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MOLECULAR ASPECTS

Utility of propidium monoazide viability assay as a biomarker for a tuberculosis disease

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SUMMARY

Reliable laboratory diagnosis of tuberculosis (TB), including laboratory biomarkers of cure, remains a challenge. In our study we evaluated the performance of a Propidium Monoazide (PMA) assay for the detection of viable TB bacilli in sputum specimens during anti-TB chemotherapy and its potential use as a TB biomarker.

The study was conducted at three centres on 1937 sputum specimens from 310 adult bacteriologically confirmed pulmonary TB patients obtained before commencing anti-TB treatment and at regular intervals afterwards. Performance of the PMA assay was assessed using various readout assays with bacteriology culture results and time to positivity on liquid media used as reference standards.

Treatment of sputum with N-acetyl-cysteine was found to be fully compatible with the PMA assay. Good sensitivity and specificity (97.5% and 70.7–80.0%) for detection of live TB bacilli was achieved using the Xpert® MTB/RIF test as a readout assay. Tentative Ct and ΔCt thresholds for the Xpert® MTB/RIF system were proposed. Good correlation ($r = 0.61$) between Ct values and time to positivity of TB cultures on liquid media was demonstrated.

The PMA method has potential in monitoring bacterial load in sputum specimens and so may have a role as a biomarker of cure in TB treatment.

1. Introduction

Tuberculosis (TB) still poses a major health problem globally causing over 10 million new cases and 1.7 million deaths annually [1]. Despite recent advances in the development of new diagnostic tools, based largely on molecular techniques [2–4] reliable laboratory diagnosis of TB remains a challenge. Similarly reliable measures of non-infectivity/cure remains problematic [5] especially in drug resistant cases and high-burden settings where multiple re-infection episodes may occur. Viability assays distinguishing between viable and dead mycobacterial cells, could serve as good biomarkers for a TB disease and cure [6–8]. Existing rapid molecular tools based on the detection of relatively stable DNA fragments, including nucleic acids amplification tests (NAAT), line-probe assays

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(LPAs) and Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, USA) cannot always differentiate viable and non-viable cells [2,9,10].

Since mRNA molecules are produced only by metabolically active bacteria, assays based on detection of specific mRNAs were amongst the first assays developed for distinguishing between live and dead cells, for example detection of 85B RNA and other RNAs in sputum [7,11,12]. However, working with the RNA could pose a challenge especially in resource-poor settings lacking adequate laboratory and transport conditions due to the RNA unstable nature and other methodological issues [13,14].

Permeability assays utilising DNA-intercalating dyes selectively binding to the DNA within damaged cells constitute an alternative approach [15–17]. Photolysis of DNA-intercalating dyes containing azide group, including ethidium monoazide (EMA) and propidium monoazide (PMA) produces nitrene molecules which form covalent links with DNA subsequently inhibiting PCR; DNA within live cells is not affected by the dyes and therefore could be amplified [17]. A variety of readout assays, including any PCR-based techniques (NAAT, LPAs, real-time PCR) could be used for result detection.

Of these two compounds, PMA proved to be more specific for bacterial cells (probably due to a presence of two positive charges) and has been applied to a variety of bacterial species, including *Mycobacterium tuberculosis* and non-tuberculosis *Mycobacteria* (NTM) [8,18] as well as *Listeria* [19], *Vibrio vulnificus* [20], *Helicobacter pilori* [21], *Streptococcus* spp [22]. Although PMA-based assays proved feasible for the differentiation viable from non-viable cells, performance varied by bacterial species and also was affected by the presence of inhibitors as well as other assay-related factors including dye concentration, length and intensity of light exposure resulting in incomplete signal suppression and false positive results [13,23]. For *Mycobacteria*, increasing the concentration improved specificity [8]. Overall studies produced contradictory results indicating a need for further improvement of these promising methodologies.

In our study conducted within a multi-center project on TB biomarkers, we aimed to evaluate the performance of the PMA assay for the detection of viable TB bacilli in sputum specimens during anti-TB chemotherapy and its potential use as a TB biomarker.

2. Materials and methods

The study was conducted by a research network comprising sites in UK, Italy, Russia, Lithuania, and Latvia. Ethics approvals were received at all participating sites. All patients gave signed informed consent before the recruitment.

2.1. Initial optimization

We optimized the previously developed protocol [8] using different liquefying agents on artificial sputum specimens spiked with *M. tuberculosis* H37Rv (NCTC7416) and a Xpert® MTB/RIF as a readout assay. Optimization also included development of the assay reading rules using qPCR Ct values. Details of the optimization protocol are available online as Supplementary material.

2.2. Validation and evaluation of the assay performance on clinical specimens

2.2.1. Study population

For the subsequent validation and evaluation phases, sputum specimens were obtained from 310 adult (≥18 y.o.) patients with culture-confirmed pulmonary TB recruited across three field sites (see Supplementary Table 1). These were new non-MDR TB cases and MDR TB cases commencing first- or second-line antimicrobial therapy as well as re-treatment non-MDR TB (relapses, treatment after default or failure) and MDR TB cases commencing a new cycle of first-line or second-line antimicrobial therapy. All patients received routine treatment in accordance with WHO and local guidelines.

From each patient, sputum was collected before commencing treatment and then at the following time points: 2 weeks; 1st, 2nd, 3rd, penultimate and ultimate months of treatment (for non-MDR patients), and at 1st, 2nd, 3rd, 4th, 5th, 6th, 12th, 15th, 18th, 24th, and ultimate months of treatment (for those infected with the MDR TB). Total number of sputum specimens available for analysis in validation and evaluation phases was 1937.

2.2.2. Specimen collection, treatment, microscopy and bacteriology

Three sputum specimens were collected from each patient at each time point. The first specimen was decontaminated by conventional NALC-NaOH treatment (final NaOH concentration, 1%) and subsequently used for smear preparation and culturing using solid (LJ) and liquid (Bactec MGIT 960; Becton Dickinson, United States) media. Second and third specimens were pooled together and liquefied prior to the PMA treatment to preserve viability of mycobacterial cells by adding an equal volume of 0.5% NALC solution (Supplementary Material). After 15 min incubation liquefied sputum specimens were split into two portions, one of which was decontaminated by adding NaOH solution and used for culturing on solid LJ media. The second portion was diluted with sterile PBS (pH = 7.0) to 50 ml and centrifuged at 3000 g for 15 min. After centrifugation, the resulting pellet was resuspended in 2 ml of sterile PBS and used for microscopy and the PMA assay.

Microscopy and growth on solid media grading was performed on all specimens according to WHO recommendations [24]. On liquid media the time to positivity in hours was recorded; specimens displaying no growth on day 42 were considered negative.

Identification of *M. tuberculosis* complex was performed using visual assessment, microscopy and the molecular GenoType® Mycobacterium CM assay (Hain Lifescience GmbH, Nehren, Germany).

2.2.3. PMA assay

PMA treatment, subsequent DNA extraction and readout were performed on liquefied sputum pellets resuspended in PBS according to the PMA optimized protocol (Supplementary material 1). PMA solution in sterile deionised water was added to 200 μl of liquefied sputum samples to a final concentration of 100 μM or 500 μM (Table 1) and processed as described. After 15 min light exposure specimens were used for DNA extraction or loading into

Table 1
Specimens, readout assays and PMA concentrations used in the study.

Field site	Readout assay and PMA concentration				
	Hain MTBDR*plus* v 1.0	Hain MTBDR*plus* v 2.0	GeneXpert MTB/RIF G3 v.3	GeneXpert MTB/RIF G4 v.5	
	100 μM	100 μM	500 μM	100 μM	500 μM
Samara	531	694	–	–	–
Vilnius	357	–	81	100	242
Riga	208	221	–	–	–

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