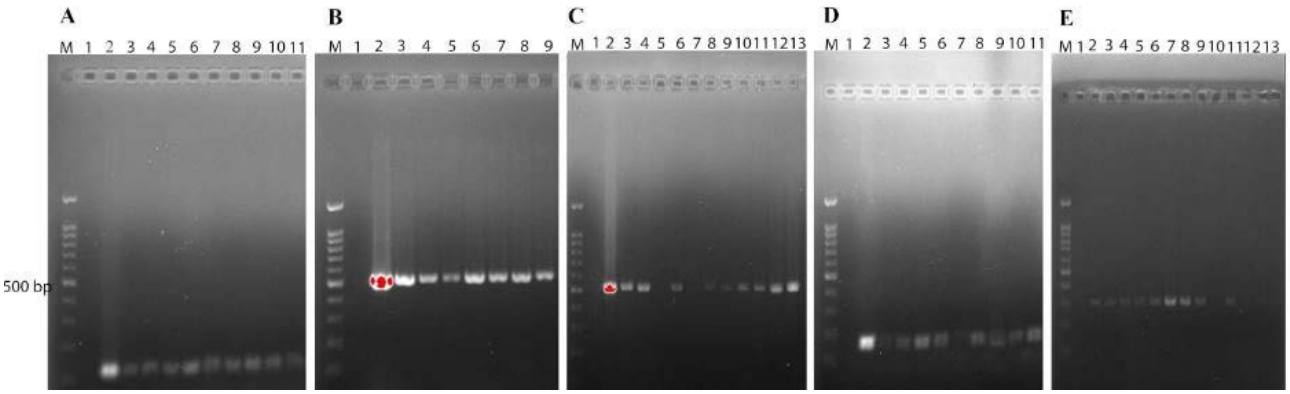

Figure 2. Gel Electrophoresis of PCR Products Amplified Using the *IS6110* Gene of *M. tuberculosis*. *Note*. PCR: Polymerase chain reaction; *M. tuberculosis*: *Mycobacterium tuberculosis*; M: 100 bp ladder; 1: Negative control; 2: Positive control; A: Nimesh primer pair; 3 to 11: Clinical samples; B: Kim primer pair; 3 to 9: Clinical samples; C: The Kox primer pair; 3 to 13: Clinical samples; D: The Gouvenia primer pair; 3 to 11: Clinical samples; E: PCR products amplified with the Alvandi primer pair for the *IS6110*; 3 to 13: Clinical samples

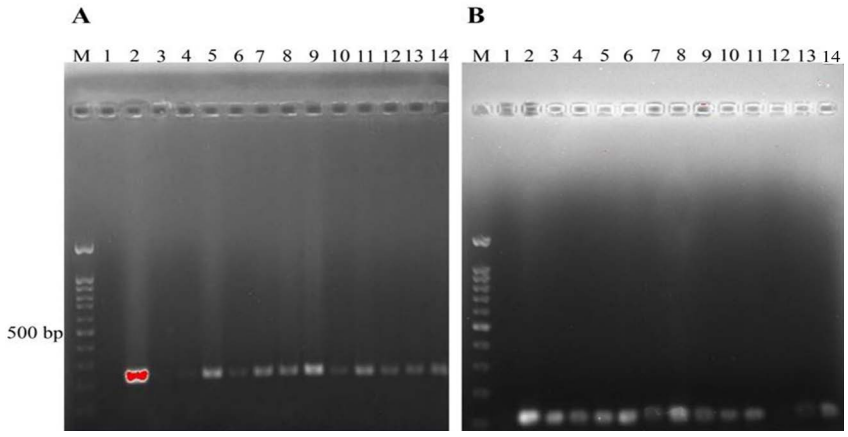

Figure 3. Gel Electrophoresis of PCR Products Amplified Using the *rpoB* Primer. *Note*. PCR: Polymerase chain reaction; M: 100 bp ladder; 1: Negative control; 2: Positive control; A: PCR products amplified using the Nimesh *rpoB* primer pair; B: PCR products amplified using the Alvandi *rpoB* primer pair

Table 5. Diagnostic Value Criteria for the PCR Tests With Selected and Designed Primer Pairs

		Primer Name						
		Nimesh IS6110	**Kim IS6110**	**Kox IS6110**	**Gouvenia IS6110**	**Alvandi IS6110**	**Nimesh rpoB**	**Alvandi rpoB**
Result No. (%)	Positive	77 (41.4)	81 (43.5)	79 (42.5)	77 (41.4)	78 (41.9)	67 (36)	69 (37.1)
	Negative	109 (58.6)	105 (56.5)	107 (57.5)	109 (58.6)	108 (58.1)	119 (64)	117 (62.9)
Sensitivity % (CI 95%)		91.07 (80.38-97.04%)	91.07 (80.38-97.04%)	87.50 (75.93-94.82%)	87.50 (75.93-94.82%)	96.3 (89.56-99.23%)	86.15 (75.34-93.47%)	89.29 (78.12-95.97%)
Specificity % (CI 95%)		80.00 (72.08-86.50%)	76.92 (68.72-83.86%)	76.92 68.72-83.86%)	78.46 (70.40-85.19%)	80.77 (72.93-87.15%)	84.62 (77.25-90.34%)	85.38 (78.12-90.97%)
Accuracy % (CI 95%)		83.33% (77.19-88.39%)	81.18% (74.81-86.53%)	80.11% (73.64-85.95%)	81.18% (73.64-85.59%)	84.95% (78.98-89.76%)	84.41% (78.33-89.30%)	86.56% (80.80-91.11%)
Kappa coefficient		0.642	0.603	0.577	0.596	0.678	0.649	0.700
Kappa coefficient interpretation (agreement)		Good	Moderate	Moderate	Moderate	Good	Good	Good
Concordance %		64.2	60.3	57.7	59.6	67.8	64.9	70
Diagnostic odds ratio		40.8	34	23.33	25.5	74.2	28.72	48.68

Note. PCR: Polymerase chain reaction; CI: Confidence interval.

al, while our developed *rpoB* primer pair displayed that 37.1% of the samples were positive.

Sensitivity and specificity are critical factors in evaluating diagnostic methods for diseases. In this study, the molecular identification of suspected TB sputum samples using the designed *IS6110* primers demonstrated a sensitivity of 94.6% and a specificity of 80.8%, which outperformed the other tested primers. The detection using the designed *rpoB* primers exhibited a sensitivity of 85.3% and a specificity of 85.4%, surpassing the primers from the study performed by Nimesh et al, which had a sensitivity of 83.9% and a specificity of 84.6%.

To the best of our knowledge, no *in silico* and *in vitro* study, like our study, has so far evaluated multiple primers, including the specifically designed ones, for identifying TB (26,27).

Henriques et al conducted a study on vaginal bacteria using the PCR method. They emphasized that the *in silico* specificity of primers differs from their *in vitro* specificity. The researchers developed primers with high specificity and sensitivity and compared their results with those from earlier studies. Their findings highlighted the importance of *in silico* analysis in predicting *in vitro* specificity, indicating that conducting *in silico* assessments before *in vitro* experiments can lead to noticeably improved outcomes (28).

One of the recognized target genes used for TB detection through PCR analysis is the *IS6110* gene, which has been the focus of investigation in several studies (15,16,25). The sensitivity and specificity of these primers exceed those of many primers employed in previous research (29).

The *rpoB* gene has also been utilized in other studies for the identification of TB (30-32). The sensitivity and specificity of the PCR technique detecting the *rpoB* gene ranged from 11.3% to 31.2% and from 69% to 100%, respectively (32), which were lower than those observed in our study.

The evaluation of different primers for *IS6110* and *rpoB* genes revealed variable diagnostic performances in terms of sensitivity, specificity, and overall accuracy. Among the *IS6110*-based primers, the Alvandi *IS6110* primer pair demonstrated the highest sensitivity (96.3%) and acceptable agreement ($\kappa = 0.678$), underscoring its potential utility in reliably detecting positive cases. In contrast, primers such as Kim *IS6110* and Kox *IS6110* showed comparatively lower accuracy and moderate

agreement, which may limit their diagnostic reliability in routine practices.

When comparing *rpoB* primers, the Alvandi *rpoB* assay outperformed the Nimesh *rpoB* in terms of both accuracy (86.56% vs. 84.41%) and concordance (70% vs. 64.9%), with the highest kappa value (0.700, acceptable agreement). These findings suggest that the Alvandi *rpoB* primer provides a more consistent and robust diagnostic tool.

Notably, the diagnostic odds ratio (DOR) further emphasizes these differences. The Alvandi *IS6110* displayed the strongest association between test results and true disease status (DOR = 74.2), followed by Alvandi *rpoB* (DOR = 48.68). This indicates that primers designed by Alvandi consistently achieve superior discriminative power compared to other evaluated assays.

From a clinical perspective, high-sensitivity primers are especially valuable in initial diagnostic screening, where missing cases can have serious public health consequences. Conversely, primers with higher specificity may reduce the risk of overdiagnosis and unnecessary treatment, thereby making them preferable for confirmatory testing. The integration of both types (e.g., in a multiplex PCR assay) may, therefore, provide an optimal diagnostic approach. Future research should explore combined or multiplex strategies to maximize diagnostic yield and should also address practical considerations (e.g., cost, turnaround time, and scalability) in routine clinical laboratories.

One limitation of *IS6110*-targeting assays is the variability in copy number across different MTB strains, particularly in certain geographic regions where strains may harbor few or no copies of the element. This factor may reduce the generalizability of results and warrants validation in diverse epidemiological settings.

Conclusion

In summary, the findings demonstrated that while several *IS6110* and *rpoB* primers could provide acceptable diagnostic performance, the Alvandi *IS6110* and *rpoB* primers had higher sensitivity, accuracy, and agreement levels. These primers, particularly Alvandi *IS6110*, may, therefore, represent the most reliable options for diagnostic applications. Accordingly, PCR using these primers can be highly beneficial for the diagnosis and management of TB in low-resource settings. It is recommended that future studies use larger and more diverse populations to validate these findings and explore their integration into clinical diagnostic workflows.

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Competing Interests

The authors declare they have no conflict of interests.

Ethical Approval

This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (and ethical code IR.KUMS.REC.1396.427).

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Supplementary files

Supplementary file 1 contains Table S1.

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