ORIGINAL ARTICLE

Comparison of False Positive Rates and Invalid Results between Automated and Manual Nucleic Acid Extraction Methods for Real-Time PCR Detection of *Mycobacterium tuberculosis*

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Background: High volumes of formalin-fixed paraffin-embedded (FFPE) samples are processed for *Mycobacterium tuberculosis* (MTB) real-time polymerase chain reaction (RT-PCR) testing, making manual DNA extraction prone to human error and cross-contamination. Automated nucleic acid extraction offers a more efficient alternative.

Objective: To compare false positive rates, invalid results, DNA yield, and purity between automated and manual DNA extraction methods for MTB RT-PCR.

Materials and Methods: One thousand three hundred eighteen FFPE samples were evaluated by a pathologist for tissue reactions and classified into histologic scores. Scores of 0, 1, 2, and 3 indicated no reaction, non-specific inflammation, non-necrotizing granuloma or caseous necrosis without granuloma, and necrotizing granuloma, respectively. Of these, 767 (58.19%) underwent manual extraction, and 551 (41.81%) underwent automated extraction. RT-PCR was performed to detect MTB, with false positives identified by reviewing PCR-positive samples that did not align with the histological scores. False positivity due to cross-contamination was confirmed if a repeat PCR test, performed on newly extracted DNA, yielded a negative result. DNA yield and purity were compared between the methods using a Mann-Whitney U test.

Results: False positive rates were 1.69% for manual extraction and 0.91% for automated extraction, with invalid result rates of 2.09% and 3.27%, respectively. The manual method yielded higher median (IQR) DNA concentration and yield than the automated method at 334.60 (113.00 to 862.20) versus 120.80 (30.40 to 382.60) ng/μL and 10,038.00 (3,390.00 to 25,866.00) versus 6,040.00 (1,520.00 to 19,130.00) ng, respectively. DNA purity was also higher with the manual method with A260/A230: 2.22 (2.12 to 2.27) versus 2.02 (1.39 to 2.23), A260/A280: 1.94 (1.90 to 1.97) versus 1.90 (1.85 to 1.93). All differences were statistically significant ($p<0.0001$).

Conclusion: Automated nucleic acid extraction reduced false positive rates by 0.78% but increased invalid result rates by 1.18%. It yielded lower DNA yield and purity compared to manual extraction. Despite these limitations, automation remains a practical option for high-throughput processing, offering substantial time and resource savings with manageable invalid result rates.

Keywords: Automated nucleic acid extraction; Manual nucleic acid extraction; False positive rates; Invalid results; RT-PCR testing; *Mycobacterium tuberculosis*; FFPE samples

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Mycobacterium tuberculosis (MTB) infection is a prevalent infectious disease in Thailand, with an incidence rate of 153 cases per 100,000 population[1]. Consequently, granulomatous inflammation in surgical resection specimens processed as formalin-fixed paraffin-embedded (FFPE) blocks is frequently encountered and often associated with MTB infection. Despite this association, granulomatous inflammation can also result from other infectious and non-infectious causes[2].

MTB detection from FFPE blocks is typically performed using Ziehl-Neelsen staining and real-time polymerase chain reaction (RT-PCR). However, the sensitivity of Ziehl-Neelsen staining is low at 37.0%. While culture remains the gold standard for pathogen identification, FFPE specimens are unsuitable for this method. Consequently, RT-PCR provides a faster, more sensitive alternative, with a 74.6% sensitivity,

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while maintaining high specificity of 98.5%[3].

At the authors' institution, over 1,000 RT-PCR tests for MTB detection in FFPE specimens are conducted annually. Due to the high volume of samples, manual DNA extraction is labor-intensive, which increases the risk of human error and cross-contamination. In contrast, automated DNA extraction offers advantages, including higher throughput, reduced hands-on time, elimination of variability caused by human error, and a minimized risk of contamination[4-7]. However, studies have shown that automated extraction may result in lower DNA quality and quantity than manual extraction[8,9].

Due to the lack of comparative data on positive and invalid results between the two nucleic acid extraction methods, the present study aimed to determine whether automated DNA extraction could reduce false positives in RT-PCR detection of MTB caused by cross-contamination, which was particularly important since false positive results could negatively affect patients by leading to unnecessary treatment. Since invalid result rates might be associated with DNA quality, the present study would also examine the rate of invalid results, compare DNA yield and purity between the methods, and explore the relationship between tissue reaction and RT-PCR outcomes.

Materials and Methods

Histological evaluation

One thousand three hundred eighteen FFPE samples submitted for RT-PCR detection of MTB were examined for tissue reactions by a single pathologist (PP) before RT-PCR analysis, and the pathologist was blinded to the molecular test results. The reactions were categorized using a four-tier histologic scoring system to assess their concordance with MTB infection with no inflammation as a score 0, non-specific inflammation as score 1, non-necrotizing granuloma or caseous necrosis without granuloma as score 2, and necrotizing granuloma as score 3, as outlined in Table 1. This histologic assessment was used as a practical reference to identify potential false positive RT-PCR results in routine diagnostic practice.

DNA extraction, concentration, yield, and purity analysis

Among the total samples, 767 (58.19%), underwent DNA extraction using the manual QIAamp® DNA FFPE Tissue Kit (QIAGEN, Germany), while 551 (41.81%) were processed using the automated QIAsymphony® SP instrument with the QIAsymphony® DSP DNA Mini Kit (QIAGEN, Germany), as shown in Table 1. DNA extraction was carried out according to the manufacturers' protocols. DNA concentration and purity from both methods were assessed using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc., United States).

DNA was extracted in volumes of 30 µL for the manual method and 50 µL for the automated method. The DNA yield was calculated by multiplying the concentration by the volume. Purity was assessed based on absorbance ratios at 260 to 280 nm (A260/A280) and 260/230 nm (A260/A230). An A260/A280 ratio of approximately 1.8 was considered acceptable, while an A260/A230 ratio between 2.0 and 2.2 was regarded as optimal.

RT-PCR detection of MTB

Samples with high DNA concentrations were

Table 1. Sample characteristics

Variables	n (%)
Total samples	1,318
Manual method	767 (58.19)
Automated method	551 (41.81)
Samples underwent one repeat RT-PCR	107 (8.12)
Manual method	62 (4.70)
Automated method	45 (3.41)
Samples underwent two repeat RT-PCR	20 (1.52)
Manual method	5 (0.38)
Automated method	15 (1.14)
Histologic scores	
0: No inflammation (unlikely)	275 (20.86)
1: Non-specific inflammation (unlikely)	233 (17.68)
2: Non-necrotizing granuloma or caseous necrosis without granuloma (possible)	309 (23.44)
3: Necrotizing granuloma (suspicious)	501 (38.01)
Initial RT-PCR results	
Invalid	34 (2.58)
Negative	929 (70.49)
Positive	355 (26.93)
Results of the first repeat RT-PCR	
Invalid	0 (0.00)
Negative	97 (76.38)
Positive	30 (23.62)
Results of the second repeat RT-PCR	
Invalid	0 (0.00)
Negative	14 (70.00)
Positive	6 (30.00)

RT-PCR=real-time polymerase chain reaction

diluted to 500 ng/µL before RT-PCR to be used as working DNA. The working DNA was used in RT-PCR detection of MTB with the Anyplex™ MTB/NTM Real-time Detection V2.0 kit (Seegene Inc., Korea), targeting the IS6110 and mpb64 genes, and run on the CFX96 IVD Real-Time PCR System (Bio-Rad Laboratories, Inc., United States). Results were interpreted according to the kit's specifications. If the initial RT-PCR result was invalid, as indicated by failure of internal control to amplify, the working DNA was further diluted, typically by a one-to-two ratio, to reduce the concentration of PCR inhibitors and enhance reaction efficiency. In cases where the initial working DNA concentration was below 100 ng/µL, a 9:10 dilution was performed prior to repeating RT-PCR.

Detection of false positives

Samples that tested positive by RT-PCR but exhibited histological features inconsistent with MTB infection, with a histological score of 0 or 1, underwent repeat RT-PCR for confirmation. Since cross-contamination of the original DNA was suspected, new DNA extraction was performed prior to the repeat RT-PCR to prevent contamination. If the repeat test resulted in a negative outcome, the initial positive result was classified as a false positive, due to cross-contamination.

Statistical analysis

Normality of continuous variables was assessed using the Shapiro-Wilk test. DNA concentration, yield, and purity between the two extraction methods were compared using the Mann-Whitney U test due to non-normal data distribution. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 10.0.2 (GraphPad Software Inc., United States).

Ethical approval

The present study was approved by the Institutional Review Board (Institute of Pathology, Thailand; IOP-KM-R67-004). The present study was a retrospective study, and informed consent from each participant was not required.

Results

The occurrence of false positives in RT-PCR detection of MTB was observed in 13 samples (1.69%) using the manual method, compared with five samples (0.91%) with the automated method. This represented a reduction of 0.78% in false positives with the automated method. In contrast, invalid results were recorded in 16 samples (2.09%) for the manual method and 18 samples (3.27%) for the automated method, indicating a 1.18% increase in invalid results with the automated approach (Table 2).

Table 2. Comparison of the occurrence of invalid and false positive results between manual and automated nucleic acid extraction methods

	Manual method n (%)	Automated method n (%)	Total n (%)
Invalid	16 (2.09)	18 (3.27)	34 (2.58)
False positive	13 (1.69)	5 (0.91)	18 (1.37)

One hundred twenty-seven samples required repeat RT-PCR, of which 34 samples (2.58%) initially yielded invalid results. After dilution of the working DNA, as described previously, and repetition of RT-PCR, all these samples were successfully processed without invalid results (Table 1).

Table 3 presents the DNA concentration and yield obtained using both methods. The manual method yielded a median DNA concentration of 334.60 ng/µL (IQR 113.00 to 862.20), while the automated method showed a median concentration of 120.80 ng/µL (IQR 30.40 to 382.60). The median DNA yield from the manual method was 10,038.00 ng (IQR 3,390.00 to 25,866.00), compared with 6,040.00 ng (IQR 1,520.00 to 19,130.00) obtained using the automated method.

DNA purity, as measured by the A260/A230 ratio, showed a median value of 2.22 (IQR 2.12 to 2.27) for the manual method and 2.02 (IQR 1.39 to 2.23) for the automated method. The median A260/A280 values were 1.94 (IQR 1.90 to 1.97) for the manual method and 1.90 (IQR 1.85 to 1.93) for the automated method. All comparisons between the manual and automated extraction methods were statistically significant (all $p<0.0001$).

Tissue reactions correlated with MTB detection rates (Table 4). Samples with necrotizing granulomas (score 3) had a PCR-positive rate of 50.10%, while those with non-necrotizing granulomas or caseous necrosis without granulomas (score 2) showed a positive rate of 23.30%. Samples exhibiting non-specific inflammation (score 1) demonstrated a positive rate of 9.01%, and those with no tissue reaction (score 0) had a positive rate of 4.00%. The PCR-positive rate increased with higher histological scores, as indicated by these data.

Table 3. Comparison of the concentration, yield, and purity of DNA extracted by manual and automated nucleic acid extraction methods

	Manual method; median (IQR)	Automated method; median (IQR)	p-value
DNA concentration (ng/μL)	334.60 (113.00 to 862.20)	120.80 (30.40 to 382.60)	<0.0001
DNA yield (ng)	10,038.00 (3,390.00 to 25,866.00)	6,040.00 (1,520.00 to 19,130.00)	<0.0001
A260/A230	2.22 (2.12 to 2.27)	2.02 (1.39 to 2.23)	<0.0001
A260/A280	1.94 (1.90 to 1.97)	1.90 (1.85 to 1.93)	<0.0001

IQR=interquartile range

Comparisons between groups were performed using the Mann-Whitney U test, $p<0.05$ was considered statistically significant.

Table 4. The correlation between histologic score and initial RT-PCR results

Histologic scores	Invalid; n (%)	Negative; n (%)	Positive; n (%)
0	9 (3.27)	255 (92.73)	11 (4.00)
1	7 (3.00)	205 (87.98)	21 (9.01)
2	5 (1.62)	232 (75.08)	72 (23.30)
3	13 (2.59)	237 (47.31)	251 (50.10)

Discussion

The authors' study demonstrated that the automated DNA extraction method reduced the false positive rate by 0.78%, but it also resulted in a 1.18% increase in the rate of invalid results. The small reduction in the false positive rate observed in the present study may be attributable to normal variability inherent to FFPE-based MTB RT-PCR testing. In addition, the reported false positive cases reflect only detectable incidents and may not capture all false positive events, which should be considered when interpreting these findings. False positives may also arise from steps outside the automated process, including sectioning, deparaffinization, pipetting before sample loading, DNA dilution, and mixing DNA with the master mix prior to RT-PCR. Furthermore, cross-contamination during automated DNA extraction may occur because of aerosol formation, improper instrument movement, or technical malfunction[7,10].

All initially invalid samples became valid after diluting the working DNA and repeating the RT-PCR assay. This finding suggests that dilution effectively reduces PCR inhibitors, thus resolving invalid results. In a study by McKee et al., a 10-fold dilution was found to reduce PCR inhibition effectively[11]. The authors' study demonstrated that DNA extracted using the automated method exhibited significantly lower purity compared to the manual method. Moreover, the mean A260/A230 ratio for the automated method fell below the acceptable range of 2.0 to 2.2. Only three samples showed negative absorbance values, which are more likely attributable to true sample-to-sample variability rather than systematic measurement error. This lower purity indicates the presence of PCR inhibitors, which contributed to the increased occurrence of invalid results.

A likely PCR inhibitor in the present study is guanidine thiocyanate, a component of the automated DNA extraction reagents. This compound exhibits strong absorbance at approximately 230 nm, leading to a reduced A260/A230 ratio compared with guanidine hydrochloride used in the manual extraction kit at equivalent concentrations. In addition, guanidine thiocyanate is known to inhibit Taq polymerase activity. Nevertheless, despite a low A260/A230 ratio, the extracted DNA often remains suitable for RT-PCR analysis and can be further improved by additional chloroform purification or sample dilution to reduce inhibitor concentration[12].

In addition to DNA purity, DNA yield is equally important. PCR requires a sufficient amount of templated DNA to initiate and sustain the amplification process. Insufficient DNA may lead to failed amplification or false negatives[13]. Thus, ensuring an adequate DNA yield is essential for providing enough template for the PCR reaction to proceed effectively. A study by Riemann et al. on nucleic acid extraction from whole-blood samples found that manual extraction yielded significantly higher quality and quantity of DNA compared to the automated method[8]. Similarly, a study by Domínguez-Vigil et al. on liquid-based cytology samples found that the automated method produced lower DNA quantities[9].

Despite the reduction in false positives and the increase in invalid results with the automated method, these changes may not be significant when considering the overall benefits, such as reduced hands-on time. As shown in Table 5, laboratories processing up to 24 samples per run can reduce hands-on time by up to eightfold using the automated method, enabling scientists to allocate time to other tasks. This method also reduces the total time per run by 20 minutes, although the cost per sample

Table 5. Comparison of time and cost between manual and automated nucleic acid extraction methods

	Manual method	Automated method
Maximum number of sample/run	12	24
Total time/run (minutes)	40	60
Total time/24 samples (minutes)	80	60
Hand-on time/run (minutes min)	40	10
Hand-on time/24 samples (minutes)	80	10
Cost/sample (THB)	214	250

increases by 36 THB. Riemann et al. also found that although automated DNA extraction required less hands-on time, it was associated with higher costs[8]. Nevertheless, the automated method is cost-effective and well-suited for laboratories processing high volumes of samples.

Histological evaluation plays a crucial role in determining whether there is sufficient tissue for RT-PCR and in minimizing false positives, especially in cases where the tissue reaction is inconsistent with MTB infection. The authors' study showed that samples without tissue reaction or those exhibiting non-specific inflammation had RT-PCR positivity rates of only 4.00% and 9.01%, respectively. Thus, to ensure that the DNA used for RT-PCR is free from cross-contamination, it is essential to perform new DNA extraction before conducting repeat RT-PCR to confirm the results in such cases.

The present study found that RT-PCR positivity rates were as high as 50.10% and 23.30% for histologic scores of 3 and 2, respectively. In comparison, a study by Lee et al. reported that samples exhibiting histological features of MTB demonstrated an RT-PCR positivity rate of 65.8%, with a sensitivity of 74.6%. In contrast, AFB staining had a markedly lower sensitivity of only 37.0%[3]. Therefore, in cases of suspicious tissue reactions or reactions consistent with MTB infection but negative AFB staining, RT-PCR can significantly enhance diagnostic sensitivity.

A limitation of the present study is that cycle threshold (Ct) values were not analyzed or reported. As the assay was used as a qualitative test according to the manufacturer's instructions, interpretation of weak-positive results was limited. Without Ct values, it is difficult to distinguish true low-level positivity from borderline detections. In addition, histologic score assessment in the present study was not subjected to interobserver validation, which may introduce observer-related variability.

Conclusion

Automated DNA extraction offers a modest reduction in the false positive rates for MTB RT-PCR compared to manual extraction. However, it is associated with a slight increase in the invalid result rate, which is manageable through strategies such as DNA dilution to overcome PCR inhibition. Furthermore, automated extraction typically yields DNA with lower quantity and purity than manual methods. This reduced purity contributes to the higher rate of initial invalid results observed with automation. While manual extraction is time-consuming, particularly for high-throughput settings, automation significantly reduces hands-on time, albeit at a higher per-sample cost. Therefore, automated nucleic acid extraction presents a practical and efficient alternative for laboratories processing large volumes of FFPE samples for MTB detection, balancing a small increase in potentially resolvable invalid results with substantial savings in time and labor.

What is already known about this topic?

Manual DNA extraction, which requires extensive hands-on processing, is prone to human error, and carries a higher risk of cross-contamination, whereas automated extraction offers reduced manual processing time and improved workflow efficiency.

What does this study add?

This study demonstrates that automated DNA extraction modestly reduces false positive rates in MTB RT-PCR detection compared with the manual method but leads to a slightly higher frequency of invalid results. Importantly, invalid results were correctable through DNA dilution, indicating that automated extraction remains practical for high-throughput laboratories.

Authors' contributions

PP conceptualized and designed the study, developed the methodology, performed the statistical analysis, evaluated the histologic scores, drafted the manuscript, reviewed and edited the manuscript, and supervised the study. SP performed the laboratory analyses and interpreted the laboratory results. CU was responsible for data collection. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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