Letter to the Editor

The Retrospective Diagnostic Potential of GeneXpert MTB/RIF for the Analysis of Formalin-Fixed Paraffin-Embedded Tissue from Extrapulmonary Tuberculosis Patients*

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Tuberculosis (TB) is a chronic disease caused by infection with *Mycobacterium tuberculosis*. Laboratory confirmation of this infection is challenging due to the paucibacillary nature of extrapulmonary tuberculosis (EPTB). Bacteriological confirmation of EPTB generally requires collection of invasive specimens using needle aspiration biopsy to allow histopathological analysis, *M. tuberculosis* culture, smear microscopy, and molecular testing. *M. tuberculosis* culture remains the gold standard for a definitive diagnosis; however, its turnaround time is approximately two to four weeks. The GeneXpert MTB/RIF system has comparable sensitivity to *M. tuberculosis* culture and can be used to determine EPTB diagnosis from fresh specimens[1].

Compared with formalin-fixed paraffin-embedded (FFPE) tissues, the direct use of fresh tissue carries certain biosafety risks and is also not suitable for laboratories with limited resources. Using histological analysis as a reference for diagnosis, prior studies have reported that the use of the GeneXpert MTB/RIF assay for FFPE tissues had variable sensitivities[2]. In our study, the gold standard was established as a TB diagnosis based on clinical, radiological, microbiological, pathological, and therapeutic criteria. The case definition for TB in this study was determined as any of the following: 1) a positive *M. tuberculosis* culture result; 2) any two positive diagnostic tests from three methods (staining, pathological, and radiological); 3) a single positive test with clinical suspicion; or 4) a single positive test with a positive outcome on treatment.

FFPE tissues are non-infectious and have better long-term preservation of cellular structure, making them useful for retrospective studies. In this study, we assessed the potential for GeneXpert MTB/RIF in the diagnosis of TB using FFPE tissue samples taken from clinically diagnosed EPTB patients.

Sixty-four archival FFPE tissue samples from EPTB patients were taken between February 2019 and June 2021. The DNA extraction from FFPE tissues was conducted using a commercially available TaKaRa DEXPAT Easy DNA kit (Takara Bio Inc, Shiga, Japan) in accordance with the manufacturer's instructions[3]. The paraffin-embedded tissue was cut to a thickness of 5 μm; then, three sections were placed into 1.5 mL microtube with sterilized tweezers. The size of the embedded tissue samples used in this study were determined to be at minimum of 6 mm × 6 mm. The DEXPAT reagent was mixed upside down and 0.5 mL (approximately 20 drops) of this reagent was added into each microtube containing a tissue section. The microtubes were mixed upside down to prevent the resin from settling when the DEXPAT reagent was added. The microtubes were covered and heated at 100 °C for 10 min; then, the microtubes were centrifuged at 12,000 rpm at 4 °C for 10 min immediately after heating. Next, the supernatant layer was removed with a pipette, avoiding absorption of the paraffin film, resin, and tissue residue. The supernatant layer could then be used directly as a DNA template for PCR reactions. Samples with 0.5 mL supernatant alongside 1.5 mL

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of sterile phosphate-buffered saline (pH 6.8) or the referenced sample reagent were transferred to GeneXpert MTB/RIF cartridges. The cartridges were then loaded into the GeneXpert MTB/RIF instrument, and the corresponding assay was performed automatically by the instrument and associated software. The final test results were obtained approximately 2 hours later. Tissue specimens were analyzed using the standard four-module GeneXpert MTB/RIF instrument with automated readout. Among 64 FFPE tissues, the GeneXpert MTB/RIF assay detected 37 *M. tuberculosis* positive samples, corresponding to 57.81% (37/64). In the semi-quantitative test on *M. tuberculosis* levels, 17 showed "EXTREMELY LOW", 18 showed "LOW" or "MEDIUM", and only two specimens showed "HIGH". One specimen was determined to possess rifampicin resistance, indicated by "Rif Resistance", whereas 27 were "Rif Resistance Not Detected", and 9 were "INDETERMINATE" (Table 1). The specimens that were both positive in acid-fast staining and negative in GeneXpert MTB/RIF testing were utilized in subsequent analysis for nontuberculous *mycobacterial* (NTM) identification.

Next, we utilized 30 random normal tissue samples as negative controls to analyze the diagnostic power of GeneXpert testing. The corresponding GeneXpert analysis of the 64 FFPE tissues from clinically diagnosed EPTB patients and the 30 normal tissues were as follows: 37 "true positives", 30 "true negatives", "zero false positives", and 27 "false negatives". The sensitivity, specificity, positive predictive value, and negative predictive value of this GeneXpert analysis were 57.81% (95% *CI:* 44.82%–70.06%), 100% (95% *CI:* 88.43%–100.00%), 100%, and 52.63% (95% *CI:* 45.48%–59.68%), respectively (Table 2).

Table 1. The GeneXpert MTB/RIF results of 64 formalin-fixed paraffin-embedded tissues of EPTB

Category	GeneXpert readouts	Outcomes
Mtb detection	*Mtb* detected	37
	Mtb not detected	24
	Invalid*	3
Mtb semiquantitative analysis	Extremely low	17
	Low	9
	Medium	9
	High	2
Rif resistance	Rif resistance	1
	Rif resistance not detected	27
	Indeterminate	9

Note. EPTB: extrapulmonary tuberculosis; *Mtb*: *Mycobacterium* tuberculosis; Rif: Rifampin; *The results were *Mtb* not detected ultimately after repeated twice.

Table 2. Diagnostic performance of the GeneXpert MTB/RIF on formalin fixed paraffin embedded tissues (*N* = 94)

Category	TP	TN*	FP	FN	SE, % (95% *CI*)	SP, % (95% *CI*)	PPV (%)	NPV, % (95% *CI*)
Lymph nodal tissues (*n* = 25)	6	15	0	4	60 (26.24–87.84)	100 (78.20–100.00)	100	78.95 (63.71–88.90)
Non-lymph nodal tissues (*n* = 69)	31	15	0	23	57.41 (43.21–70.77)	100 (78.20–100.00)	100	39.47 (32.36–47.06)
Total	37	30	0	27	57.81 (44.82–70.06)	100 (88.43–100.00)	100	52.63 (45.48–59.68)

Note. TP: True Positive, TN: True Negative; FN: False Negative; FP: False Positive; SE: Sensitivity; SP: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value. *Thirty normal tissue specimens were as negative controls. Reference: TB diagnosis based on clinical, radiological, microbiological, pathological and therapeutic criteria.

Furthermore, we aimed to use 16S rRNA sequencing (Forward primer: 5'-TGGAGAGTTTG-ATCCTGGCTCAG-3'; Reverse primer: 5'-TACCGCGGC-TGCTGGCAC-3') to identify specimens with positive staining and negative GeneXpert MTB/RIF to exclude those with NTM infection. Therefore, two specimens were included in this 16S rRNA sequencing analysis; however, the corresponding sequencing results for these samples were still negative after two repeats of this PCR procedure. Both of the two specimens were taken from the irrigation fluid of joints. Therefore, it is possible that the negative PCR results were caused by the samples containing too few cells to be detected.

In this study, we analyzed the potential of GeneXpert MTB/RIF on FFPE tissues for the detection of *M. tuberculosis*. Overall, we concluded that GeneXpert MTB/RIF has the potential to detect M. tuberculosis in such archival FFPE tissue; however, the sensitivity of this system is not high enough due to paucibacillary nature of EPTB and uneven distribution of features in FFPE tissue specimens. Therefore, it is challenging to obtain sufficient nucleic acid samples from FFPE tissue specimens.

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Histologically-confirmed TB was extremely prevalent among FFPE biopsy specimens that were submitted for routine TB diagnosis. FFPE preserves tissue morphology and enables immunohistochemical analysis for clinical analysis. Nevertheless, DNA extraction from FFPE blocks is difficult because the FFPE procedure reduces the quality and quantity of DNA compared to the original fresh surgical specimen.

The microbiological culture of *M. tuberculosis* remains the gold standard test for clinical diagnosis. However, even the most sensitive liquid media are imperfect and have low sensitivities[4]. *Mycobacterium* culture typically requires the use of several antibiotics to limit other fast-growing microbiota in the culture medium; this is one of the critical reasons why the process of mycobacterial culture, especially liquid culture, is cumbersome and costly. In addition, mycobacterial culture requires elevated levels of laboratory biosafety, protection, and technical skills, which hinders its advancement and implementation in developing areas with high TB burden.

Compared with other studies that only used histopathology as the gold standard for comparison[2], our results showed higher sensitivity (57.81% *vs*. 28.57%). However, the sensitivity in the current study was relatively lower than other studies that used *M. tuberculosis* culture as the gold standard (57.81% *vs.* 65.7%)[5]. The Chi-square test was used to determine the positive detection rate of lymph nodal and non-lymph nodal tissue. Our corresponding results demonstrated that the detection rate of lymph nodes was slightly higher than that of non-lymph nodal tissue (60% *vs.* 57.41%; χ^2 = 3.367, P = 0.067). The reason for this difference may be attributed to the use of Auramine O (AO) staining; further, recent studies revealed that AO staining increases the smear positivity in lymph nodal TB[6]. Romdhane et al. have reported that GeneXpert MTB/RIF Ultra use on FFPE is a reliable tool for the detection of *M.* tuberculosis complex species, particularly for cases where microbiological investigations have not been performed[7]. They demonstrated that the GeneXpert MTB/RIF Ultra possessed sensitivity of 63% across FFPE samples, which was a slightly higher sensitivity than what was found in the present study. The GeneXpert MTB/RIF Ultra, as a next-generation product, showed excellent specificity and extreme sensitivity in paucibacillary specimens of EPTB[8]. Unfortunately, the high cost of this equipment has hindered its widespread applications. Although FFPE tissue specimens have a paucibacillary nature, efficient extraction and scientific evaluation methods are beneficial in overcoming this defect[9]. Therefore, we concluded that GeneXpert MTB/RIF has the potential to be used to detect *M. tuberculosis* in archived FFPE tissue.

Prior studies recommended that EPTB diagnosis must be reviewed prudently and NTM disease must be ruled out[10]. However, no NTM-infected specimens were found in any of the suspect specimens in the current study. Nonetheless, there are notable limitations to our study. First, to use FFPE tissues as the starting samples on PCR-based assays, pretreatment procedures for deparaffinization, and mitigation of the effects of PCR inhibitors are necessary. These additional procedures would, to some extent, defeat the intention of using the GeneXpert MTB/RIF to detect *M. tuberculosis* rapidly and easily in FFPE tissues. Furthermore, the GeneXpert cartridge contains a filter membrane that facilitates the trapping of *M. tuberculosis* and, thus, increases detection rates.

Additionally, we extracted DNA from FFPE samples and added them to the GeneXpert cartridge for the reaction This process avoids errors or potential damage to the instrument; however, it sacrifices the detection rate of *M. tuberculosis*. Finally, our study did not follow up with these EPTB patients to obtain more information about their treatment history and medications; further, this study did not differentiate the more drug-resistant *M. tuberculosis* variants from more susceptible variants in these FFPE tissues.

In conclusion, we evaluated the GeneXpert MTB/RIF assay for the detection of *M. tuberculosis* in FFPE specimens. Further research on larger collections of EPTB tissue is required to confirm whether GeneXpert MTB/RIF is an appropriate diagnostic test which can be used to supplement traditional histopathological methods.

Contributors JIA Qing Jun, ZENG Mei Chun and SHU Li Ping designed this study. JIA Qing Jun supervised all the experiments; CHENG Qing Lin, HUANG Yin Yan, AI Li Yun and WU Yi Fei acquired the data; JIA Qing Jun drafted the manuscript; LI Qing Chun and WANG Le critically revised the manuscript for important intellectual content; and FANG Zi Jian performed the statistical analysis assisted by CHENG Shi.

Competing Interests None declared.

Patient Consent Obtained.

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REFERENCES

1. Allahyartorkaman M, Mirsaeidi M, Hamzehloo G, et al. Low diagnostic accuracy of Xpert MTB/RIF assay for extrapulmonary tuberculosis: A multicenter surveillance. Sci Rep, 2019; 9, 18515.
2. Njau AN, Gakinya SM, Sayed S, et al. Xpert((R)) MTB/RIF assay on formalin-fixed paraffin-embedded tissues in the diagnosis of extrapulmonary tuberculosis. Afr J Lab Med, 2019; 8, 748−52.
3. Begum S, Prabhu V, Mohanty V, et al. Unveiling the arcanum of formalin-fixed paraffin-embedded archival tissue blocks: A valuable resource for genomic DNA extraction. J Oral Maxillofac Pathol, 2022; 26, 289−95.
4. Park M and Kon OM. Use of Xpert MTB/RIF and Xpert Ultra in extrapulmonary tuberculosis. Expert Rev Anti Infect Ther, 2021; 19, 65−77.
5. Sarfaraz S, Iftikhar S, Memon Y, et al. Histopathological and microbiological findings and diagnostic performance of GeneXpert in clinically suspected tuberculous lymphadenitis. Int J Infect Dis, 2018; 76, 73−81.
6. Gulati HK, Mawlong M, Agarwal A, et al. Comparative Evaluation of Clinical, Cytological and Microbiological Profile in Abdominal vs. Cervical Lymph Nodal Tuberculosis with Special Emphasis on Utility of Auramine-O Staining. J Cytol, 2021; 38, 191−7.
7. Romdhane E, Arfaoui A, Benabdessalem C, et al. Performance of GeneXpert ultra in the diagnosis of Tuberculous Cervical lymphadenitis in formalin fixed paraffin embedded tissues. Tuberculosis (Edinb), 2020; 125, 102012.
8. Perez-Risco D, Rodriguez-Temporal D, Valledor-Sanchez I, et al. Evaluation of the Xpert MTB/RIF Ultra Assay for Direct Detection of Mycobacterium tuberculosis Complex in Smear-Negative Extrapulmonary Samples. J Clin Microbiol, 2018; 56, e00659−18.
9. McDonough SJ, Bhagwate A, Sun Z, et al. Use of FFPE-derived DNA in next generation sequencing: DNA extraction methods. PLoS One, 2019; 14, e0211400.
10. Sharma SK, Mohan A and Kohli M. Extrapulmonary tuberculosis. Expert Rev Respir Med, 2021; 15, 931−48.