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RESEARCH ARTICLE

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Direct detection from clinical sputum samples to differentiate live and dead *Mycobacterium Tuberculosis*

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Background: In this study, we aimed to optimize the condition of propidium monoazide (PMA) treatment for direct detection of *Mycobacterium tuberculosis* (MTB) from clinical specimens.

Methods: The light exposure time, dark incubation time, bacterial load, and PMA concentration were varied to determine the optimal condition of PMA treatment.

Results: Overall, the maximum ΔCq value was observed in the group receiving a light exposure time of 20 minutes, which was significantly higher than the others ($P < 0.05$). The prolongation of dark incubation time seemed more likely to result in greater ΔCq value, and the ΔCq values were 2.0, 4.1, 6.5, 10.1, and 12.7 cycles under dark incubation time of 10, 20, 40, 60, and 120 minutes, respectively. Alternatively, the 4+ samples exhibited favorable detection results at the application of 10^4-fold dilution by PMA assay with Cq values higher than 35 cycles. Further evaluation revealed that the PMA assay showed an accordance rate of 98.0% (98/100) among clinical sputa.

Conclusions: we develop an acceptable method to directly identify the live bacteria from sputum samples. Our data demonstrate that the dark incubation plays a crucial role in the efficacy of PMA treatment for MTB.

KEYWORDS
Mycobacterium tuberculosis, diagnosis, propidium monoazide, live MTB, dead MTB

1 | INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), remains a serious global health concern, with 10.5 million new cases and 1.4 million deaths in 2015.[1] Rapid and accurate diagnosis is essential for timely initiation of antituberculosis treatment and prevention of further spread in the community.[2] Although smear microscopy and culture are still the major tool for the diagnosis of TB patients worldwide, especially in high TB prevalent settings, recent advances in molecular diagnostics provide highly sensitive and rapid options for detection of MTB directly from specimens, which is challenging the current TB diagnostic landscape.[2,3]

World Health Organization has recommended several commercial assays for molecular detection of MTB and drug-resistant TB (eg, GenoType MTBDR from Hain Lifescience, GeneXpert MTB/RIF from Cepheid, and TB-LAMP from Eiken Chemical).[4,5] Despite providing high sensitivity and short turnaround time, the molecular diagnostics has unsatisfactory sensitivity in smear-negative specimens and high cost that impedes their implementations in resource-limited settings. Another obvious limitation of the current molecular tools is that they fail to discriminate live and dead MTB on the basis of detecting the chromosomal DNA.[6,7] As a result, conventional culture is superior to molecular methods in regard to this aspect, which allows it to be used in monitoring therapeutic efficacy of TB patients during follow-up period in spite of taking at

least 2 weeks.[5] This instinct drawback highlights the urgent need to develop a promising molecular assay for distinguishing living and dead MTB, which extend the scope for molecular diagnostics by detection of follow-up patients.

Compared with genomic DNA, mRNA is turned over rapidly in living bacterial cells, and the target mycobacterial mRNA therefore is a good indicator of living mycobacterial cells.[8,9] Because of the thick and waxy mycobacterial cell wall, MTB is virtually recalcitrant to routine lysis procedures. As a result, the isolation of mRNA from MTB becomes more difficult than that from other bacteria.[6] In addition, most of mRNA species exhibit a half-life of only a few minutes.[10,11] Taken together, the application of mRNA-based assay for reflecting mycobacterial viability may be associated with the increased proportion of false-negative samples. Recently, several studies have demonstrated that the combination between DNA-binding chemicals and PCR would inhibit PCR amplification of DNA derived from dead cells.[12–15] Of these DNA-binding chemicals, propidium monoazide (PMA) is considered as an optimized agent, which penetrates only dead bacterial cells rather than living cells with intact membrane, thus resulting in better specificity.[15] The usefulness of PMA-based assay to differentiate live bacilli from dead ones was proven in both clinical MTB isolates and sputum specimens from TB patients.[7,16,17] However, the interpretation of results in previous studies on MTB was always reliant on the signal reduction of ~5 cycles by quantitative PCR,[18] which was significantly lower than 10 cycles' signal reduction from other bacteria species.[12,14] This difference indicated that the pretreatment condition of PMA was not optimal for blocking all the gene amplification from dead MTB, thereby possibly resulting in false-positive results due to inadequate treatment of PMA. In this study, we aimed to optimize the condition of PMA treatment for direct detection of MTB from clinical specimens, and further evaluate its performance for discrimination of live and dead MTB in the clinical sputum samples.

2 | METHODS

2.1 | Preparation of live and dead bacteria

The reference MTB strain H37Rv was subcultured on the Löwenstein-Jensen (L-J) medium for 4 weeks at 37℃. The freshly grown MTB colonies were scraped from the surface of L-J medium and transferred to a sterilized screw cap tube including glass beads and 2 mL normal saline. After vigorous agitation for 1 minute, the turbidity of suspension was adjusted to a 1.0 McFarland turbidity standard. The standards containing 10^3, 10^4, 10^5, and 10^6 bacilli/mL were prepared by serial dilution of the suspension at a 1.0 McFarland turbidity (the proposed bacteria concentration of 10^8 bacilli/mL), respectively. One hundred microliters standards were transferred to a 1.5-mL light-transparent microcentrifuge tube and then heated for 60 minutes at 80°C for the preparation of dead bacteria.

2.2 | Optimization of PMA treatment

Propidium monoazide (Biotium, Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide to yield a stock concentration of 20 mmol/L and stored at −20℃ refrigerator.[14] On the basis of previous findings,[14,18] the light exposure time, dark incubation time, bacterial load, and PMA concentration were varied to determine the optional condition of PMA treatment. Briefly, light exposure times were 10, 20, 40, and 80 minutes; dark incubation times were 10, 20, 40, 60, 120, and 240 minutes; the bacterial loads were 10^2, 10^3, 10^4, and 10^5 CFU/mL; and the PMA concentrations were 100, 200, 400, and 800 μmol/L. PMA was added into the light-transparent microcentrifuge containing 100 μL of live or dead bacteria, and incubated in the dark at 4℃,[17] followed by light exposure to blue light-emitting diode light (BBI, Beijing, China) at room temperature. The PMA-treated bacteria were further analyzed by GeneXpert assay.

2.3 | GeneXpert MTB/RIF assay

Having the PMA-treated bacteria, we adapted it to the GeneXpert MTB/RIF automated assay (Cepheid, Sunnyvale, CA, USA). The Cq value of *rpoB* amplification was used to evaluate the efficacy of PMA treatment by comparing the difference between live and dead samples. In view of five probes yielded by GeneXpert, the Cq values of Probe A were selected to record the Cq value of amplification for the purpose of comparison. The ΔCq was calculated by the difference in amplification yield between live and dead samples treated with the same condition. All the experiments were performed in triple.

2.4 | Specimen collection

A total of 50 smear-positive sputum specimens were collected from newly diagnosed TB patients seeking health care in Changping TB dispensary from January 2015 to January 2016. All clinical specimens were examined by fluorescence staining for acid-fast bacilli (AFB), liquid culture, and the novel molecular method described in this study.

2.5 | Laboratory examinations

Direct smear of each sputum specimen was performed with fluorescence staining for AFB.[19] Grading of the density of AFB on a slide was followed the national guidelines for TB laboratories in China: negative (0 AFB/50 fields), scanty (1-9 AFB/50 fields), 1+ (10-49 AFB/50 fields), 2+ (1-9 AFB/field), 3+ (10-99 AFB/field), and 4+ (⩾100 AFB/field).[19] In addition, the sputum specimens were digested with NALC [N-acetyl-L-cysteine]-NaOH for 15 minutes and then neutralized with phosphate-buffered saline (PBS; pH 6.8) to 45 mL. Followed by the centrifugation at 4000 × *g* for 15 minutes, the sediment was resuspended with 4 mL of PBS. In order to inactivate the tubercle bacilli, 2 mL of the above resuspension was incubated at 80°C for 60 minutes, while the other 2 mL without further treatment was considered as live sample. Then, 0.5 mL of the inactivated sample

and live sample was inoculated into a Bactec MGIT tube according to the manufacturer's instructions, respectively (Becton Dickinson, Sparks, MD, USA). In addition, the residual 1.0 mL of the sediment was treated with PMA following the optimized condition and then detected by GeneXpert assay, respectively.

2.6 | Statistical analysis

Student's *t* test was used for statistical comparisons for determining the optimal conditions of PMA assay. All calculations were performed in SPSS 14.0 (SPSS Inc, USA), and the difference was declared as significant, if *P* value was less than 0.05.

2.7 | Ethics approval

This study was approved by the Ethics Committee of Beijing Children's Hospital (Reference number 2014-47). The methods used in this study were performed in accordance with relevant guidelines and regulations. All the patients taking part in this study signed the informed consent forms prior to enrollment.

3 | RESULTS

3.1 | Determination of optimal light exposure time

We firstly evaluate the correlation between light exposure time and ΔCq. As shown in Figure 1, along with the prolongation of light exposure time, the Cq value of dead bacteria treated with 100 μmol/L PMA was increased from 28.5 to 34.2 cycles, and that of live bacteria was also increased from 24.7 to 27.3 cycles. When the ΔCq was calculated by the comparison between dead and live bacteria treated with PMA, the maximum ΔCq value was observed in the group receiving a light exposure time of 20 minutes, which was significantly higher than the others ($P < 0.05$). Thus, the light exposure time was set as 20 minutes in the forthcoming experiments.

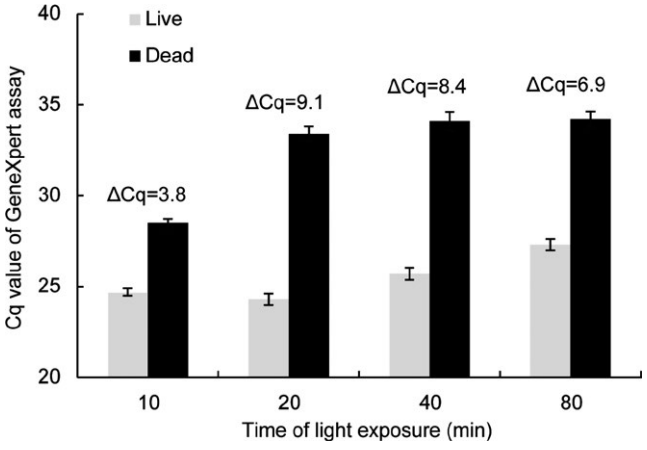

FIGURE 1 Comparison between the mean difference in ΔCq values of PMA-treated samples with different light exposure time. The figure shows the Cq difference between PMA-treated live and dead bacteria with the same light exposure time

3.2 | Determination of optimal dark incubation time

Different dark incubation times were also assessed in the present study (Figure 2). Overall, the prolongation of dark incubation time seemed more likely to result in greater ΔCq value, and the ΔCq values were 2.0, 4.1, 6.5, 10.1, and 12.7 cycles under dark incubation time of 10, 20, 40, 60, and 120 minutes, respectively, while there was no significant difference in ΔCq value between 120 and 240 minutes groups ($P = 0.14$), indicating that the dark incubation time of 120 minutes was optimal for PMA assay in MTB.

3.3 | Effect of bacterial load on ΔCq results

The performance of PMA assay seemed to be influenced by the bacterial load (Figure 3). With the increase in bacterial load, the ΔCq value was significantly decreased, from 12.7 cycles for 10^3 CFU/mL to 7.7 cycles for 10^5 CFU/mL, except for a relative low ΔCq value [ΔCq = 8.7] observed for 10^2 CFU/mL, which may be attributed to the high Cq value of sample containing live bacteria. Notably, the Cq value of sample with a high dead bacteria load of 10^5 CFU/mL was 25.3 cycles, resulting in a "low positive" result by GeneXpert. Hence, another main issue regarding PMA assay was how to yield a favorable result at a high bacteria load, such as sputum samples at a smear grade of 4+.

3.4 | Effect of PMA concentration on ΔCq results

Propidium monoazide concentration is a potential factor influencing the ΔCq results according to previous reports.[17,18] Thus, we investigated whether the addition of high concentrations of PMA provided sufficient efficacy to distinguish dead bacteria from live bacteria at a high load. As shown in Figure 4, the Cq values of dead bacteria samples increased along with the additional supplement of PMA for bacterial loads of both 10^4 and 10^5 CFU/mL. However, we observed that the addition of 800 μmol/L PMA in the reaction mixture showed a switch of color from transparent to red, which led to the invalid result by GeneXpert. As a

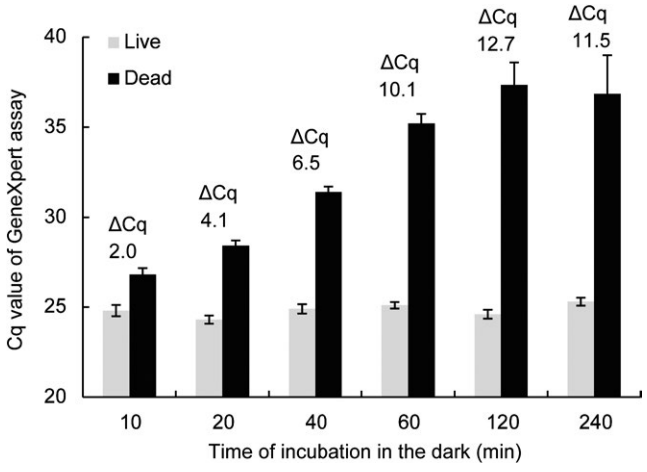

FIGURE 2 Comparison between the mean difference in ΔCq values of PMA-treated samples with different dark incubation time. The figure shows the Cq difference between PMA-treated live and dead bacteria with the same dark incubation time

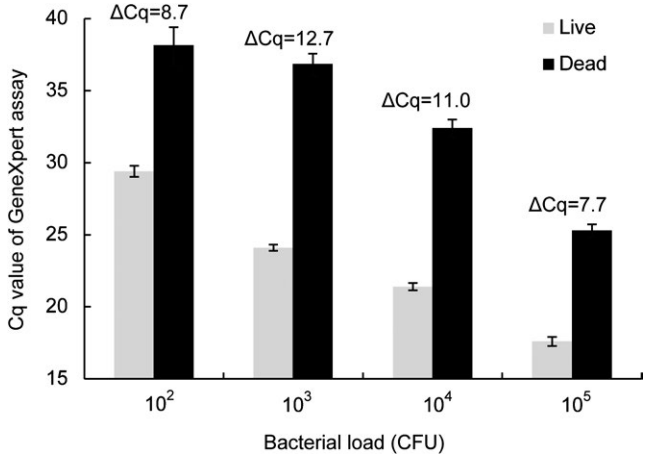

FIGURE 3 Influence of bacterial load on ΔCq values of PMA-treated samples. The figure shows the Cq difference between PMA-treated live and dead bacteria with the same bacterial load

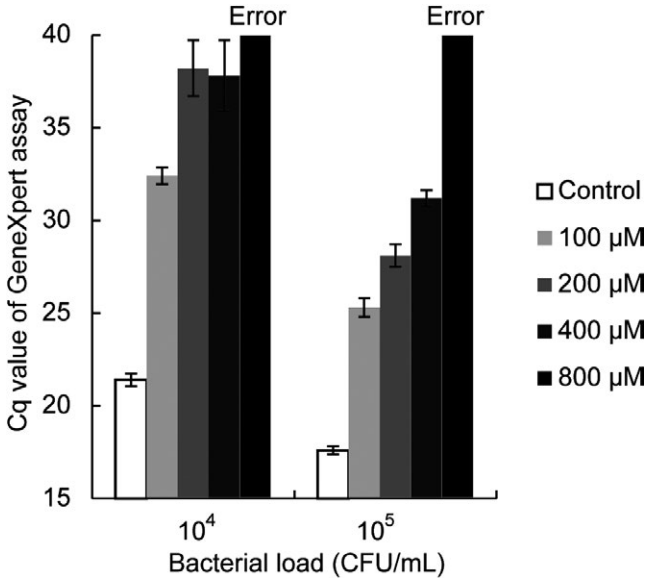

FIGURE 4 Influence of PMA concentration on ΔCq values of PMA-treated samples. The figure shows the Cq difference between PMA-treated live and dead bacteria with the same concentration of PMA. Error represents the invalid result reported by GeneXpert

consequence, 400 μmol/L of PMA seemed to be the highest concentration suitable for PMA assay. We also found that even if 400 μmol/L of PMA was added in the reaction mixture, the sample with a bacterial load of 10^5 CFU/mL yielded a Cq value of 31.2 cycles. Considering the potentially higher bacterial concentration of MTB in the sputum samples with 4+ smear positivity grading, another method rather than only increasing the concentration of PMA was required to improve the detection efficacy of PMA assay for sputum samples with high positivity grading.

3.5 | Dilution of sputum sample with high smear-positive grade

We further assessed whether the dilution of sputum samples with 4+ or 3+ grading provided an alternative for PMA assay among these samples. Five 4+ sputum samples were serially diluted 10-fold in PBS. As shown in Figure 5, all these samples exhibited favorable detection results at the application of 10^4-fold dilution by PMA assay with Cq values higher than 35 cycles. For sputum samples with 3+ grading, similar results were observed in the 10^2-fold dilution, the Cq values of which were higher than 35 cycles.

3.6 | Performance of the PMA assay for detection of live MTB in sputum samples

A total of 50 inactivated/dead samples were collected for evaluating the performance of the PMA assay for detection of live MTB in sputum samples. The treatment of sputum samples with PMA was performed according to the sputum positivity grading (Table 1). The presence of Cq value higher than 35 cycles was considered as an indicator of the detection of dead MTB in the sputum sample. As shown in Table 2, when setting the liquid culture as gold standard, the PMA assay showed an accordance rate of 98.0% (98/100). In addition, all the PMA-treated samples with scanty, 1+ and 2+ positivity grading exhibited 100.0% agreement compared with liquid culture, whereas each inactivated sample from 3+ and 4+ group had a Cq value higher than 34.5 and 33.3 cycles by GeneXpert, respectively, yielding an accordance rate of 90.0% (9/10) and 87.5% (7/8).

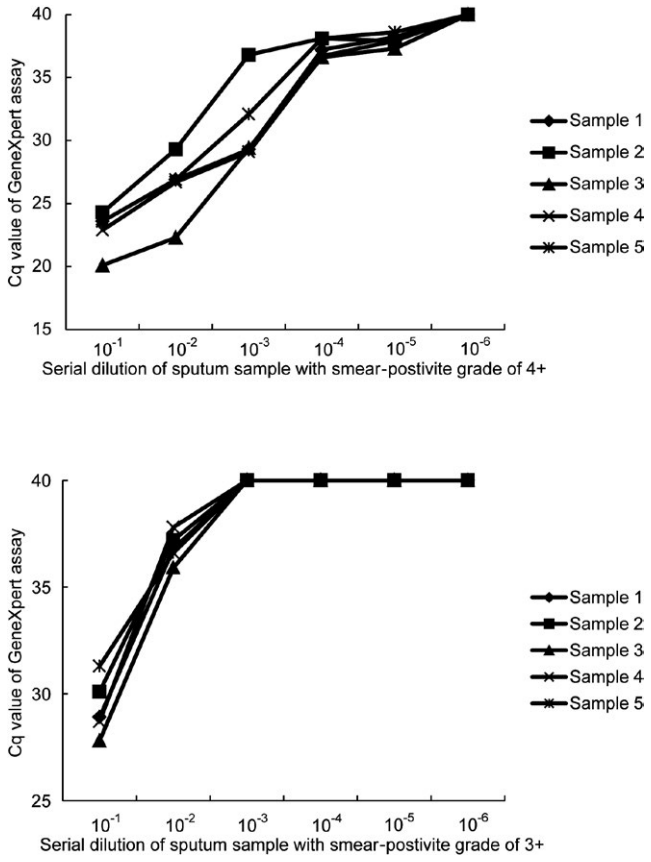

FIGURE 5 Performance of PMA assay on serial dilution samples of clinical sputa at smear grading 4+ and 3+

TABLE 1 PMA treatment condition for sputum samples at different smear positivity grades

Positivity grading	Dilution fold	Concentration of PMA (μmol/L)	Dark incubation time (min)	Exposure time (min)
Scanty	NA	100	120	20
1+	NA	100	120	20
2+	NA	100	120	20
3+	10^2	100	120	20
4+	10^4	100	120	20

NA, Not available.

TABLE 2 Performance of the PMA assay for detection of viable MTB in sputum samples

Smear	No. of sputum samples	C(t) range		Accordance rate[a] (%)
		Viable	Dead	
Scanty	6	29.3-34.2	36.8-38.9	100.0% (12/12)
1+	21	22.5-27.2	35.9-38.1	100.0% (42/42)
2+	14	20.2-24.8	36.1-37.2	100.0% (28/28)
3+	5	16.5-21.3	34.5-38.0	90.0% (9/10)
4+	4	14.3-16.2	33.3-37.9	87.5% (7/8)
Total	50	14.2-34.2	33.3-38.9	98.0% (98/100)

[a]Results of liquid culture were set as gold standard to calculate the accordance rate between PMA assay and liquid culture.

4 | DISCUSSION

The application of PMA has been widely used to distinguish live and dead bacteria.[13–15] The present study confirms previous observations that PMA could be an alternative to predict the treatment outcome of TB patients. Compared with previous studies, our data provide an optimized condition for PMA treatment suitable for detection of live MTB in clinical practice.[17,18] The most important finding of this study is the crucial role of dark incubation in the efficacy of PMA treatment for MTB. Different from other bacterial species, MTB requires 120 minutes of dark incubation time to produce the acceptable efficacy of PMA treatment, which is significantly higher than that of other bacteria.[13,14] During the dark incubation period, PMA penetrates the permeable cell wall and membranes and chemically modifies the DNA of dead bacteria.[12] It is well known that the strikingly thick cell wall of MTB is an efficient permeability barrier that makes MTB naturally resistant to the entrance of chemical substances.[20] As a consequence, despite dead bacteria, it may take longer dark incubation time to allow the sufficient PMA to get access to the DNA of dead MTB. Although the prolongation of dark incubation has no influence on the live bacteria, nearly 2.5 hours of pretreatment with PMA does not meet the criteria for point-of-care diagnostic algorithms. The great challenge of PMA treatment for MTB is its poor permeability. In addition to longer dark incubation time, we speculate that the addition of low concentration of surfactant,[21] such as Tween-80 and Triton X-100, may serve as an alternative for shortening the incubation time. Further study is required to evaluate the potential role of surfactant in the PMA assay for MTB.

In addition, we observed that even 400 μmol/L of PMA could not effectively differentiate live and dead bacteria for sputum samples with 4+ positive grading. On the basis of a recent report, a high proportion of the specimens with high grading were bloody or caseous sputum samples.[19] Hence, the high foreign protein contents may inhibit the efficiency of digested procedure,[19] thereby affecting the penetration of PMA into the dead mycobacterial cells. In addition, several previous studies have demonstrated that the addition of high concentration of PMA not only improves the exclusion of dead bacteria in clinical samples, but also negatively leads to the increase of false-negative results, which is associated with the toxicity of chemical dyes for living cells.[18] Hence, the dilution of clinical specimens with high positive grading may serve as an alternative for application of PMA assay in these samples. Based on our experimental data, we propose to dilute 10^4 and 10^2 for 4+ and 3+ sputum samples in the clinical practice, respectively.

Overall results of this study confirmed that PMA assay could reliably detect the live MTB in sputum, while analysis of paired specimens also revealed that two samples with inactivated bacteria could not be identified by PMA assay, suggesting the dilution ratio may be not efficient to allow the genomic DNA of dead bacteria to be inhibited by PMA for these samples. According to our observations mentioned above, the dilution ratio relies on the smear-positive grades of clinical specimens. However, the small sample volume used in smear microscopy may be responsible for sampling variations and thus lead to quantification error of smear microscopy.[22] The potential quantification error of sputum samples still challenges the clinical application of PMA assay in the future. Fortunately, the high-grade smears only account for a small proportion of smears,[19] which could not appear to play an important role in determining the performance of PMA assay in clinical practice.

We also acknowledge the limitations of this study. First, the recommended cutoff Cq value in this study still has overlap with positive results at very low bacterial load,[23] indicating that the false-negative results may occur in the paucibacillary specimens. Hence, we should pay more attention to the Cq results higher than 35 cycles yielded from samples with scanty grade. Second, we used the harsh condition for the inactivation of tubercle bacilli to avoid the interruption from live bacteria. Although a previous study revealed that the incubation at 80°C did not affect the integrity of the DNA for subsequent molecular investigations,[24] it is difficult to conclude whether this harsh condition has great effect on the permeability of dead bacteria. Third, the study only assessed the PMA assay in a small number of sputum samples. Further studies in clinical samples from follow-up TB patients should be performed to evaluate the performance of

PMA assay in predicting treatment outcome during follow-up period. Fourth, the light intensity and lamp geometry to light sources would affect the efficacy of PMA treatment. Hence, the diversity of blue LED system used by different researchers might weaken the universality of the optimal light exposure time determined in this study.

In conclusion, we develop an acceptable method to directly identify the live bacteria from sputum samples rather than comparing the Cq values of PMA-treated and naïve clinical samples. Our data demonstrate that the dark incubation plays a crucial role in the efficacy of PMA treatment for MTB. In addition, the appropriate dilution of specimens with high grading is essential for differentiation of live and dead bacteria in these samples.

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AUTHORS' CONTRIBUTIONS

Jie Lu and Zuosen Yang designed the experiments. Jie Lu, Huiwen Zheng, Hui Yang, Zhongdong Wang, and Jin Shi conducted the experiments. Jie Lu, Ping Chu, and Shujing Han interpreted the results. Jie Lu and Zuosen Yang wrote the manuscript.

AVAILABILITY OF DATA AND MATERIAL

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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