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Early tuberculosis treatment monitoring by Xpert® MTB/RIF

To the Editors:

Progress made to improve laboratory capacity for tuberculosis (TB) diagnosis led to the development of molecular assays that are now replacing conventional microscopy and culture-based methods on a large scale [1, 2]. Unfortunately, current molecular techniques detect both live and dead bacteria, and a positive result does not imply the viability of the pathogen. Indeed, DNA can persist for a long period after bacterial death and nucleic acid from dead bacteria is equally amplifiable. Therefore, molecular assays are unsuitable for treatment monitoring and/or for infection control purposes.

We report an innovative approach to selectively amplify DNA derived from viable *Mycobacterium tuberculosis* in clinical specimens, which is useful for monitoring mycobacterial load in pulmonary TB patients during anti-TB treatment.

The protocol is based on pre-treatment of samples with propidium monoazide (PMA; Biotium Inc., Hayward, CA, USA), a chemical compound that can intercalate the DNA of non-viable (or membrane-damaged) organisms but is excluded from viable bacteria. After light activation, PMA binds covalently to the DNA, preventing its amplification by PCR [3]. After light exposure, unbound PMA is not able to interact further with DNA molecules.

The assay was first optimised using acid-fast bacilli (AFB)-negative sputum samples spiked with dead or live mycobacteria at different concentrations. In brief, live *Mycobacterium fortuitum* cells were added to *N*-acetyl-cysteine decontaminated sputum specimens negative for AFB by smear microscopy at a final concentration of 10^6 bacteria·mL^{-1}. An aliquot of this laboratory-made sample was treated to heat kill the *M. fortuitum* cells. PMA stock solution was prepared and stored at -20°C and protected from light exposure, until use, as recommended by the manufacturers. PMA was added as a pre-treatment at a final concentration of 500 µM and incubated for 30 min at 4°C in the dark, followed by light exposure to blue light-emitting diode (LED)-active light (GenIUL, Terrassa, Spain) for 15 min at room temperature. DNA was then extracted using a standard phenol chloroform procedure. As a control to evaluate the efficacy of the light exposure step, an aliquot of naked DNA extracted from an *M. fortuitum* culture was treated with 500 µM PMA previously exposed to the LED light for 15 min. The commercial line-probe assay (GenoType® Mycobacterium CM; Hain Lifescience, Nehren, Germany) was then performed in order to identify clinically relevant mycobacterial species [4]. Samples containing live *M. fortuitum* showed a normal hybridisation profile on a nitrocellulose strip, whereas samples treated to kill bacteria did not show any amplification, except for the internal control (data not shown). Since naked DNA treated with light-inactivated PMA showed a normal hybridisation profile, the light exposure step was efficient and the PCR was not further inhibited by residual PMA.

Having optimised the protocol for the inactivation of DNA derived from dead bacteria, we adapted it to the Xpert® MTB/RIF automated assay (Cepheid, Sunnyvale, CA, USA). The real-time PCR performed by the Xpert® MTB/RIF provides threshold cycles (Ct) that can be used to calculate the difference in amplification yield between samples with and without PMA pre-treatment (ΔCt): a low ΔCt indicates the presence of amplifiable DNA from live bacteria, whereas a high ΔCt indicates that target DNA in the sample originated from dead or damaged bacteria and, therefore, PMA pre-treatment significantly affects its amplification. To calculate the ΔCt value we considered the mean between the Ct values provided for each probe included in the Xpert® MTB/RIF test (A to E) [5].

We tested the PMA protocol using the Xpert® MTB/RIF assay on a Ct calibration curve of AFB-negative clinical specimens with heat-killed/live mycobacteria added in different ratios. High dead–live ratios resulted in a maximal difference in ΔCt values between heat-treated and live portions with PMA, whereas samples containing a similar percentage of killed and live bacteria could not be differentiated from one another by the use of PMA

pre-treatment (data not shown). Similar data have been reported by KRALIK *et al.* [6].

Finally, we tested the approach on clinical specimens. 10 patients diagnosed with active pulmonary TB (sputum smear positive and culture positive) were included in the first validation study. Samples were collected at the time of diagnosis (*t*0) and after 10–20 days of therapy (*t*1), *N*-acetyl-cysteine decontaminated and processed for MGIT-960 liquid culture (BD Diagnostic Systems, Sparks, MD, USA) according to international guidelines [7]. All patients were still AFB-positive by sputum smear microscopy at *t*1. In parallel, 250 μL of decontaminated samples were processed for molecular analysis by Xpert® MTB/RIF assay with and without PMA pre-treatment. Samples were then processed as recommended by the manufacturer's instructions for the Xpert® MTB/RIF assay.

As shown in figure 1, PMA did not significantly affect PCR yield of specimens collected at *t*0 (mean±SEM ΔCt 2.3±0.5), whereas specimens collected during therapy at *t*1 showed a ΔCt of 10.5±0.9 after PMA treatment (p=0.0003) confirming that the sputum smear positivity of these samples was mostly due to highly damaged bacteria. Moreover, ΔCt calculated between *t*0 and *t*1 in PMA-untreated samples was found to be too low (ΔCt 2.9±0.9) to appreciate a real decrease in bacterial load due to the therapy.

Two patients rated ''low'' at *t*0 by the Xpert® showed a negative culture at *t*1, whereas all the other patients rated ''medium'' or ''high'' remained culture positive by MGIT. Although an exact time to positivity cannot be assessed, we observed that cultures from samples collected at *t*1 became positive approximately 7–10 days later compared with cultures from samples collected at *t*0, suggesting a severe reduction in the live bacterial load.

All patients were successfully treated and cured at the end of therapy, and this was consistent with the reduction of live bacteria detected by the PMA assay.

As a negative control, we retrospectively also included a treatment failure case (AFB positive and culture positive after 5 months of standard treatment). In this case, PMA pre-treatment of both decontaminated sputum specimens collected at 1 month and two during treatment did not significantly affect PCR yield (ΔCt ~2), thus supporting inefficacy of anti-TB treatment.

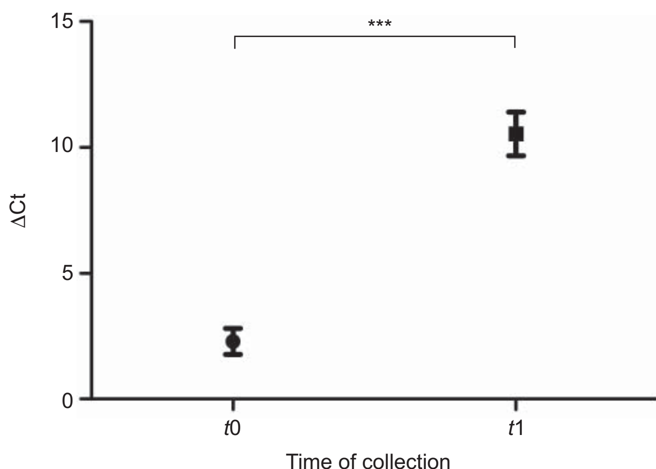

FIGURE 1. Comparison between the mean difference in threshold cycle (ΔCt) (propidium monoazide (PMA) treated minus PMA untreated) obtained from sputum samples collected before starting treatment (*t*0) and 10–20 days after the beginning of anti-tuberculosis therapy (*t*1). Errors bars represent ± SEM values. ***: p<0.001.

The same protocol should, in principle, be compatible with other CE-approved molecular tests and line probe assays endorsed by the World Health Organization for the diagnosis of TB; further studies are needed to rule out this possibility.

Previous studies [3, 6] have demonstrated the possibility of using PMA to distinguish between live and dead mycobacteria using a concentration of 25–50 μM. During protocol set up, a final concentration of 100 μM fully avoided amplification of DNA derived from heat-killed *M. fortuitum*; a weak background due to the amplification of DNA from heat-killed *M. tuberculosis* was observed (data not shown). We can speculate that the different cell wall composition somehow affects the capacity of PMA to penetrate damaged mycobacteria. Indeed, KRALIK *et al.* [6] observed that the PMA-induced signal reduction between live and dead *Mycobacterium avium* subsp. *paratuberculosis* were lower compared with those found for other bacterial species. In addition, considering the amount of debris that could potentially sequester PMA molecules in decontaminated sputa, we increased the final concentration to 500 μM. Further studies evaluating differences of PMA treatment effect on strains showing different cell wall composition (*e.g.* Beijing strains) and/or in sputa with different characteristics (*e.g.* blood-containing sputum) could better elucidate the usefulness of this test in different clinical settings.

According to LØVDAL *et al.* [8], a small proportion of cells presumed to be dead or heavily injured are not able to grow. The presence of a small number of cells that are heavily injured but non-culturable could explain why we still have a positive signal in samples collected at *t*1 despite the PMA pre-treatment. PMA pre-treatment does not avoid the detection of a small proportion of dead cells (false positive for a viability assay): the test might, therefore, overestimate viable mycobacteria. However, from a clinical perspective, this would represent a minor error compared with the risk (very small for this test) of underestimating viable mycobacteria.

Our data indicate, for the first time, that quantitative molecular techniques combined with the PMA method could be an alternative to direct microscopy and culture for monitoring early treatment response and for preliminary evaluation of personalised regimens. The use of this assay can allow earlier evaluation of treatment efficacy, showing a clear decrease in the vital mycobacterial load. However, the absence of the response to therapy might also be promptly identified by the test allowing a regimen change and limiting the spread of infection and further resistance development [9].

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New opportunities in tuberculosis control

To the Editors:

The main objective of a tuberculosis (TB) control programme is to break the chain of transmission in the community [1, 2]. Early identification and effective treatment of infectious cases is key. It is assumed that active TB may develop in ~10% of infected patients, usually within 2 yrs of infection [3].

In our centre (Hospital Center of Vila Nova de Gaia/Espinho, Vila Nova de Gaia, Portugal), reflecting the Portuguese policy, the focus of TB contact investigations used to be only on the persons named by the index case. In 2004, we decided to include a routine evaluation of every patient's home and work environment with the co-operation of public health teams [4].

According to Portuguese guidelines, a close contact is defined as a subject exposed to the index case for >8 h daily or >40 h of cumulative exposure time. All these identified contacts are requested to undergo a screening programme, which includes a symptoms questionnaire, clinical examination, a tuberculin skin test (TST) with 2 TU of purified protein derivative RT 23, an interferon-γ release assay (IGRA) and chest radiography. TST reactions >10 mm in diameter are confirmed with IGRAs. 6 months of chemotherapy with isoniazid is offered to contacts with latent TB infection (LTBI) [5]. The rate of compliance with this preventive treatment, which is not compulsory, has been described to be between 19% and 96% [6].

In this letter, we present a report where failing to identify at-risk contacts and the refusal of preventive therapy resulted in a family cluster.

In 2001, a 28-yr-old female was diagnosed with TB in our outpatient TB clinic. The sputum was smear- and culture-positive for *Mycobacterium tuberculosis*, and the isolate was resistant to streptomycin. The patient began treatment with isoniazid, rifampicin, pyrazinamide and ethambutol in the form of directly observed treatment (DOT). The clinical, radiological and bacteriological courses were favourable.

At that time, contact investigation was based only on clinical interview. The patient named one contact, her husband. The husband was asymptomatic, had normal chest radiography and a positive TST of 21 mm diameter. He was offered preventive treatment but refused. Follow-up was proposed and scheduled, but he failed to attend.

In 2008, the husband came to our outpatient TB clinic, complaining of cough, dyspnoea and asthenia. Chest radiography revealed an infiltrate with cavities in the right apical region. The sputum was smear- and culture-positive for *M. tuberculosis*, and the isolate was resistant to streptomycin. He began treatment with isoniazid, rifampicin, pyrazinamide and ethambutol in the form of DOT. The clinical, radiological and bacteriological courses were favourable.

During clinical interview, this male named only two contacts, both of them cohabitants: his wife and his 6-yr-old son. The wife did not present any sign of recurrent TB. The child presented growth retardation, cough and low-grade fever. Chest computed tomography revealed mediastinal adenopathy and subpleural consolidation at the left lung apex. He began treatment with