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Ultrasensitive enzyme-linked immunosorbent assay for the detection of MPT64 secretory antigen to evaluate *Mycobacterium tuberculosis* viability in sputum

Kentaro Sakashita[a,b,c,f], Rikiya Takeuchi[d], Keita Takeda[b,e,f], Mikio Takamori[a], Kensuke Ito[d], Yuriko Igarashi[b,f], Eiji Hayashi[c], Mari Iguchi[c], Masahiro Ono[c], Tetsuya Kashiyama[c], Masatoshi Tachibana[a], Jun Miyakoshi[a], Koichi Yano[a], Yu Sato[a], Miyake Yamamoto[a], Kengo Murata[a], Akihiko Wada[a], Kinuyo Chikamatsu[f], Akio Aono[f], Akiko Takaki[f], Hideaki Nagai[e], Akira Yamane[e], Masahiro Kawashima[e], Mariko Komatsu[d], Kazunari Nakaishi[d], Satoshi Watabe[d], Satoshi Mitarai[b,f,*]

[a] *Department of Respiratory Medicine, Tokyo Metropolitan Tama Medical Center, Tokyo, Japan*
[b] *Department of Basic Mycobacteriology, Graduate School of Biomedical Science, Nagasaki University, Nagasaki, Japan*
[c] *Department of Internal Medicine, Tokyo Metropolitan Matsuzawa Hospital, Tokyo, Japan*
[d] *Department of Research and Development, TAUNS Laboratories Inc., Shizuoka, Japan*
[e] *Department of Respiratory Medicine, National Hospital Organization Tokyo National Hospital, Tokyo, Japan*
[f] *Department of Mycobacterium Reference and Research, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan*

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ABSTRACT

Objectives: This study examined *Mycobacterium tuberculosis* (MTB)-secreted MPT64 as a surrogate of bacterial viability for the diagnosis of active pulmonary TB (PTB) and for follow-up treatment.
Methods: In this proof-of-concept prospective study, 50 PTB patients in the Tokyo metropolitan region, between 2017 and 2018, were consecutively included and 30 healthy individuals were also included. Each PTB patient submitted sputum on days 0, 14 and 28 for diagnosis and follow-up, and each healthy individual submitted one sputum sample. The following were performed: smear microscopy, Xpert MTB/RIF, MGIT and solid culture, and MPT64 detection on the sputum samples. Ultrasensitive ELISA (usELISA) was used to detect MPT64. The receiver operating characteristic analyses for diagnosis and follow-up revealed the optimal cut-off value of MPT64 absorbance for detecting culture positivity at multiple intervals.
Results: The sensitivity of MPT64 for diagnosing PTB was 88.0% (95% CI 75.7–95.5) and the specificity was 96.7% (95% CI 82.8–99.9). The specificity of MPT64 for predicting negative culture results on day 14 was 89.5% (95% CI 66.9–98.7). The sensitivity of MPT64 for predicting positive culture results on day 28 was 81.0% (95% CI 58.1–94.6).
Conclusions: This study revealed that MPT64 is useful for diagnosing active PTB in patients and predicting treatment efficacy at follow-up.
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1. Introduction

Tuberculosis (TB) remains a major life-threatening disease, but early diagnosis and treatment can improve patient outcomes. Bacteriological detection of *Mycobacterium tuberculosis* (MTB) is the gold standard of TB diagnosis, and many tests are performed to diagnose TB. In many TB epidemic settings, an acid-fast bacilli (AFB) smear remains the only available and feasible test to diagnose active TB. However, smear microscopy sensitivity is low

* Corresponding author. Department of Mycobacterium Reference and Research, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3-1-24 Matsuyama, Kiyose, Tokyo, Japan. Tel.: +81 42 493 5711 ext 395; fax: +81 42 492 4600.
E-mail address: mitarai@jata.or.jp (S. Mitarai).

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and it cannot discriminate between viable and dead bacilli. To cope with the shortcomings of the AFB smear, a nucleic acid amplification test (NAAT) has been introduced to more efficiently diagnose MTB. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) can identify MTB with a higher sensitivity and yield results within two hours (Boehme et al., 2010). However, NAAT cannot evaluate MTB viability since it also detects DNA from dead tubercle bacilli. Thus, NAAT is not useful for follow-up diagnosis.

Positive MTB culture is the gold standard for diagnosing active TB. In addition, time to detection (TTD) in liquid culture and the number of colonies on solid media reflect the burden of viable MTB bacilli. Culture examination is important for sensitive TB diagnosis and evaluating anti-TB treatment efficacy, especially in multidrug-resistant TB (MDR-TB) patients (WHO, 2011). However, access to culture examinations is limited in many developing countries. In addition, culture examination is time-consuming due to the slow-growing nature of MTB (Kanchana et al., 2000) and results cannot be obtained in a timely manner. Therefore, an alternative method must be developed to determine negative culture results with a short turnaround time (TAT) to confirm efficacy of MTB treatment. MPT64 is a secretory immunogenic protein, which is highly specific for MTB and several substrains of the *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), and not found in non-TB mycobacteria (Cole et al., 1998; Roche et al., 1996). In fact, MPT64 detection has been applied for rapid MTB identification in AFB culture-positive specimens (Abe et al., 1999) such as Capilia TB-Neo (TAUNS Laboratories, Izunokuni-shi, Shizuoka, Japan). MPT64 is excreted during the active growth phase of MTB (Andersen et al., 1991). MPT64 detection would enable rapid identification of viable MTB in clinical specimens.

The ultrasensitive, enzyme-linked immunosorbent assay (usELISA) method was developed to overcome the relatively low sensitivity of conventional ELISA. This method combines alkaline phosphatase (ALP)-induced hydrolysis of a novel substrate–17β-methoxy-5β-androstan-3-ol-3-phosphate (A3P)–with a 3α-hydroxysteroid dehydrogenase (3α-HSD)-mediated thio-NAD cycling reaction. The usELISA introduces a coenzyme in addition to a substrate and can amplify the metabolite signal with higher detection sensitivity (Watabe et al., 2014). For example, a previous study has indicated that the usELISA improves detection of the HIV p24 antigen (Nakatsuma et al., 2015).

The current study used the usELISA to detect MPT64 in sputum specimens and predict MTB culture results. It aimed to establish the optimal MPT64 cut-off values with the usELISA system for both TB diagnosis and treatment follow-up as a surrogate of culture examination.

2. Materials and methods

2.1. Study design and participants

A prospective study was designed by enrolling 50 patients who were bacteriologically diagnosed with PTB. PTB patients were consecutively enrolled, from three TB hospitals located in the Tokyo metropolitan region, between 01 February 2017 and 31 May 2018. The study sites were Tokyo Metropolitan Tama Medical Center, Tokyo Metropolitan Matsuzawa Hospital and National Health Organization Tokyo National Hospital. The laboratory procedures were performed at the Research Institute of Tuberculosis in Tokyo, Japan. PTB patients enrolled in the study were AFB smear-positive and NAAT-positive and/or culture-positive for MTB. Patients who were treated with standard anti-TB drugs for > 6 days were ineligible (to avoid false negative microbiological examination results). As the non-PTB control, commercially available sputum specimens (NOVA Biologics, Inc., Oceanside, CA, USA), consecutively collected from 30 healthy adults, were purchased. In the non-PTB group, the participants were negative for hepatitis C and B viruses and HIV.

2.2. Sputum collection procedure

Each PTB patient submitted three early morning sputum specimens. The patients who started anti-TB treatment before enrolment submitted the sputum specimens on days 0 (within 5 days after administration), 14 and 28. The patients who could not submit three sputum specimens were excluded from the analysis (Fig. 1).

2.3. Conventional laboratory examinations

Sputum volume and quality were evaluated using the Miller & Jones classification to confirm the usefulness of the specimens (Miller, 1963). The sputum was homogenized with a 3X volume of semi-alkaline protease (SAP, Sputazyme, Kyokuto Pharmaceutical Industrial. Co., Ltd., Tokyo, Japan). After 15 minutes, it was equally divided into two vials. One specimen was used for the AFB smear, mycobacterial growth indicator tube (MGIT) (Becton Dickinson, Sparks, MD) culture, solid culture on 2% Ogawa medium (Kyokuto Pharmaceutical Industrial. Co., Ltd., Tokyo, Japan), and Xpert MTB/RIF. The other specimen was used for MPT64 detection with usELISA. The operators performing the usELISA were unaware of the culture examination results. The index test results were only available to the data analyzer.

The first half of the specimen was digested and decontaminated using the standard N-acetyl-*L*-cysteine (NALC)-2% NaOH method. After centrifugation (3000 *g* for 20 minutes at 4 °C), the supernatant was discarded, and the sediment was resuspended in 2 mL of phosphate buffer (PB, pH 6.8). The AFB smear examination was performed using auramine O (FUJIFILM Wako Pure Chemical Co., Japan) fluorescent staining. Aliquots of 500 μL and 100 μL of suspension were added to MGIT and two 2% Ogawa tubes, respectively. A positive culture was identified as MTB with Capilia TB-Neo. Xpert MTB/RIF was performed with 500 μL of the suspension, according to the manufacturer's instructions.

The AFB smear-positivity grade was recorded using the standard smear grade system in Japan (Mitarai, 2016). Culture positivity and TTD in MGIT were recorded. Xpert MTB/RIF results were recorded for MTB detection (including cycles of threshold: Ct) and rifampicin resistance. The minimum Ct of all detected probes in each Xpert MTB/RIF test was recorded.

2.4. Sputum treatment for ultrasensitive ELISA

The other half of the specimen was centrifuged at 11,000 *g* for 6 minutes. After discarding supernatant, 4 M urea was added to the sediment at half the original volume of the sputum and further solubilized. NALC solution (0.5%) of 12% of the original volume of the sputum was added and thoroughly mixed. The mixture was incubated for 15 minutes at ambient temperature. After incubation, the specimen was mixed with 10 mL of washing solution (33 mM phosphate buffer, pH 6.8, 0.05% Tween 80) and centrifuged at 11,000 *g* for 6 minutes. The supernatant was removed, and the sediment was resuspended in 200 μL of heat-treatment buffer (33 mM phosphate buffer, pH 6.8, 2% Tween 20). Finally, 200 μL of the suspension was transferred to a 1 mL polypropylene tube and heated at 46 °C for 60 minutes to promote the release of MPT64 from MTB, as previously reported (Nakaishi et al., 2019). After heating, the suspension was filtered (0.1 μm pore size) and the filtrate was subjected to usELISA.

2.5. Ultrasensitive ELISA

The recombinant MPT64 antigen was used for basic system analysis. The primary and secondary anti-MPT64 mouse monoclonal antibodies were purified at TAUNS Laboratories (Shizuoka,

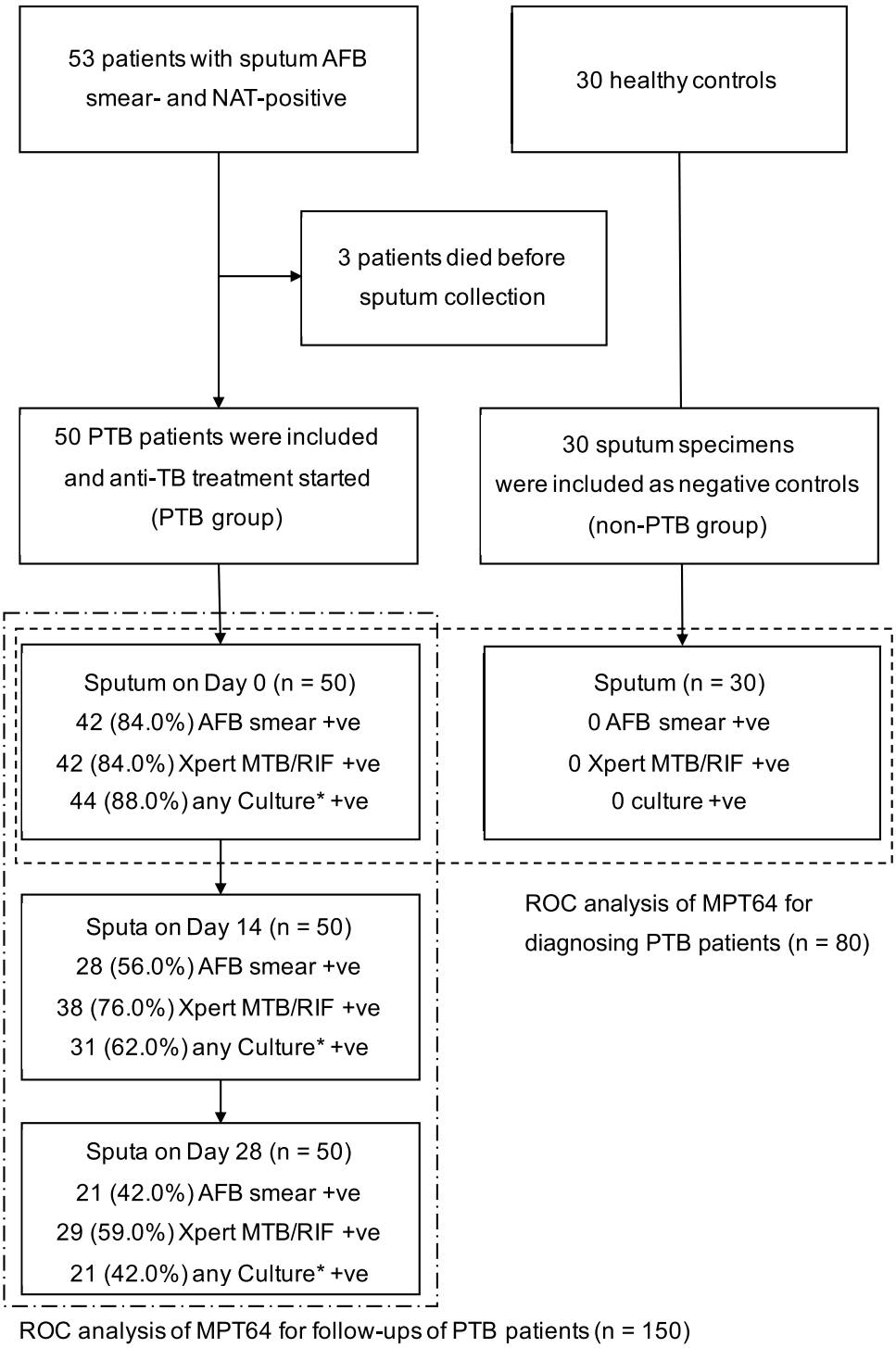

Fig. 1. Process of enrolment and final inclusion.

Japan). The specificity of the antibody was reported 100% tested to 96 *Mycobacterium* species (four MTB complex and 92 non-TB Mycobacteria) and three other genus bacteria (Chikamatsu et al., 2014). The primary anti-MPT64 antibody was immobilized on a 96-well microplate. The secondary anti-MPT64 antibody was conjugated with ALP. The color reagent used a new principle by enzyme cycling reaction that can be detected 10^{-19} moles/assay of protein. This principle was used to detect MPT64 (Figure S1) (Watabe et al., 2014). The usELISA procedure was performed as previously reported (Ito and Watabe, 2012; Watabe et al., 2014). In short, 50 μL of each pretreated specimen was added to the wells, and 50 μL of the ALP-labeled anti-MPT64 antibody solution was added. The plate was incubated for 1 hour at 37 °C. After washing five times with Tris-hydroxymethyl aminomethane buffered saline containing surfactant using the ImmunoWash 1575 automated plate washer (Bio-Rad, Hercules, CA, USA), 100 μL of the coloring reagent (a mixture of thio-NAD, NADH, 3α-HSD, and A3P) was added to each well. MPT64 absorbance was measured with the

SH-9000 microplate reader (Corona Electric, Ibaraki, Japan) for 90 minutes at 37 °C. By using the serially diluted recombinant MPT64 antigen, the LOD/LLOQ was determined as 0.15 pg/mL as the qualitative method. The precision (CV) was 20% with 30 repeated measures of negative specimens. The recovery was 87.3% using MPT64 spiked negative sputum specimens. The linearity (R^2 = 0.9674) and calibration curves are shown in Figure S2. The measurable range that was tested was 0–200 pg/mL. The final MPT64 incremental absorbance (MPT64 ΔAbs) was calculated according to the following formula:

[(sample absorbance$_{405nm}$ at 90 min - sample absorbance$_{620nm}$ at 90 min) - (sample absorbance$_{405nm}$ at 0 min - sample absorbance$_{620nm}$ at 0 min)] - [(blank absorbance$_{405nm}$ at 90 min - blank absorbance$_{620nm}$ at 90 min) - (blank absorbance$_{405nm}$ at 0 min - blank absorbance$_{620nm}$ at 0 min)].

The total turnaround-time was approximately 5 hours and actual hands-on time was 5 minutes for this assay.

2.6. Clinical data collection in patients with and without PTB

Basic information was collected on the participants in the PTB group, including: age, sex, nationality, comorbidity, HIV infection status, anti-TB treatment regimen, drug susceptibility testing (DST) result, and chest X-ray findings (the presence of a cavity). In the non-PTB group, clinical information about age and sex was collected.

2.7. Statistical analysis

The receiver operating characteristics (ROC) curves were drawn for the MPT64 ΔAbs based on disease status (PTB or non-PTB) for PTB diagnosis and follow-up. For diagnosing PTB, the optimal cut-off value for the usELISA was calculated as maximizing the Youden's index. For the follow-up at day 14, the cut-off value was set as maximizing the positive likelihood ratio (PLR) to predict the most positive culture results because many PTB patients are still culture-positive. Inversely, on day 28, another cut-off value was set as maximizing the negative likelihood ratio (NLR) to predict the most culture-negative results because many negative culture results were expected. The Cochran-Armitage trend test was used to compare each diagnostic test's positivity rate during the anti-TB treatment courses. Spearman's rank correlation coefficient was applied to evaluate the correlation of two non-parametric data groups. The Kruskal-Wallis test was applied to compare the difference in non-parametric data among more than three groups. The Mann-Whitney U test compared two continuous variables that followed a non-normal distribution. Cochran's Q test was applied to compare the categorical variable for more than three methods among the same participants. Dunn's test was used as a post-hoc test for two method comparisons if a statistical difference was found with the Cochran's Q test. McNemar's test was applied to the evaluation for two methods with the same participants. A difference was regarded as statistically significant at p-value < 0.05. A 95% confidence interval (CI) was estimated by using the Clopper Pearson interval method for a binominal proportion. STATA version 14.1 (StanCorp, College Station, Texas, USA) was used for the statistical analysis.

3. Results

3.1. Basic characteristics of study participants

The inclusion process and microbiological examination results are shown in Fig. 1. The participants' basic characteristics are summarized in Table 1. There was a significant difference in age distribution between the PTB and non-PTB groups, but no significant differences in the proportion of males, median sputum volume or sputum qualities. No rifampicin resistance was observed with Xpert MTB/RIF and MGIT DST. The most common comorbidity was diabetes mellitus followed by malignant neoplasm and chronic kidney disease.

3.2. Evaluation of the diagnostic accuracy of MPT64 for active pulmonary TB

The ROC curve of MPT64 ΔAbs for differentiating PTB from the non-PTB (n = 80) group demonstrated that the area under the curve (AUC) was 0.94 (95% CI 0.90–0.99) (Fig. 2). Based on the maximum Youden's index, the optimal cut-off was calculated as 0.004. There was no significant difference in diagnostic sensitivity or specificity between MPT64 ΔAbs, AFB smear and Xpert MTB/RIF (Table 2).

3.3. MPT64 ΔAbs trend in PTB patients during anti-TB treatment

MPT64 ΔAbs in the PTB group significantly decreased after anti-TB treatment (Fig. 3A). The MPT64 ΔAbs over time for each PTB patient is shown in Fig. 3B. Three patients showed persistently higher MPT64 ΔAbs value over the cut-off (ΔAbs = 0.004) and were positive for the AFB smear, Xpert MTB/RIF and cultures throughout the 28-day treatment. One patient had severe and uncontrolled diabetes mellitus and a persistent cough over 4 months before TB diagnosis. Another patient had untreated advanced lung cancer. The other patients had no comorbidities. Those three patients had multiple cavitary lesions discovered on chest X-ray. Among all PTB patients, the positivity of the sputum smear, Xpert MTB/RIF and culture significantly decreased over the course of anti-TB treatment (Fig. 4).

3.4. MPT64 for predicting sputum MTB culture positivity in the follow-up period

This study analyzed the follow-up sputum specimens separately from the diagnostic samples and prepared ROC curves of MPT64 ΔAbs on day 14 (n = 50) and day 28 (n =50) to predict MTB culture positivity, while taking into account the changes in MPT64 excretion after treatment. The sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of MPT64 for follow-up specimens are shown in Table 3. The AUC of the ROC curve was 0.80 (95% CI 0.67–0.93) on day 14 (Fig. 5) and 0.73 (95% CI 0.58–0.88) on day 28 (Fig. 6). The cut-off value was set as 0.025 for day 14 follow-up sputum to maximize the PLR. For sputum collected on day 28, the cut-off value was set as–0.007 to minimize the NLR. On day 14, the sensitivity of Xpert MTB/RIF was significantly higher than that of the MPT64 ΔAbs ($p < 0.001$) and smear microscopy ($p = 0.008$). The specificity of the MPT64 ΔAbs was significantly higher than that of Xpert MTB/RIF ($p = 0.014$). On day 28, the sensitivity of Xpert MTB/RIF was significantly higher than that of the smear ($p = 0.002$) and significantly different from that of the MPT64 ΔAbs ($p = 0.045$). MPT64 ΔAbs tended to show higher sensitivity than that of the smear ($p = 0.058$). However, no significant difference was observed in this specificity.

Correlation between MPT64 absorbance, smear grades, and Ct values of Xpert MTB/RIF in MTB culture-positive sputum specimens

Among culture-positive specimens, the $\log_{10}$ scale of the MPT64 ΔAbs ($\log_{10}$ MPT64 ΔAbs) and AFB smear grades (n = 81) had a significant positive correlation (rs = 0.768, $p < 0.0001$; Fig. 7A), whereas the $\log_{10}$ MPT64 ΔAbs and Ct (n = 77) had a significant inverse correlation (rs = −0.833, $p < 0.0001$; Fig. 7B). In liquid culture-positive cases, $\log_{10}$ MPT64 ΔAbs and $\log_{10}$ TTD (n = 81) had a significant inverse correlation (rs = −0.623, $p < 0.0001$; Fig. 7C). The AFB smear and TTD among liquid culture-positive cases (n = 90) had a significant inverse correlation (rs = −0.625, $p < 0.0001$). In addition, the Ct and TTD among liquid

Table 1
Basic characteristics and microbiological results of the participants (n = 80).

		PTB group (n = 50)	non-PTB group (n = 30)	*p*-value
Median age [IQR] years		62 [44–81]	41 [32–50]	0.0001*
Men (%)		33 (66.0%)	23 (76.67%)	0.450**
Patients with HIV		1 (1.89%)	0 (0%)	1.00**
Nationality	Japanese	42 (84.0%)	unknown	NA
	Non-Japanese	8 (16.0%)	unknown	NA
Microbiological sputum results of patients with PTB on day 0 and from healthy controls				
Sputum volume, mL	Median [IQR]	2 [1,2]	2 [2]	0.271*
Miller & Jones classification	M1	12 (24.0%)	3 (10.0%)	
	M2	5 (10.0%)	4 (13.3%)	
	P1	15 (26.0%)	10 (33.3%)	0.364*
	P2	13 (24.0%)	7 (23.3%)	
	P3	8 (16.0%)	6 (20.0%)	
AFB smear	Positive	42 (84.0%)	0 (0%)	< 0.001**
Xpert MTB/RIF	Positive	42 (84.0%)	0 (0%)	< 0.001**
MGIT	Positive	41 (82.0%)	0 (0%)	< 0.001**
Solid culture	Positive	41 (82.0%)	0 (0%)	< 0.001**
Any culture	Positive	44 (88.0%)	0 (0%)	< 0.001**
Drug-susceptible	for INH, RIF, EMB, PZA	43 (86.0%)	NA	
Drug-resistant	for STR	4 (8.0%)	NA	
	for INH and STR	2 (4.0%)	NA	
	for PZA	1 (2.0%)	NA	
Chest radiograph findings	Cavity lesion(s)	23 (46.0%)	NA	
Comorbidity	Diabetes mellitus	12 (24.0%)	NA	
	Malignant tumor	5 (10.0%)	NA	
	Chronic kidney disease	4 (8.0%)	NA	
	Alcoholism	2 (4.0%)	NA	
	Rheumatoid arthritis with immunosuppressive therapy	2 (4.0%)	NA	
	Gastrectomy	1 (2.0%)	NA	
	None	24 (48.0%)	NA	

Abbreviations: EMBethambutol; INHisoniazid; IQRinterquartile range; MGITmycobacterial growth indicator tube; NAnot applicable; PTBpulmonary tuberculosis; PZApyrazinamide; STRstreptomycin.
* Mann-Whitney U test
** Fisher's exact test

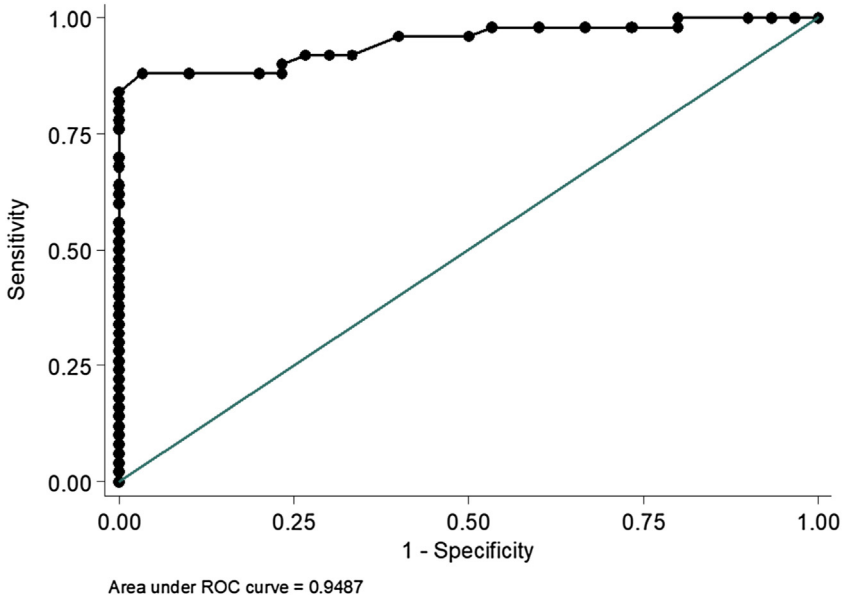

Fig. 2. ROC curve used to diagnose pulmonary tuberculosis with MPT64 absorbance at 0.004 (n = 80).

Table 2
Diagnostic performance of MPT64, sputum AFB smear and Xpert MTB/RIF to diagnose active PTB (n = 80).

	TP	FN	FP	TN	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]
MPT64 ΔAbs Cut-off: 0.004	44	6	1	29	88.0 [75.7–95.5]	96.7 [82.8–99.9]
AFB Smear	42	8	0	30	84.0 [70.9–92.8]	100 [91.6–100]
Xpert MTB/RIF	42	8	0	30	84.0 [70.9–92.8]	100 [91.6–100

Abbreviations: AFB, acid-fast bacilli; NA, not applicable; PTB, pulmonary tuberculosis; 95% CI, 95% confidence interval
* p-values of sensitivity and specificity comparison were 0.713 and NA, respectively

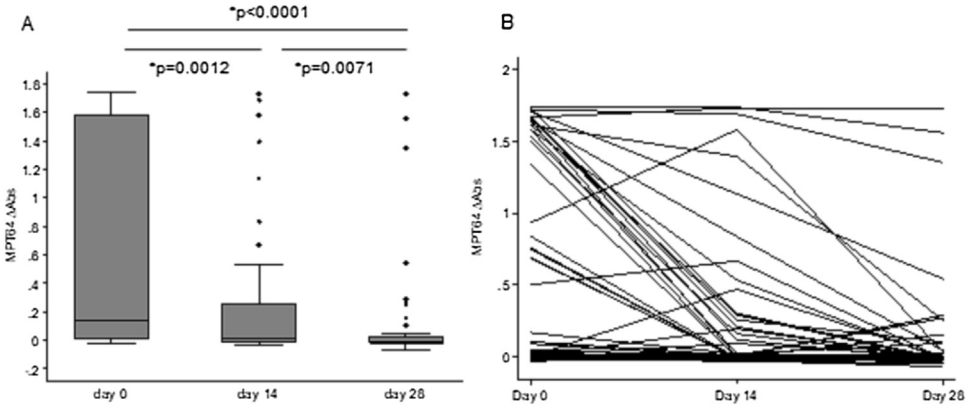

Fig. 3. MPT64 production during anti-TB treatment (A) Boxplot of MPT64 ΔAbs in the PTB group during anti-TB treatment (n = 50); (B) MPT64 ΔAbs over time for each PTB patient during anti-TB treatment (n = 50).

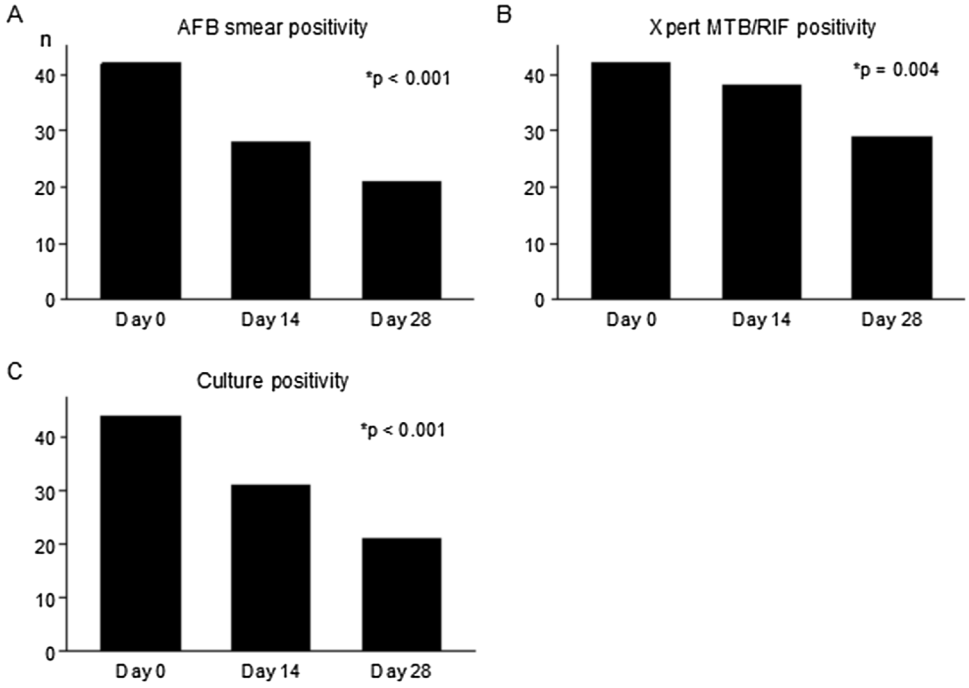

Fig. 4. The number of PTB patients positive for AFB smear (A), Xpert MTB/RIF (B), and culture (C) (n = 50). *Cochran-Armitage trend test.

culture-positive cases (n = 86) had a significant positive correlation (rs = 0.700, $p < 0.0001$).

4. Discussion

This study was designed as a proof of concept and evaluated MPT64 for the diagnosis and follow-up assessment of PTB in heat-treated sputum using the usELISA method. It was demonstrated that MPT64 in sputum samples had comparable sensitivity to smear and Xpert MTB/RIF to diagnose active PTB with an optimal cut-off value, and that MPT64 values decreased as anti-TB treatment progressed. These findings supported this study's hypothesis.

The MPT64 ΔAbs in the culture-positive cases showed a significant inverse correlation with the TTD of the liquid culture and Ct of Xpert MTB/RIF. A previous study showed a positive

Table 3
Diagnostic performance of MPT64, sputum AFB smear and Xpert MTB/RIF at follow-up after anti-TB treatment (n = 50).

	MPT64 ΔAbs (Cut-off: 0.025)	AFB smear	Xpert MTB/RIF	*p*-value
	Sputum on day 14 (n = 50)			
Sensitivity (%) [95% CI]	54.8 [36–72.7] $p < 0.001$**	74.19 [55.4–88.1] $p = 0.008$**	96.77 [83.3–99.9]	< 0.001*
Specificity (%) [95% CI]	89.5 [66.9–98.7] $p = 0.014$**	73.68 [48.8–90.9]	57.8 [33.5–79.7]	0.034*
Positive likelihood ratio	5.21 [1.35–20.1]	2.82 [1.29–6.15]	2.3 [1.35–3.91]	
Negative likelihood ratio	0.50 [0.33–0.76]	0.35 [0.18–0.67]	0.05 [0.007–0.398]	
	MPT64 ΔAbs (Cut-off: −0.007)	AFB smear	Xpert MTB/RIF	*p*-value
	Sputum on day 28 (n = 50)			
Sensitivity (%) [95% CI]	81.0 [58.1–94.6]	57.14 [34.0–78.2] $p = 0.002$**	100.0 [83.9–100]	0.002*
Specificity (%) [95% CI]	72.4 [52.8–87.3]	69.0 [49.2–84.7]	72.4 [52.8–87.3]	0.948*
Positive likelihood ratio	2.93 [1.57–5.48]	1.84 [0.95–3.55]	3.63 [2.01–6.54]	
Negative likelihood ratio	0.26 [0.10–0.65]	0.62 [0.35–1.08]	NA	

Abbreviations: MPT64mycobacterial protein from species tuberculosis-64; PTBpulmonary tuberculosis; 95% CI95% confidence interval
* Cochran's Q test
** McNemar's test after Dunn's test

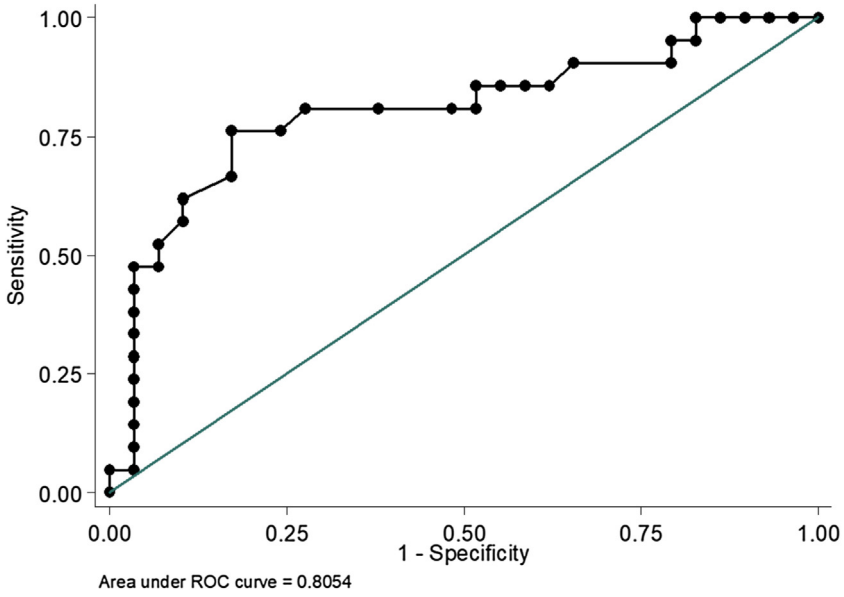

Fig. 5. ROC curve used to follow-up MTB culture positivity on day 14 (n = 50).

correlation between the increasing MTB Ct of Xpert MTB/RIF and TTD (Nabeta et al., 2011). The current study also found that the Ct and TTD had a significant linear correlation in culture-positive cases at day 0 (for diagnosis). These findings suggest that MPT64 production reflects the mycobacterial load in sputum.

A general trend was observed in decreasing MPT64 levels with effective anti-TB treatment. Furthermore, in accurately predicting the culture positivity at day 14, the MPT64 ΔAbs showed a significantly higher specificity than Xpert MTB/RIF and smear microscopy with the optimal cut-off value. Similarly, to accurately predict the culture negativity at day 28 by applying the other optimal cut-off value from this study, the sensitivity of MPT64 ΔAbs tended to be higher than that of Xpert MTB/RIF and smear microscopy. These findings support the idea that MPT64 ΔAbs in the follow-up period could be useful for predicting MTB culture results with optimal cut-off values on days 14 and 28.

MTB-specific antigens–including MPT64, early secretory antigenic target-6, culture filtrate protein-10 (CFP-10), and CFP-21–have previously been measured in clinical sputum samples (Kalra et al., 2010; Mehta et al., 2012). In one study the conventional sandwich ELISA method for detecting antigens was applied and demonstrated a diagnostic sensitivity of 56% and specificity of 90% in the AFB smear-positive sputum samples (Kalra et al., 2010). In another study, an immuno-polymerase chain reaction was applied to detect TB-specific antigens and showed that the sensitivity improved from 56% to 77.5% in the sputum AFB smear-positive samples (Mehta et al., 2012). Although these studies measured MPT64 directly mixed with other TB-specific antigens in sputum, the sensitivity was lower than the current study for diagnosing active PTB cases, which was probably due to the low sensitivity of conventional ELISA. Ji et al. established a quantitative ELISA method to detect MPT64 in cultured MTB samples and determined that 1.7×10^4 CFU/mL MTB was the lower limit of MTB detection with the conventional sandwich ELISA method (Ji et al., 2014). This finding suggests that the threshold level for detecting viable MTB with the conventional ELISA method is similar to the threshold level of the direct sputum smear (Rouillon et al., 1976; Smithwick, 1979). In another recent study, selective, single-stranded DNA aptamers against MPT64 protein were tested on clinical sputum samples using an enzyme-linked oligonucleotide assay (ELONA) and had a sensitivity and specificity of 91.3% and 90%, respectively (Sypabekova et al., 2017). This study showed that

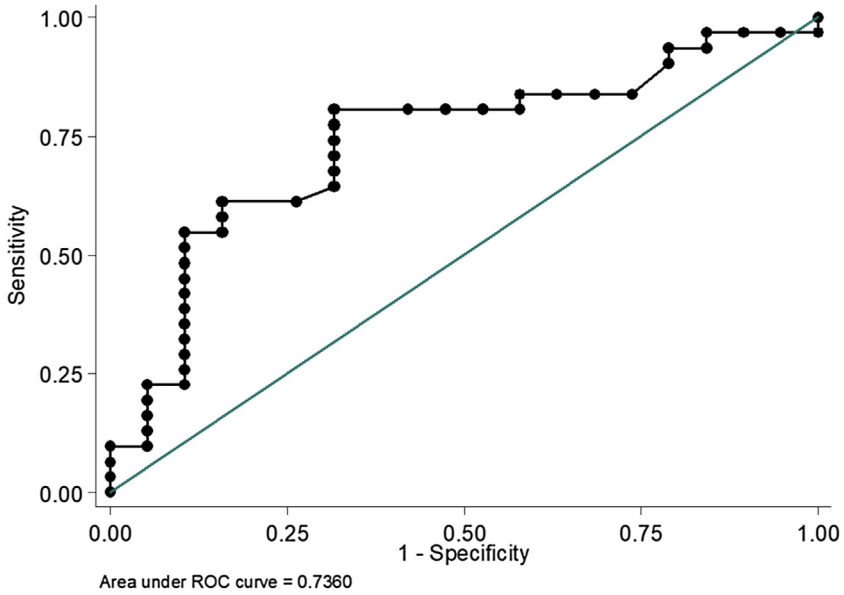

Fig. 6. ROC curve used to follow-up MTB culture positivity on day 28 (n = 50).

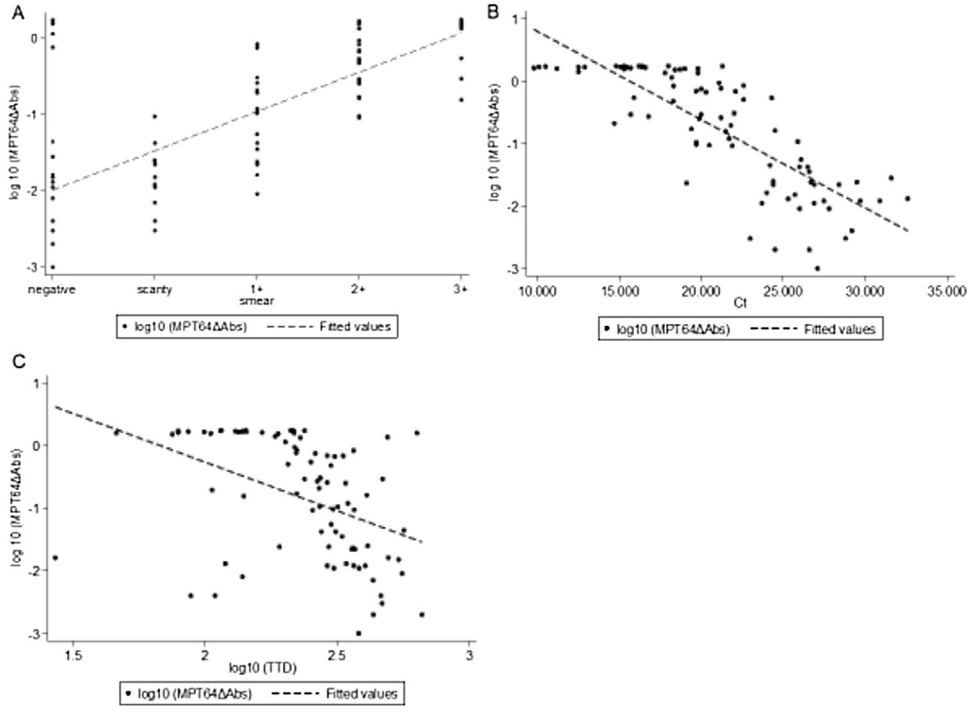

Fig. 7. Correlation between MPT64 absorbance (ΔAbs) and smear grades (A), Ct (B), and TTD (C) in MTB culture-positive cases (n = 81, 77, and 77, respectively).

an aptamer-based diagnostic method with ELONA can be accurate. Thus, these studies show that MPT64 is a potential surrogate marker for the diagnosis of PTB. Kawasaki et al. reported that the highest limit of detection in their ELISA system was 8.5 pg/mL (Kawasaki et al., 2019). In the current study, the limit of detection in usELISA was approximately 0.15 pg/mL. The difference was approximately 60 times. As additional information, the correlations of MPT64 concentrations with AFB smear positivity, Ct values and $\log_{10}$ TTD are shown in Figure S3 for comparison of data with Kawasaki et al. (Kawasaki et al., 2019).

The novelty of this study was to determine whether MPT64 could be assayed during follow-up to assess response to treatment. The conventional culture examination takes days to weeks for a positive result and approximately 6–8 weeks to determine a negative result. This delay interferes with effective treatment strategies for patients. If MPT64 in sputum is measured by usELISA, the short TAT (within 1 day) will be useful to quickly evaluate treatment response.

As an alternative method for evaluating treatment response in PTB patients, lipoarabinomannan (LAM) has been reported as a candidate marker (Kawasaki et al., 2019). Kawasaki et al. reported that the decline in sputum LAM concentrations correlated with increases in MGIT TTD in individual patients, and sputum LAM measured by the assay may be used as a biomarker of bacterial load

prior to and during TB treatment. However, LAM is a common structural component of mycobacterial species; thus, it is not bacteriologically specific for MTB. Although it could be used as a biomarker of bacterial load in cases of confirmed MTB disease, it may not be used for the diagnosing PTB due to its non-specificity. A recent report for Fujifilm SILVAMP TB LAM keeps high specificity even with increased sensitivity, and it could change the conventional thought about LAM antigen (Bulterys et al., 2019), but still needs more prospective cohorts to demonstrate diagnosis accuracy. It is also unclear why the organic component of MTB declined so quickly after anti-TB treatment.

This study had several limitations. First, it did not include patients with MDR-TB and had one patient with HIV infection. In future studies, it will be necessary to evaluate the usefulness of MPT64 detection in these populations. Second, several studies have reported that MPT64 gene mutations produced a false negative in MTB detection tests using anti-MTB64 monoclonal antibodies (Basu et al., 2015; Hirano et al., 2004; Muyoyeta et al., 2013; Qiu et al., 2015). Chikamatsu et al. reported two (0.4%) false-negative results for MTB and one (0.2%) false-negative result for *Mycobacterium bovis* BCG Connaught from 500 clinical MTB complex isolates (Chikamatsu et al., 2014). Although the false-negative rate was low, MPT64-negative results must be critically interpreted. Third, a few patients exhibited high MPT64 production throughout the 28-day anti-TB treatment. This may have been due to a sustained high bacillary load in diabetes mellitus and advanced PTB patients. Such cases may necessitate an extended follow-up period to evaluate MPT64 compatibility with other standard reference methods, including smear and culture. Fourth, although the usELISA method needs minimal handling with pretreatment, co-enzymes and a heating process for specimen analysis, and can be performed with conventional ELISA devices, the TAT and hands-on-time will be a practical problem. Further improvement in sensitivity for the detection of MPT64 and the development of an automated device (in progress) is necessary. Fifth, a case-control design using healthy controls, use of a research assay that requires further validation and no pre-specified threshold is required. Further assessment work is needed to demonstrate accuracy in a prospective cohort as a next step.

5. Conclusions

This study revealed the accuracy of MPT64 detection by usELISA to diagnose active PTB from sputum specimens. Furthermore, it demonstrated the predictive capability of secreted MPT64 levels to assess changes in MTB culture during treatment. MPT64 reflected viable bacterial loads in sputum. Detecting MPT64 in clinical specimens will allow healthcare workers to estimate the viability of MTB within a day and could replace culture examination. This method could reduce the time needed to determine the efficacy of an anti-TB drug and improve rapid diagnostic measurements.

Ethical Approval

The institutional review boards of all participating facilities approved this study (protocol number: 201782). All PTB patients provided written informed consent before enrolment. The purchased MTB-negative sputum specimens were collected after obtaining consent for research use from each healthy volunteer. The authors have met the ICMJE authorship criteria.

Declaration of interests

KS has received a research grant from the Tokyo Metropolitan government. The other authors do not have commercial or other associations that might pose a conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2020.04.059.

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