

Preliminary Evaluation of *Mycobacterium tuberculosis* Detection in Culture and Artificial Sputum Using a BioNanoPore Membrane and Real-time PCR

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Abstract

The rapid detection and diagnosis of *Mycobacterium tuberculosis* is critical to evaluate disease severity, efficacy of treatments and therapeutics, and public health monitoring. This study evaluated a BioNanoPore technology (BNP™ Middlebrook agar) to detect and quantitate *M. tuberculosis* in less time than traditional plate counting methods. BNP™ Middlebrook enabled visual detection of *M. tuberculosis* from actively-growing cultures and inoculated artificial sputum within 5 days; however, colonies were not visible on Middlebrook 7H10 agar. For cultures incubated in the presence or absence of artificial sputum for 19 days on BNP™ Middlebrook, *M. tuberculosis* ranged from 5.81-5.86 log₁₀CFU/mL from liquid culture and 6.39-6.50 log₁₀CFU/mL in artificial sputum; counts for *M. tuberculosis* in liquid culture ranged from 5.70-5.85 log₁₀CFU/mL on Middlebrook 7H10. All colonies from 19 day-old cultures evaluated from the BNP™ Middlebrook and Middlebrook 7H10 media were positive for the *Mycobacterium* insertion sequence (IS) 6110 by real-time PCR. This study demonstrates that BNP™ Middlebrook can detect *M. tuberculosis* faster than standard plating techniques in the presence or absence of a simulated biological matrix (artificial sputum). Moreover, the BNP™ Middlebrook color development step does not interfere with real-time PCR detection of IS 6110. This study provides a preliminary assessment of the potential use of BNP™ Middlebrook for a more rapid screening and detection of viable *M. tuberculosis* with respect to clinical specimen evaluation, therapeutic treatment/vaccine efficacy, or epidemiological surveillance.

Keywords: *Mycobacterium tuberculosis*; BioNanoPore membrane; Quantitation

Introduction

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* that typically affects the lungs, although extrapulmonary (e.g., lymphadenitis) TB disease can manifest as well [1]. The symptoms of active TB of the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats. Transmission of TB occurs from person-to-person via droplets expelled from the throat and lungs during coughing in individuals harboring the disease. In healthy people, TB infection often causes no symptoms; however, in immune compromised individuals, such as those positive for human immunodeficiency virus (HIV), the probability of developing the disease is higher. The World Health Organization (WHO) has reported an estimated 8.8 million incident cases of TB globally in 2010 in which 1.1 million estimated deaths were among HIV-negative individuals and 0.35 million deaths occurred in HIV-positive people [2]. Since 2002, the global number of annual incident rate for TB per 100,000 individuals within a population has been falling by 1.3% [2].

A key element in the successful treatment and control of disease is proper diagnosis within a time window enabling the opportunity or successful treatment. Diagnostic tests aiding in appropriate treatment strategies need to be rapid, sensitive, and specific for the identification of the causative agent. In the case of TB, diagnosis can be complexed by factors such as the clinical sample matrix (e.g., sputum) and Multidrug Resistance (MDR) strains that can be difficult to distinguish using basic historical methods such as microscopic smear evaluations and culturing.

Within the last decade, various methods and approaches have been developed to increase the time to detection as well as increase sensitivity and specificity for *M. tuberculosis* and MDR strains compared to the

traditional standard of culturing for up to 12 days on semi-solid media. Such approaches include real-time PCR, denaturing gel electrophoresis, nested PCR, dot blot hybridization, sequencing, fluorescence microscopy, adenosine deaminase and interferon-γ assays, surface plasmon resonance, nitrate reductase activity, gas chromatography-mass spectrometry, luminescence assays, immunoswabs, and time to positivity assays [1,3-14]. Each method has been evaluated in some capacity for turnaround time, limit of detection, sensitivity, dynamic range, and reproducibility.

The BioNanoPore (BNP™) Ultra Fast Identification Technology (NanoLogix, Inc., Hubbard, OH) is a dual-plate system (nutrient and chromagen) designed for a more rapid detection of microorganisms. A cellulose membrane containing a thin film of nutrient agar is on the surface of the nutrient agar plate that enables exchange of water and nutrients. The chromagen plate contains 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in an agarose matrix that leads to dark purple to black coloration of the bacterial colonies upon metabolism.

The BNP™ technology has been evaluated for the more rapid detection and quantitation of virulent *Bacillus anthracis* and *Yersinia*

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pestis compared to traditional culture methods [15]. Both *B. anthracis* and *Y. pestis* were detected and quantified at 6 and 24 hr, respectively, in phosphate-buffered saline (PBS) and naïve rabbit blood, which was faster than the standard culture times for *B. anthracis* (e.g., 18-24 hr) and *Y. pestis* (e.g., 48-72 hr) on semi-solid medium [15]. When compared to growth of *B. anthracis* and *Y. pestis* on tryptic soy agar, similar results were obtained using BNP™ for the total colony forming units (CFU)/mL [15]. Based on these results, this study was conducted to determine whether *M. tuberculosis* could be detected more rapidly using BNP™ than the standard approach of culturing on Middlebrook 7H10 agar for 2-3 weeks. In addition to this viability assessment, real-time PCR was used to confirm the isolated microcolonies on BNP™ Middlebrook were positive for the insertion sequence (IS) 6110 for *M. tuberculosis*.

Methods

M. tuberculosis detection and quantitation

M. tuberculosis ATCC 35801 was used for this study. Actively growing liquid cultures were prepared from streak plates made from frozen cultures. Briefly, frozen stock cultures obtained in-house were thawed and a sample was streaked onto Middlebrook 7H10 agar (Hardy Diagnostics, Santa Maria, CA) in triplicate. The plates were incubated at $37 \pm 1^\circ\text{C}$ under a 95% air and 5% CO_2 humidified atmosphere for 19-21 days. An individual colony was used to inoculate 25 mL of Middlebrook 7H9 broth (Hardy Diagnostics) containing 0.1% glycerol and 10% Oleic Acid Albumin Dextrose Complex. The culture was incubated at $37 \pm 1^\circ\text{C}$ while shaking at 200 rpm for approximately 14 days and used for subsequent tests.

The growth evaluation for *M. tuberculosis* on BNP™ Middlebrook was performed in two phases. Phase I was conducted to determine whether *M. tuberculosis* could be detected in a shorter period of time on BNP™ Middlebrook nutrient plates (NanoLogix, Inc.) compared to standard culturing on Middlebrook 7H10. Aliquots (100 μL) of liquid stock culture were serially-diluted ten-fold in PBS and plated in triplicate on BNP™ Middlebrook and Middlebrook 7H10. Parallel negative controls (sterile Middlebrook 7H9 broth) were prepared on both BNP™ Middlebrook and Middlebrook 7H10. All plates were incubated at $37 \pm 1^\circ\text{C}$ under a 95% air and 5% CO_2 humidified atmosphere for 3, 4, 5, or 7 days ($N=3/\text{time point}$). For BNP™ Middlebrook at each time point, the membrane was aseptically removed, transferred to the chromagen indicator plates, and incubated at room temperature for 20-30 min (Figure 1). For BNP™ Middlebrook and Middlebrook 7H10, all plates were visually inspected for growth; if possible, colonies were counted and CFU/mL determined.

For Phase II, the growth and detection of *M. tuberculosis* in a simulated biologically-relevant matrix was conducted in which artificial sputum was prepared as previously described [16]. Briefly, one emulsified egg was added to 1 L of 1% (w/v) aqueous methylcellulose. Triplicate samples of stock *M. tuberculosis* liquid cultures were prepared in the artificial sputum by adding 100 μL culture to 900 μL artificial sputum. These samples were then serially diluted ten-fold in PBS and each dilution plated in triplicate on BNP™ Middlebrook. Negative control blanks were prepared and processed in parallel in which sterile Middlebrook 7H9 broth alone ($N=3$), or sterile Middlebrook 7H9 broth was spiked into the sputum ($N=3$) rather than bacteria. Cultures were incubated at $37 \pm 1^\circ\text{C}$ under a 95% air and 5% CO_2 humidified atmosphere for up to 21 days. All plates were visually inspected for growth, colonies counted, and CFU/mL determined.

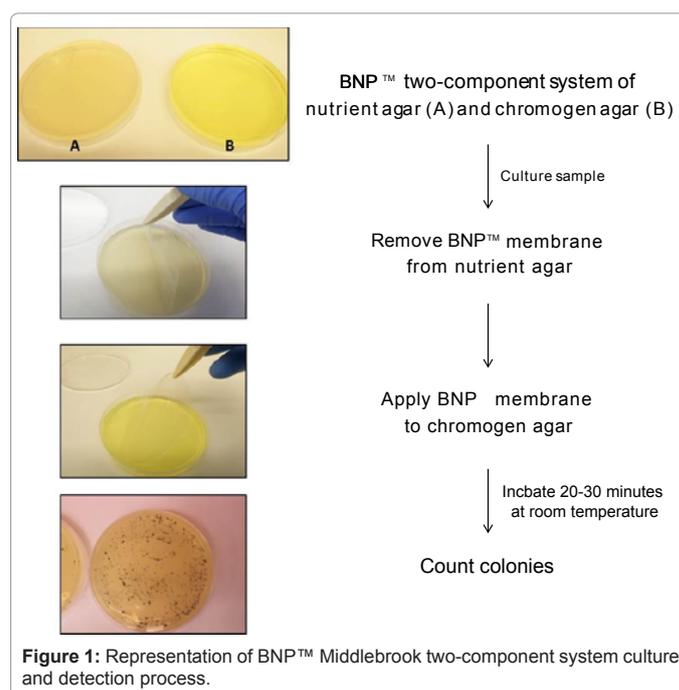
All data were expressed as mean \log_{10} CFU/mL. For the streak plates, samples were expressed as being positive or negative for growth. The t-test (MS Excel; Microsoft Corporation, Redmond, WA) was used to compare the mean \log_{10} CFU/mL for liquid cultures plated on BNP™ Middlebrook and Middlebrook 7H10. $P \leq 0.05$ was used as the level for significance.

Real-Time PCR confirmation of *M. tuberculosis*

To demonstrate the ability to utilize PCR confirmation with culturing on BNP™ Middlebrook, *M. tuberculosis* liquid culture was plated and incubated for at least 21 days as described above. Samples evaluated included colonies obtained from BNP™ Middlebrook before and after color development, as well as Middlebrook 7H10.

For PCR detection, the target amplicon (52 bp) was designed from the IS 6110 multicopy element sequence. The IS 6110 sequence is present among multiple *Mycobacterium* species and has been used as a PCR target for detection, diagnosis, and measurement of bacterial tissue burden [1,17-22]. Following culture for up to 21 days on BNP™ Middlebrook and Middlebrook 7H10, nucleic acid was extracted from a single isolated *M. tuberculosis* colony using the NucliSENS® easyMAG® (bioMérieux, Inc., Durham, NC) according to the manufacturer's instructions. For BNP™ Middlebrook, nucleic acid was isolated from colonies prior to and after the 20-30 min color development step. Each nucleic acid sample was eluted in 25 μL elution buffer in which 5 μL was added to TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA) containing 900 nM of each primer and 250 nM of labeled probe. Each PCR reaction was conducted in a total volume of 25 μL .

The PCR primers and probe were designed using the Custom TaqMan® Gene Expression Assays (Applied Biosystems) in which the sequences were as follows: forward 5'-TCATGTGGGCCCGTGA-3'; reverse 5'-TCCTCGATGCCCCAGATC-3'; probe 5'-FAM-AGTTCGGCCTCGAGC-MGBNFQ-3'. Amplification was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System in



which the run conditions included a 2 min incubation at 50°C; 10 min incubation at 95°C; 40 cycles at 95°C for 15 sec and 60°C for 1 min. Assays were performed in duplicate and threshold cycle (C_t) for each sample determined using the Sequence Detection Systems Automation Controller Software v2.3 and mean value reported. All DNA extractions and real-time PCR reactions were performed in a blinded fashion with respect to sample identification.

Results

M. tuberculosis detection and quantitation

For Phase I, bacterial colonies were observed visually on both streak plates and spread plates as early as 5 days on BNP™ Middlebrook (Table 1) in which the dark blue colonies were ≤ 0.5 mm in diameter (Figure 2). *M. tuberculosis* colonies were not observed visually on Middlebrook 7H10 at any period of time up to 7 days in culture. Since colonies could not be counted on the Middlebrook 7H10, a quantitative comparison of samples cultured on BNP™ Middlebrook to that of the Middlebrook 7H10 was not possible.

Based on the results of *M. tuberculosis* detection in Phase I, a total culture time on BNP™ Middlebrook and Middlebrook 7H10 chosen for Phase II was 19 days, corresponding to two additional weeks of growth beyond the earliest detection time of 5 days. At 19 days incubation, *M. tuberculosis* colonies were visible on BNP™ Middlebrook and Middlebrook 7H10, enabling a quantitative comparison. Liquid culture samples plated on BNP™ Middlebrook (Figure 3) and Middlebrook 7H10 ranged from 5.81-5.86 \log_{10} CFU/mL (CV=0.5%) and 5.70-5.85 \log_{10} CFU/mL (CV=1.4%), respectively (Table 2). No significant difference was observed ($P=0.45$) for the mean \log_{10} CFU/mL when comparing growth of *M. tuberculosis* on BNP™ Middlebrook and Middlebrook 7H10. All streak plates of *M. tuberculosis* liquid cultures on BNP™ Middlebrook and Middlebrook 7H10 were positive for growth; all blank samples were negative for growth (Table 2). Following incubation of spiked artificial sputum samples for 19 days on BNP™ Middlebrook, colony counts ranged from 6.39-6.50 \log_{10} CFU/mL having a %CV of 1.0; all corresponding streak plates of spiked artificial

Media	Number of Days Cultured ^a			
	3	4	5	7
BNP™ Middlebrook	0 (3)	0 (3)	3 (3)	3 (3)
Middlebrook 7H10	0 (3)	0 (3)	0 (3)	0 (3)

^aNumber positive (Number tested)

Table 1: Qualitative assessments of *Mycobacterium tuberculosis* ATCC 35801 on BNP™ Middlebrook and Middlebrook 7H10 media (N=3/time period).

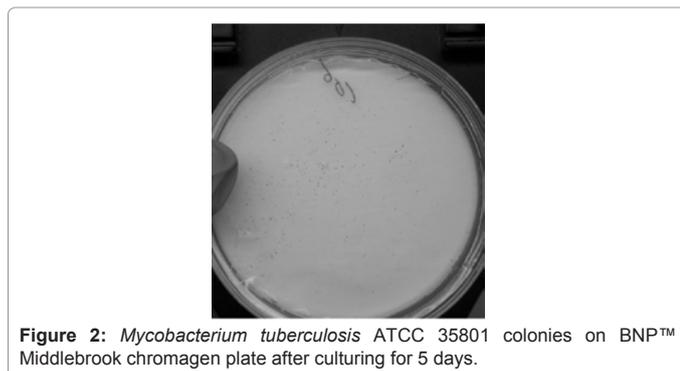


Figure 2: *Mycobacterium tuberculosis* ATCC 35801 colonies on BNP™ Middlebrook chromagen plate after culturing for 5 days.

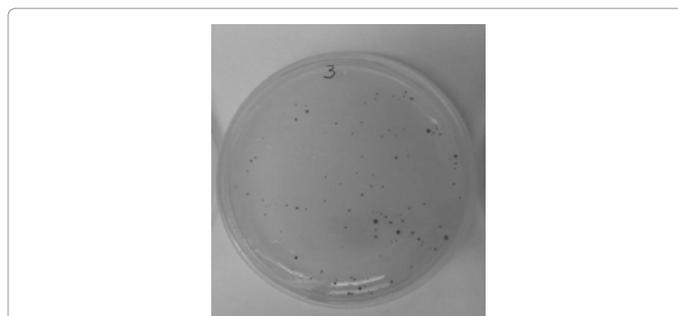


Figure 3: *Mycobacterium tuberculosis* ATCC 35801 colonies on BNP™ Middlebrook chromagen plate after culturing for 19 days.

sputum were positive for growth; all blank samples were negative for growth (Table 2).

Real-Time PCR confirmation of *M. tuberculosis*

For the colonies selected for PCR analysis, every attempt was made to ensure similar sized-colonies were selected for the DNA isolation step. All colonies evaluated from the BNP™ Middlebrook and Middlebrook 7H10 media were positive for IS 6110 in which the mean C_t values were all less than 20. For BNP™ Middlebrook, amplification of the IS 6110 target sequence was not affected when PCR was performed on DNA isolated from a colony either prior to (mean $C_t=17$) or after the color development step (mean $C_t=19$). DNA amplification for IS 6110 from *M. tuberculosis* grown on Middlebrook 7H10 yielded a mean C_t of 18. Amplification of the target gene was not observed in the negative (no DNA template) controls.

Discussion

Tuberculosis is a global public health concern in which early diagnosis is critical to enabling health care providers the ability to provide the necessary medical treatment in a timely manner. Although there have been advances in the available techniques to help diagnose *M. tuberculosis* infection, the gold standard method for laboratory is culture on semi-solid media that can require up to 3-4 weeks. Additionally, TB diagnosis relies on microscopic evaluation of acid-fast bacilli in sputum spears; however, the diagnostic sensitivity of this technique ranges from 25% to 65% compared to culturing and can vary by factors such as specimen type and number, *Mycobacterium* species, and staining technique [16].

Currently, there are many types of “rapid” diagnostic tests for TB that have undergone various levels of testing and evaluation as well as the post-research and development assessments describing strengths and weaknesses [1,8,23,24]. Of these, nucleic acid amplification tests (NAATs) and semi- and fully-automated liquid culture systems have improved the time to detection, accuracy, and sensitivity for determining TB load; however, NAATs are limited by their ability to distinguish between viable and non-viable organisms and results from liquid culture systems can take days to weeks [8]. It has been suggested that that no single mycobacterial quantitative technique has ideal performance characteristics and assay choice depends on the need and turnaround time [8]. Moreover, a definitive reference test is a critical component for assessing the performance of potential diagnostics [1,25]. However, an approach that combines more than one assay, such as culturing with NAATs can potentially improve TB diagnosis, ultimately leading to a positive impact on clinical care and treatment. Examples of such results using assay combinations have been observed

Sample	Streak plate	BNP™ Middlebrook		Middlebrook 7H10	
		Mean Log ₁₀ CFU/mL (Range; %CV)	Streak plate	Mean Log ₁₀ CFU/mL (Range; %CV)	Streak plate
<i>M. tuberculosis</i> liquid culture ^a	+	5.83 (5.81-5.86; 0.5%)	+	5.79 (5.70-5.85; 1.4%)	
Liquid culture blank	-	0	-	0	
<i>M. tuberculosis</i> in artificial sputum	+	6.45 (6.39-6.50; 1.0%)	ND	ND	
Artificial sputum blank	-	0	ND	ND	

ND=Not Determined

^aMean values between BNP™ Middlebrook and Middlebrook 7H10 not significantly different (P=0.45); t-test at P<0.05 confidence level.

Table 2: Quantitative and qualitative assessments of *Mycobacterium tuberculosis* ATCC 35801 cultured on BNP™ Middlebrook and Middlebrook 7H10 media for 19 days (N=3/sample).

for smear microscopy and NAATs [26], enzyme-linked immunospot (ELISPOT) assay and tuberculin skin test [27], and ELISPOT with adenosine deaminase levels [28].

Based on the favorable results that have been obtained using a combinational assay approach, the present study provides preliminary data for the BNP™ Middlebrook product in which viability through culturing and DNA confirmation via real-time PCR of *M. tuberculosis* can be achieved. The BNP™ Middlebrook technology enabled a more rapid detection of *M. tuberculosis* in the presence or absence of artificial sputum that was approximately 2 weeks faster when compared to the traditional culture method using Middlebrook 7H10. Moreover, the visible detection within 5 days when culturing on BNP™ Middlebrook is similar to or twice as fast as that reported for liquid cultures evaluated using semi- or fully-automated procedures [8,23,29].

Although colonies were clearly visible on BNP™ Middlebrook within 5 days, a quantitative comparison between BNP™ Middlebrook or Middlebrook 7H10 could not be performed. Therefore, we chose a time point corresponding to 2 weeks after the first day in which colonies were observed on BNP™ Middlebrook. After 19 days incubation, the mean log₁₀ CFU/mL counts were similar for *M. tuberculosis* on BNP™ Middlebrook and Middlebrook 7H10. It should be noted that the respective counts for *M. tuberculosis* in artificial sputum plated on BNP™ Middlebrook were higher (approximately 0.5 log₁₀ CFU/mL) than the non-sputum samples (liquid cultures) plated on BNP™ Middlebrook. This may be due in part to the fact that the artificial sputum contains an emulsified egg that may provide additional nutrients during culturing, and eggs have been a media component for culturing *M. tuberculosis* [30]. However, the effect of eggs and such differences in regard to the use of artificial sputum would require further investigation.

In this study, the positive PCR amplification of IS 6110 provided confirmation that the colonies visualized were *M. tuberculosis*, which was to be expected as a purified culture of *M. tuberculosis* ATCC 35801, was used for the evaluation. The results showing that PCR was able to be performed on colonies subjected to chromagen staining indicates that BNP™ Middlebrook provides the capability to assess both viability and genetic confirmation from the same sample within a homogeneous preparation. Although IS 6110 has been used as a potential NAAT-based diagnostic, there is the potential for false positives to be identified as this insertion sequence in present various *Mycobacterium* species [20]. Moreover, this technology will need to be further evaluated to determine the feasibility of accurately performing quantitative counts and PCR on known mixed bacterial populations or clinical samples plated onto BNP™ Middlebrook.

Ideally, highly accurate diagnostic tests for TB would be simple to perform, not require specialized equipment or facilities, provide

rapid results for decreased time to treatment, are readily available, and cost-effective for the end user. These qualities are especially important in developing or high-TB burdened countries where the access and ability to purchase specialized equipment is limited, and number of trained personnel is low. The results of the present study demonstrate a proof-of-concept for BNP™ Middlebrook in detecting TB compared to the Middlebrook 7H10 reference test and should be considered as an ideal candidate for future evaluation of clinical samples. Moreover, these testing iterations could lead to the modification of the BNP™ Middlebrook/PCR approach used in this study in which MDR strains could be selectively detected by performing multiplex PCR reactions that target specific MDR gene targets.

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