Diagnostic Microbiology and Infectious Disease 79 (2014) 228–232

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycobacteriology

Direct detection and identification of acid-fast bacteria from smear-positive broth cultures using a pyrosequencing method ☆

Jian R. Bao *, Richard B. Clark, Ronald N. Master, Arelis E. Piscitelli, Praveena R. Tummala, Lynn L. Eklund, Beatriz G. Poselero, Jackie Wright

Quest Diagnostics Nichols Institute, Chantilly, VA 20151

ARTICLE INFO

Article history:
Received 2 November 2013
Received in revised form 28 February 2014
Accepted 4 March 2014
Available online 15 March 2014

Keywords:
Acid-fast bacteria
Identification
Broth culture
Pyrosequencing

ABSTRACT

Broth culture is a standard method for detection of acid-fast bacteria (AFB) (e.g., *Mycobacterium* and *Nocardia*) from patient specimens. Direct nucleic acid-based identification from smear-positive broths expedites the infectious disease diagnosis. We developed and evaluated the performance of a pyrogram-based technique (direct-broth-pyrosequencing [DBP]) to identify AFB directly from smear-positive broths. One hundred thirteen AFB-positive broths from patient specimens were tested. Bacterial DNA was amplified by polymerase chain reaction and sequenced using the PyroMark ID system. The DBP method correctly identified the AFB species/group in 109 (97%) of the 113 broths, including 15 *Mycobacterium* species and 4 *Nocardia* species. Three broths that yielded indeterminate results were found to be AFB-AFB mixed broths and required purified colonies on solid media for definite identification. The 4th broth was repeatedly identified by sequencing to be *Mycobacterium intracellulare*, even though the organism was not isolated and the AccuProbe was negative. This method did not identify the AFB organisms from broths containing 2 AFB organisms, but did not produce false identification. No cross-reaction was observed when AFB-positive broths were spiked with non-AFB microorganisms, indicating that the DBP method was specific to AFB. The DBP method gives rapid (within 8 h), accurate AFB identification directly from broth cultures and provides another useful AFB identification tool in a clinical laboratory.

1. Introduction

Acid-fast bacteria (AFB) (e.g. *Mycobacterium* and *Nocardia* species) are usually significant microorganisms in patient specimens. Their rapid detection and identification are critical for optimal patient care. Liquid (broth) culture systems recommended by the World Health Organization (Nyendak et al., 2009), together with simultaneously culturing on solid media, have become a standard method for AFB detection in most clinical laboratories. Automated broth culture systems expedite AFB-positive detection. The subsequent AFB organism identification from AFB-positive broth cultures may still be delayed since most available AFB identification methods require developed colonies on solid media. The gold standard for AFB species identification is largely relied on DNA sequences of specific target genes, usually the 16S ribosomal RNA gene, in combination with certain phenotypic characteristics in some cases. For most clinical laboratories, the feasibility of AFB identification using sequences is likely low. Further studies are need to establish the gold standard sequencing method for identification directly from broth cultures. Direct AFB identification from AFB-positive broth has been available by using molecular or immunological techniques (Brent et al., 2011; Ichiyama et al., 1997; Lu et al., 2011; Miller et al., 2002; Moon et al., 2012; Pfyffer et al., 1994; Reischl et al., 1994; Said et al., 2011; Shen et al., 2011; Soini and Musser, 2001). The available methods are typically targeting a single species (e.g., *Mycobacterium tuberculosis* complex [MTBC]) or a limited number of AFB species (for example, AccuProbe method with only 4 *Mycobacterium* species) and thus leave other AFB organisms unidentified. The AccuProbe test, a direct DNA-probe method (Gen-Probe, San Diego, CA, USA), is a widely-used method to detect and identify AFB organisms directly from broth cultures (Miller et al., 1994; O'Sullivan et al., 2002; Telenti et al., 1994). The AccuProbe method identifies 4 groups of *Mycobacterium* species (MTBC, *Mycobacterium gordonae*, *Mycobacterium kansasii*, and *Mycobacterium avium* complex), but not other AFB organisms. The method is relative labor intensive and expensive as it requires 1 probe for 1 targeted organism. Newly emerging mass spectrometry methods, such as matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), can be a good option for rapid AFB identification (Balada-Llasat et al., 2013), but lack sufficient data in patient cultures. Certain methods, such as high performance liquid chromatography (HPLC) (Butler and Kilburn, 1998), are not practical for most clinical laboratories as they require significant expertise. A rapid detection and identification method that can simultaneously target various acid-fast organisms in the genera *Mycobacterium* and *Nocardia* and other related AFB organisms could be valuable in speeding diagnosis and thus potentially improving patient care.

☆ Conflict of interest: The authors declare that they have no conflict of interest.

* Corresponding author. Tel.: +1-703-802-6900x65217; fax: +1-703-802-7017.
E-mail address: jian.r.bao@questdiagnostics.com (J.R. Bao).

http://dx.doi.org/10.1016/j.diagmicrobio.2014.03.003
0732-8893/

We developed a rapid method using polymerase chain reaction (PCR) coupled with pyrosequencing (direct-broth-pyrosequencing [DBP]) to detect and identify various AFB directly from broth cultures. This study evaluated the feasibility of DBP in a single-shift clinical laboratory setting.

2. Materials and methods

2.1. Culture media, AFB-positive specimens, and standard AFB identification

Three types of broth media from different vendors were used for AFB culture in this study: Microbial Growth Indicator Tubes (MGIT) (BD Company, Towson, MD, USA), VersaTREK™ Myco (TREK Diagnostic Systems, Cleveland, OH, USA), and BacT/Alert™ MP (bioMerieux, Durham, NC, USA). The latter 2 broth types and some MGIT broth cultures were processed and incubated by outside laboratories and were submitted directly to the Nichols Institute for AFB identification. Only broth cultures (no-agar cultures) were used in this study. MGIT was the sole broth medium used in this laboratory for AFB cultures.

For the routine AFB culture at the Nichols Institute, patient specimens of various sources were processed according to the manufacturer's instructions (Becton Dickinson) and microbiological manual (Pfyffer and Palicova, 2011). Each processed specimen was inoculated simultaneously into both MGIT broth and Lowenstein-Jensen (L-J) slant (Becton Dickinson). The inoculated broth tubes were incubated in a BACTEC 960 system (Becton Dickinson) at 35–38 °C, and the inoculated L-J slants were incubated at 35–37 °C with 5–7% CO_2. Broth tubes identified as positive by the BACTEC 960 system were subjected to a Ziehl-Neelsen staining procedure (Atlas and Snyder, 2011) to determine the presence of AFB (smear positive). The broth cultures for this study were randomly selected from those AFB-positive culture pools and negative cultures were not used. AFB identification using the DBP method was assessed either immediately after the smear procedure or held at room temperature (20–25 °C) for 3–5 days (a few held up to 6 weeks). The DBP identification results were compared to those obtained from established standard laboratory AFB identification methods. The standard AFB identification methods used in this laboratory included AccuProbe (Gen-Probe) and a pyrosequencing/phenotypic method validated previously (Bao et al., 2010). The AccuProbe method was used to identify the 4 *Mycobacterium* species or groups (MTBC, *M. gordonae*, *M. kansasii*, *M. avium* complex) from broth cultures according to the manufacturer's instructions. The pyrosequencing/phenotypic method was used to identify AFB from colonies on L-J or Middlebrook 7H10 medium plates (Becton Dickinson). Additional cultures on solid medium were obtained by sub-culturing the positive broths onto new L-J slants, and the slants were incubated at 35–37 °C with 5–7% CO_2. Mixed AFB-AFB broth cultures were subcultured to Middlebrook plates to obtain pure isolated colonies for identification.

The organisms used in spiking experiments (AFB and non-AFB) were type strains obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) unless otherwise specified. AFB cultures were 2-week-old cultures on L-J slants (Becton Dickinson) incubated at 35–37 °C with 5–7% CO_2. Non-AFB and yeast were cultured overnight (24 h) at 35–37 °C on 5% sheep blood trypticase soy agar plates (bacteria) or on potato dextrose agar (BD Company) plates (yeast). The inoculums were prepared by suspending the organisms in sterile MGIT broth and adjusting the organism cell density to 0.5 McFarland units, followed by serial dilutions.

2.2. DNA extraction from broth cultures

Bacterial cells were harvested by centrifugation of 0.3–1.0 mL of the broth culture (Table 1) at 10,000 × *g* for 5 min in a clean 2-mL micro-centrifuge tube at room temperature. The bacterial cell pellet was washed twice with 1000 μL of sterile distilled water using the suspension-centrifuging-suspension alternation procedure. The pelleted cells were re-suspended in 300 μL DNA extract buffer (Bao et al., 2010) and incubated in a heating block at 100 ± 5 °C for 10 min. The DNA-containing supernatant was obtained by centrifugation of the suspension at 10,000 × *g* for 5 min at room temperature and used immediately for PCR or held at −20 °C for later testing.

Table 1
Broth volume used for DNA extraction based on visual bacterial density observation.

Scale	Visual description for the broth culture	Volume sampled for DNA extraction (mL)
1	Culture is clear, no significant sign of bacterial growth in the broth.	1.0
2	Culture is clear, but visible sign of bacterial growth on the bottom portion of the broth tube.	0.5
3	Culture somewhat cloudy with sign of heavy bacterial growth.	0.3

2.3. DNA amplification, pyrosequencing, and data interpretation for AFB identification

The DNA amplification, amplicon visualization, and pyrosequencing procedures have been described previously (Bao et al., 2010). In brief, 5 μL of the DNA extract was used for each 50-μL final volume PCR. The PCR was performed on an ATC 401 thermocycler (Nycktech, San Diego, CA, USA) or a Mastercycler Pro thermocycler (Eppendorf, Germany) for 40 cycles using a pair of PCR primers (forward: 5′-TGGCGAACGGGTGAGTAACA, reverse: 5′-biotin-GCTACCCGTCGTCGCCTTG; synthesized by Invitrogen, Carlsbad, CA, USA). The amplicons were visualized using the E-Gel system (Invitrogen). The PCR amplicon was bonded to sepharose beads and denatured in 0.5N NaOH buffer according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Pyrosequencing was performed on a PyroMark ID system (Qiagen) using a sequencing primer (5′-AAACTGGGTCTAATACCG; synthesized by Invitrogen) (Tuohy et al., 2005) with 60 (15 × 4) nucleotide-dispensing cycles.

The AFB identification of this method is to use short "signature" sequences (typically 30–50 base pairs) to differentiate genus and species. Resulting sequences containing a minimum of 30 nucleotide bases were aligned to an in-house-developed database and a second database developed by Michigan State University's RDP (http://rdp.cme.msu.edu). The species-calling criteria were referring to those that have been used in other related studies (Bao et al., 2010; Innings et al., 2005; Tuohy et al., 2005). Sequence homology of 95% or greater to a specific organism was considered acceptable for acid-fast organism identification from a broth culture. All identifications using this DBP method were compared to those obtained using 1 or more of the standard laboratory identification methods described above.

2.4. Impact of non-AFB microorganisms on AFB detection and identification

Cross-reactivity with non–acid-fast organisms was tested by spiking AFB-positive broths with inoculated non-AFB organisms. AFB-positive MGIT broths were obtained by inoculating with either *M. chelonae* (ATCC 35752) or *M. fortuitum* (ATCC 6841) at 10^5 bacterial cells each. The inoculated broths were incubated in a BACTEC 960 system (Becton Dickinson) at 35–38 °C. Inoculated MGIT broths that exhibited positive signals (in about 2–3 days) on the BACTEC system were spiked with 1 of 6 non–acid-fast organisms (5 bacteria and 1 yeast) (Table 2). Each MGIT tube was spiked with 500 μL of a 0.5-McFarland bacterial cell suspension prepared from MGIT broth as described above, and the final non-AFB bacterial concentration in tested MGIT tube was at approximately 5×10^6 cells/mL. The controls