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Visualizing viable *Mycobacterium tuberculosis* in sputum to monitor isolation measures

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In this *Journal*, Awoniyi and colleagues evaluated cytokine responses against novel Mtb antigens as diagnostic markers for TB disease. However a practical issue for physicians looking after patients with TB is being able to distinguish smear positive/culture negative patients during the early phases of treatment.[1] Isolation measures are mandatory for patients hospitalized with pulmonary tuberculosis (TB), in order to prevent transmission.[2] Although the average time to culture conversion is one month for patients with drug-susceptible TB,[3] 5%—30% cases require more than two months[4] and up to 6 months can be required for MDR-TB.[5] Recommendations are to maintain isolation until patients are no more infectious, which is ascertained when cultures of respiratory specimens become negative.[2] Microscopic examination of acid-fast bacilli (AFB) is mainly used as a surrogate marker[6,7] but because AFB staining do not differentiate alive from dead bacilli, the interpretation of AFB-positive smears is misleading. After 1—2 months of treatment, AFB can be considered dead while they are still alive and isolation measures are thus erroneously discontinued. Conversely, AFB can be considered viable while they have already been killed by the treatment and patients kept inadequately in isolation.[8] To overcome the misleading results, attempts have been made to develop viability biomarkers for Mtb. Studies using NAAT targeting bacterial RNA/DNA gave convincing results but their cost is not affordable in most part of the world.[9] Those using fluorescein diacetate are easiest but studies have shown either conflicting results, or have been evaluated only for a short period of time after initiation of therapy.[10]

Our objective was to develop a viability test to predict the culture results of Mtb by visualizing live bacilli in sputum of smear-positive tuberculosis patients under treatment. We evaluated a fluorescent staining able to differentiate dead bacilli from alive, which was previously used for detection of industrial environmental pathogens. We firstly adapted the kit to mycobacteriology and assessed, in *in vitro* experiments, the concordance of the test with the culture results. Then, we studied in an observational prospective study its accurateness to predict culture results in patients undergoing antituberculous therapy.

Briefly, the *Live/Dead® BacLight™ Bacterial viability test* (Invitrogen, Biocentric, France) permits to visualize the bacteria using SYTO-9 and propidium iodide (PI) fluorescent dyes, which both bind to DNA but penetrate specifically cytoplasmic membrane: SYTO-9 penetrates all bacilli, either viable or not, whereas the PI penetrates only in cells with damaged membrane. Consequently, the viable bacteria are impermeable to PI and only fluoresced due to SYTO-9 appearing green under the fluorescent microscope, whereas dead bacteria are marked by both fluorescent dyes and appear red.

A bacterial suspension (McFarland standard 1.0, approximately 10^8 CFU/ml) of *M. tuberculosis* H37Rv was submitted to heat inactivation for 5 h at 96 °C, one sample taken every hour. A same suspension was submitted to the activity of isoniazid at 1 mg/L (Sigma—Aldrich, Quentin Falavier, France), one sample was taken after 7 and 14 days of contact. Then, 10 μl of each fluorescent dye (SYTO-9: 6 mM, PI: 30 mM) were mixed with 20 μl of the bacterial suspension sample and incubated for 15 min at room temperature hidden from light. The results were visualized under a

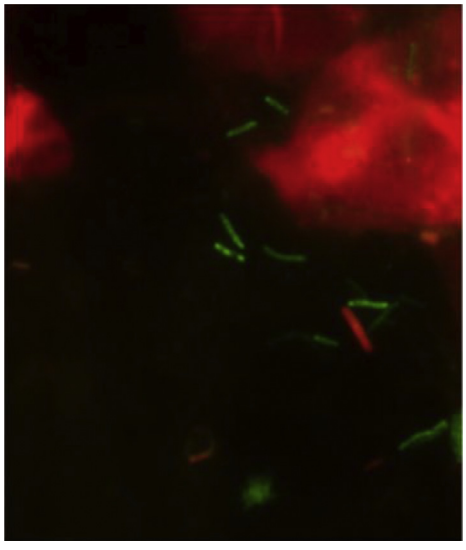

Figure 1 Viability microscopic direct test using fluorescent microscopy (kit LIVE/DEAD® *Bac*Light™) for a AFB-smear positive sputum specimen. Bacteria were incubated with SYTO-9 fluorescent dye, which penetrates all bacteria viable or not, and with propidium iodide (PI) fluorescent dye which only penetrates in cells with damaged membrane. Consequently, the viable bacteria, that are impermeable to PI, are only marked by SYTO-9 and appear green under the fluorescent microscope, whereas dead bacteria are marked by both fluorescent dyes and appear red.

fluorescent microscope (Prototype Optical LED fluorescent microscope, RAL Diagnostics, Martillac, France) at ×40 and ×100 magnification using B-2A filters and G-2A filters (excitation spectrum 450—490 and 510—560 nm, respectively) with 7 μl of the mix spotted on pre-circled slides. An example of visual results is shown in Fig. 1.

We measured the proportion of green (alive) bacilli/total green + red (dead) bacilli after heat inactivation. This proportion decreased from 90% to 40% after 1 h but 3 h were necessary to reach 0%. After isoniazid contact, the median (mean) proportion of live bacilli decreased from 80% (80% ± 0.12%) to 33% (40% ± 0.12%) after 7 days and to 13% (13% ± 0.15%) after 14 days.

We included 18 patients (mean age: 37 ± 10.5 years) admitted for smear-positive pulmonary tuberculosis and hospitalized at Bligny Hospital (Briis-sous-Forge, France). Fifteen (83%) patients were infected with a pan-susceptible *M. tuberculosis* isolate and started a standard regimen; one patient was infected with an isolate showing high-level resistance to isoniazid and two patients with MDR strains received regimens adapted to antibiotic susceptibility testing results. The patients were followed until the discharge. Respiratory specimens were routinely collected and submitted to the standard procedures[11] (Auramine smear microscopy and culture in liquid and solid media after decontamination) as well as the microscopic direct viability test. A 10-μl sample was used for viability staining according to the protocol used in the *in vitro* experiments.

After starting treatment, seven patients (39%) and three patients (17%) were still smear-positive after four and eight weeks of treatment, respectively. The number of patients who converted to culture-negative according to time is

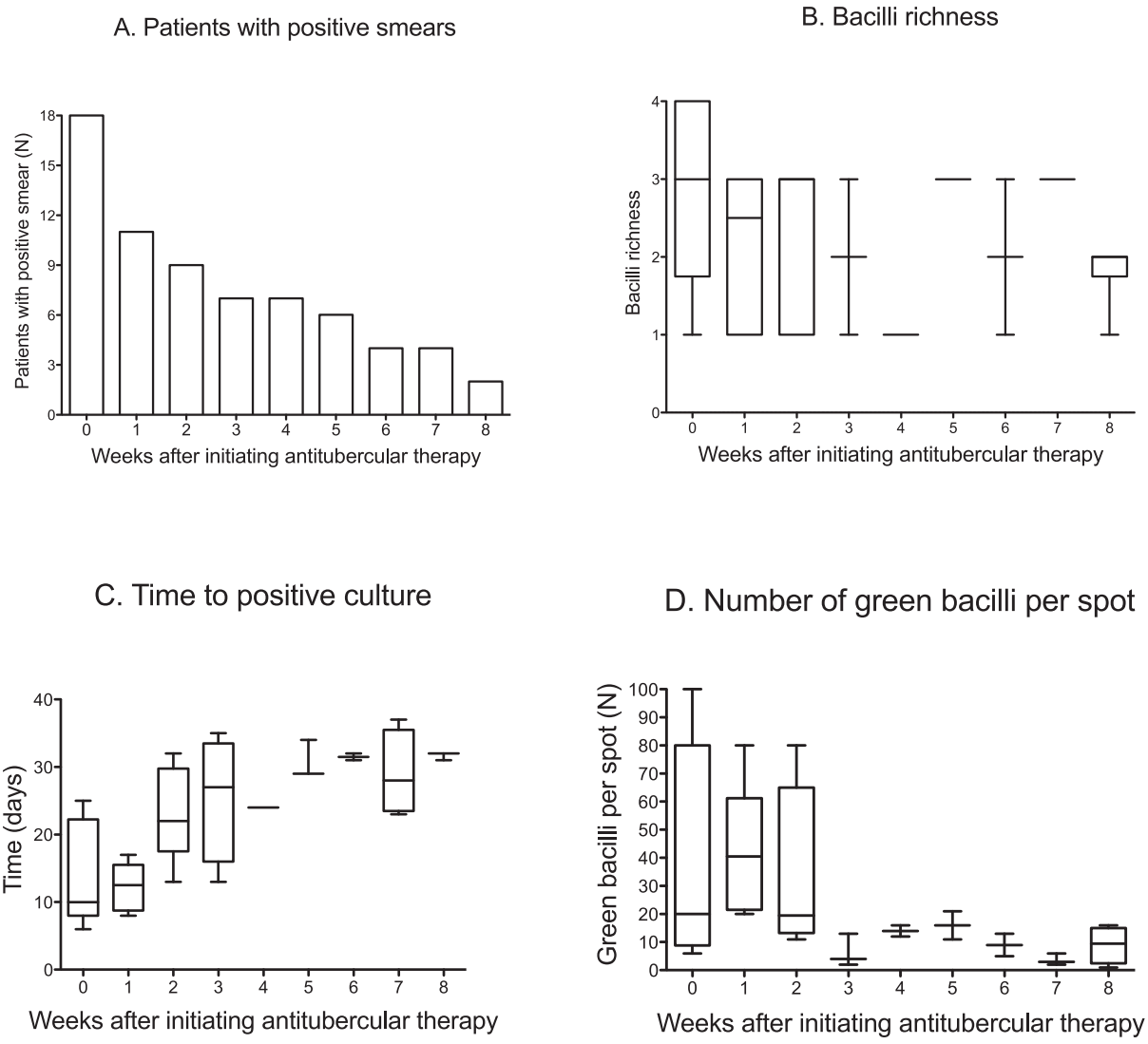

Figure 2 Clinical results of the microscopic viability test assessed for sputa collected in 18 smear-positive patients according to the weeks under antituberculous therapy. A) Number of patients treated for pulmonary tuberculosis who converted to culture-negative specimens according to time. B) AFB-positivity according to the WHO semi-logarithmic scale (<1, 1—9, 10—99 and ≥100 AFB/microscope field by Auramine staining) for all specimens collected. C) Time to culture to be positive (liquid or solid culture, the time of positivity being the first to be positive) of all specimens collected. The median time decreased significantly (Kruskall—Wallis test, $p < 0.0001$). D) Viability of *M. tuberculosis* bacilli on direct microscopic examination of sputum smears estimated by the number of green (stained by SYTO-9) bacilli per spot, in all specimens collected.