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DIAGNOSTICS

Improving acid-fast fluorescent staining for the detection of mycobacteria using a new nucleic acid staining approach

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SUMMARY

Acid fast staining of sputum smears by microscopy remains the prevalent method for detecting *Mycobacterium tuberculosis*. The sensitivity of microscopy using acid fast stains requires 10^4 bacilli per ml of sputum. Although fluorescent acid fast stains, such as Auramine-O, show improved sensitivity, almost half of culture-positive TB cases are currently estimated to remain smear-negative. These current diagnosis problems provide impetus for improving staining procedures.

We evaluated a novel fluorescent acid-fast staining approach using the nucleic acid-binding dye SYBR® Gold on mycobacterial *in vitro* cultures. The SYBR® Gold stain detected 99% of MTB in both actively replicating aerobic and non-replicating hypoxic cultures. Transmission light microscopy with Ziehl–Neelsen fuchsin, and fluorescence microscopy with Auramine-O or Auramine-rhodamine detected only 54%–86% of MTB bacilli.

SYBR® Gold fluoresces more intensely than Auramine-O, and is highly resistant to fading. The signal to noise ratio is exceptionally high due to a >1000-fold enhanced fluorescence after binding to DNA/RNA, thereby reducing most background fluorescence. Although cost and stability of the dye may perhaps limit its clinical use at this time, these results warrant further research into more nucleic acid dye variants. In the meantime, SYBR® Gold staining shows great promise for use in numerous research applications.

1. Introduction

The World Health Organization (WHO) estimated that there are more than 8.7 million new clinical cases of tuberculosis disease in 2008 [1]. Microscopy of acid-fast stained sputum smears is currently the most widely used means of identifying *Mycobacterium tuberculosis* (MTB) – infected individuals. Once stained, mycobacteria resist the dilute acid and/or ethanol-based de-colorization procedures common in staining protocols, hence the name *acid-fast*. Although microscopy is inexpensive, relatively easy to perform in resource-limited settings, and is capable of detecting MTB in less than one hour, its low sensitivity is a major problem. The sensitivity of sputum smear microscopy has been reported to range from 20% to greater than 80% [2–14], and is considerably lower when patients are co-infected with HIV [5,15,16]. The WHO reports that only 28% of MTB cases are in fact smear positive with currently used acid fast staining methods [1]. This issue alone highlights the need for improved diagnostics of MTB.

The relatively low diagnostic sensitivity of sputum smear microscopy also arises from time-pressed observers' inability to analyze more than a small portion of each sputum sample, typically no more than a fraction of a microliter [1,17,18]. Using a higher sputum volume for staining on a microscopic slide is problematic, as both background staining and decreased light transmission significantly impede the signal, thereby making even adequately stained organisms harder to detect. As a result, it has been estimated that the limit of detection of fuchsin-based acid-fast staining for MTB in unconcentrated sputum is 10^4 bacilli per milliliter [18–20]. An additional issue with current chromogenic acid-fast stains, such as the Ziehl–Neelsen (ZN) method, is that the microscopic analysis itself is labor-intensive. High magnifications are required to visually analyze sputum smears whereas the results of any of the fluorescent acid-fast stains can be visualized at far lower magnifications [21]. These issues of sensitivity and user fatigue highlight the need for improved acid-fast stains.

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The specificity of current acid-fast stains poses additional problems with regard to their use in both diagnosis and in experimental studies of MTB infection. It is known that MTB bacilli may lose their acid-fastness *in vivo* and *in vitro* under certain conditions [18,22–25]. In an earlier publication, we reported a substantial acid-fast negative bacterial population that could be visualized by other detection methods, such as immunofluorescence using antibodies against whole MTB cell lysate, and that these acid-fast negative bacilli coexisted within the same microenvironment alongside acid-fast positive bacilli in *in vitro* cultures of MTB, as well as in mouse and guinea pig lung tissue sections [26]. Although acid-fast staining of sputum samples is a common practice today, the percentage of live, acid-fast negative bacilli by current staining methods in routine TB patient sputum samples is not known with any precision.

Initial clinical diagnosis of TB is routinely performed by acid fast staining, generally followed by culture on solid or liquid media for confirmation [2]. Acid-fast staining of sputum samples can be processed within a day, whereas *in vitro* culture results are only obtained in several weeks time. Although recent efforts at improving the sensitivity of diagnostics have focused on molecular methods such as the PCR based Xpert®MTB/RIF [27] which can detect MTB at levels in the range of 100 CFU/mL sputum (like culture), these typically require more labor and material resources than does microscopy. Other approaches still in experimental stages have been reported, such as the use of fluorophages ϕ^2GFP10, which can detect and potentially identify the drug resistance profile of MTB in clinical sputum samples within days [28]. Some relatively simple modifications of the existing acid-fast staining technique have been considered more recently that might aid in lowering the limit of detection of MTB [such as thick sputum smears or membrane filtration [29–31]], potentially decreasing the current discrepancy between frequencies of smear positivity and culture positivity and allowing for better diagnosis even in resource-limited settings.

In this paper, we describe the results obtained with a new fluorescent acid-fast stain for MTB employing SYBR® Gold (Life Technologies, Eugene, OR), a dye initially developed for highly sensitive and selective staining of DNA and RNA on gels [32]. We selected this proprietary asymmetrical cyanine dye for acid-fast staining purposes based on both its target abundance and specificity (being DNA and RNA), and its unique fluorescence spectral characteristics. SYBR® Gold exhibits >1000-fold fluorescence enhancement on binding to double- or single-stranded DNA or to RNA, with a high quantum yield (~0.7), and provides low fluorescence background and strong fluorescence intensity of stained material than do other nucleic acid dyes. Excitation maxima for SYBR® Gold–nucleic acid complexes are at ~495 nm in the visible and ~300 nm in the ultraviolet wavelength ranges, compatible with excitation by blue LEDs (light-emitting diode) as well as arc and filament lamps. The emission maximum is at ~537 nm.

Our acid-fast staining protocol with SYBR Gold used a conventional acid-alcohol wash to remove any excess of stain from the microscopic slides, with the aim of providing an unbiased comparison with other dyes commonly used for MTB detection. Comprehensive microscopic analyses were performed on *in vitro* MTB cultures under actively replicating and non-replicating conditions for research purposes [33]. The specificity of the SYBR Gold staining method for mycobacteria was addressed by staining various other mycobacterial species and Gram-positive and Gram-negative bacterial strains.

2. Materials and methods

2.1. Bacterial isolates

In vitro studies were performed with the H37Rv strain of *M. tuberculosis* (ATCC number 27294), grown from low passage seed lots in Proskauer-Beck liquid medium containing 0.05% Tween 80 to early mid-log phase and frozen in aliquots at −70 °C until needed. Aliquots were subsequently thawed and grown to an optical density (OD) 0.6 at 600 nm with rapid stirring at 37 °C in DTA medium (Dubos broth base; Difco/Becton Dickinson) supplemented with Tween 80 (0.05%), bovine serum albumin (BSA) (0.5%), and glucose (0.75%).

Samples from frozen stocks of other mycobacteria species were used in this study; *Mycobacterium aurum* (ATCC#23366), *Mycobacterium flavescense* (ATCC#14474), *Mycobacterium fortuitum* (ATCC#14467), *Mycobacterium smegmatis* (ATCC#14468) and *Mycobacterium xenopi* (ATCC#19250) were obtained from ATCC, and *Mycobacterium denneng, Mycobacterium malmoense, Mycobacterium terrae and Mycobacterium vaccae* were from the Trudeau Collection.

Gram positive and Gram negative strains were obtained from ATCC and grown in media as per ATCC recommendations; *Nocardia farcinica* (ATCC 3308), *Streptococcus pneumonia* (ATCC 49619-FZ), *Enterococcus faecalis* (ATCC 29212) and *Escherichia coli* (ATCC 25922).

2.2. M. tuberculosis in vitro oxygen depletion model

The protocol used to grow *M. tuberculosis* under hypoxic conditions has been described earlier by Voskuil et al. as the rapid anaerobic dormancy (RAD) model [33]. Briefly, cultures used in the dormancy model were first grown to an OD 0.6 at 600 nm with rapid stirring at 37 °C with DTA medium supplemented with 0.05% Tween 80, 0.5% BSA, and 0.75% glucose. Cultures were diluted 1:100 and sealed with rubber gaskets and stirred rapidly (200 rpm) using a magnetic stir bar. Methylene blue was included as an indicator of oxygen depletion. Samples were collected after 7 and 11 days after culturing with sealed gaskets allowing for the cultures to become hypoxic.

2.3. Preparation of microscopy slides

For microscopy, 3 μL of *in vitro* samples from experimental mycobacterial cultures were transferred to a positively charged microscope slide (ESCO, New Hampshire), and spread within a 4 mm by 4 mm pre-designated area. Slides were allowed to air-dry for 20 min and then baked at 78 °C for one hour.

2.4. Fluorescent acid-fast staining

Samples were stained with auramine–rhodamine (AR) using the TB Fluorescent Stain Kit T (Becton, Dickinson and Company, Sparks, Maryland) per manufacturer's instructions. Briefly, AR was added to the slide and incubated in the dark at room temperature for 25 min, washed in acid-alcohol (0.5% HCL in 70% isopropanol) for no more than three minutes, followed by washing with water and counterstaining with potassium permanganate (0.5%) for four minutes. The slides were washed again in water and then mounted with Prolong Gold antifade mounting medium (Invitrogen, Carlsbad, California).

For staining of the samples on microscopy slides with SYBR Gold, the dye was diluted 1:1000 in a stain solution consisting of phenol crystals (8 g), glycerin (60 mL), isopropanol (14 mL) and distilled water (26 mL). The staining solution was dropped generously over the slide. The slides were heated on a block at 65 °C for 5 min and then allowed to cool for one minute at room temperature (use of coverslips for this step is optional). The coverslips were removed and the slides were washed in acid alcohol (0.5% HCL, 70% isopropanol) for three minutes, then washed in water and mounted using Prolong Gold antifade mounting medium. No $KMnO_4$ counterstain was used.