Direct Detection of Slow Growing Pathogens using Nanopore Technology

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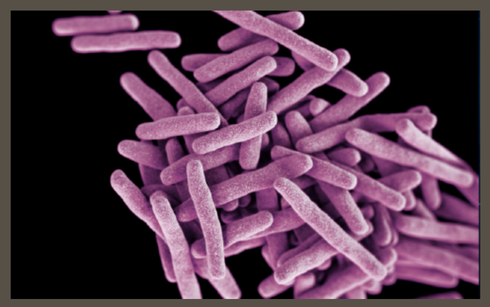
Abstract

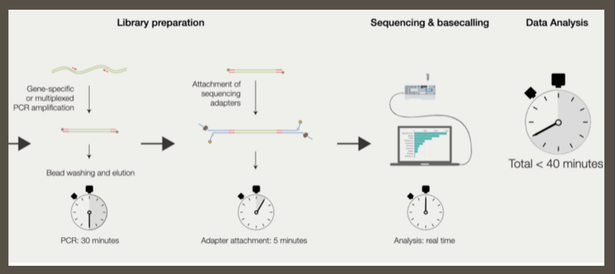
Mycobacterium is an acid-fast bacilli (AFB) that grows primarily in aerobic conditions. It is the causative agent of pathogenic Tuberculosis, Leprosy, wound infections and nosocomial infections related to implanted devices. It is a slow growing pathogen that requires 1-2 weeks to grow and identify and must be handled in a specialized biosafety level 3 (BSL-3) environment. *Aspergillus* is also a slow growing pathogen which is found ubiquitous in nature. It can infect the lungs causing aspergillosis, which manifests as flu-like symptoms. Slow growth and identification of these species coupled with specialized training required to handle these pathogens lead to long identification times and increased costs in a clinical setting. This study assessed if the new MinION sequencing technology can be used to identify *Mycobacterium* using the 16S barcoding at a lower cost. Since *Aspergillus* uses nrITS barcoding, it served as the negative control of the experiment. The MinION is a real-time portable DNA sequencing device that could be utilized in a clinical laboratory setting, and possibly in remote environments. The device can analyze organism from small viruses up to an entire human genome. DNA were extracted from *Mycobacterium, Aspergillus,* and common normal flora found on a sputum samples. Researchers then placed the purified DNA in the MinION device. This device provided real-time DNA sequencing and can sequence an entire genome in a matter of hours. To test sensitivity, various concentrations of organism were achieved through serial dilutions and the minimum detectable limit was measured in the MinION. In regards to true organism identification, the MinION correctly identified the genus. However, the MinION was inadequate with species identification.

Introduction

Rapid detection of slow growing pathogens like *Mycobacterium* is crucial in healthcare. There are many available testing methods for *Mycobacterium* species. However, these methods are either expensive, lack sensitivity and specificity, or take weeks to culture and test (Votintseva, et al., 2015). In this study, the researchers evaluated a portable fourth generation DNA sequencer called the MinION, which allows rapid real-time identification of microbes. Evaluation of the device was done in order to determine whether it could allow faster and more affordable testing for any slow growing microbes using low concentrations of microbial DNA. This could enable early detection and early treatment of the disease.

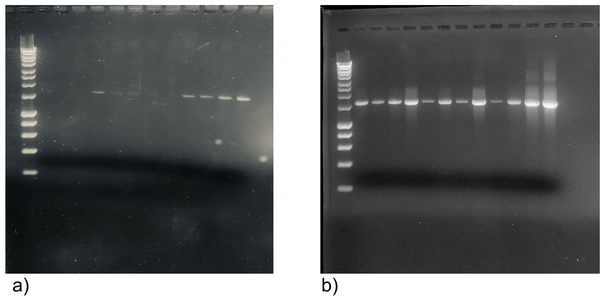


https://www.popsci.com/tuberculosis-exclusive-clips-forgotten-plague



https://nanoporetech.com/analyse/16s

Results



a) b)

PCR of 16S ribosomal DNA. Both **a** and **b** underwent 95℃(1 minute). **a** underwent 95℃(20 seconds), 55℃(30 seconds), and 65℃(2 minutes) for 25 cycles while **b** underwent the same process but with 35 cycles in order to amplify more DNA than **a**. Both **a** and **b** were amplified at about 1500kb.

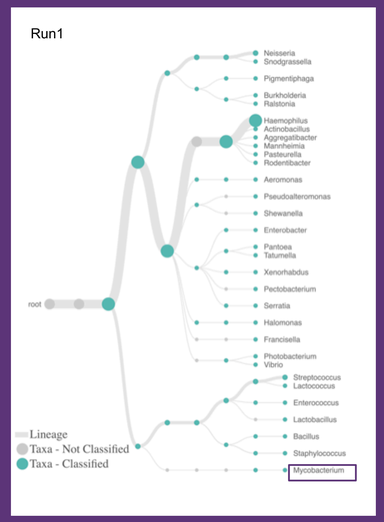
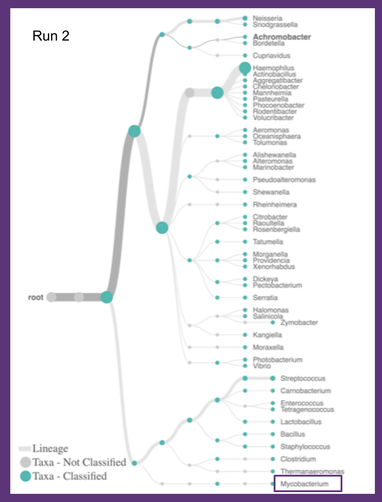


TABLE 1. MINION DETECTION AND PREDICTIVE VALUES

Mycobacterium smegmatis (CFU/mL)	Run 1	Run 2
$7.45x10^8$	*+	★+
$5.58x10^8$	★+	-
$3.69x10^8$	◆+	-
$1.80x10^8$	-	-
$0.86x10^8$	■+	-
Predictive Values (%)		
Sensitivity	80	20
Specificity	100	100
Positive Predictive Value (PPV)	100	100
Negative Predicted Value (NPV)	88	64

Samples with various concentrations of **M. smegmatis* were analyzed as well as 7 negative samples with A. niger. Run1 identified *Mycobacterium* of different species,★*M. flavescens*,■*M. stomatophae*,◆*M. saskatchewanense*, and **M. smegmatis*. The test started with 1,010 nanopores and ran for eight hours. The specificity was 1.00 meaning that 100% of the time the MinION was able to truly identify a negative sample. The sensitivity was 0.80 meaning that the MinION truly identified *Mycobacterium* infected samples 80% of the time. PPV was 1.00, meaning that of the positive mycobacterium samples identified by the minion 100% of them were truly infected with the organism. The NPV was .88, meaning that of the truly uninfected samples of *Mycobacterium* identified by the MinION only 88% of them were truly uninfected by the organism. In run 2, the MinION was able to identify *M. flavescence* in one sample. The test started with 210 nanopores and ran for eight hours. The specificity was 1.00, meaning that the MinION truly identified the negative samples 100% of the time. The sensitivity was .20, meaning that the MinION successfully identified Mycobacterium infected samples 20% of the time. The PPV was 1.00 meaning that the positive samples identified by the MinION were 100% truly infected by mycobacterium. NPV was .64, meaning that the negative samples identified by the MinION only 64% of them were truly negative samples.



Discussion

Currently, the best method for slow growing pathogen is still the culture method. During the research period, researchers encountered challenges. The first device sent by the manufacturer was broken. The study had to wait until the following week to start the data analysis. The researchers also found that the MinION does not have an internal quality control system that double checks the DNA base pairs analyzed by the device and whether it completely and accurately match the specific organism. This could explain the inadequacy of the device to accurately identify to the species level. The researchers used isolated colonies that are usually seen in respiratory samples. However, the device detected multiple genera that the researchers were not expecting. This could be due to contamination of the plates or the base pairs amplified and barcoded are closely related to the expected microorganisms. This study also noticed that several nanopores become inactive after a couple of days without usage. In addition, the data analysis presented by EPI2ME showed additional barcodes that are unexplained by the system. Further testing and experience using the device is still needed in order to determine whether the device could generate more accurate results.

Limitations

- Not enough test replication
- Not enough pores in the flow cell to run more samples
- Technical error is also possible due to lack of experience using the device

https://nanoporetech.com/how-it-works#search&

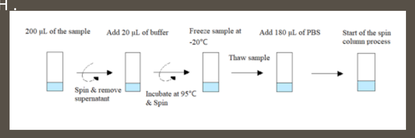
Methods

Growth Conditions and Bacteria Suspension

Mycobacterium specific culture media 7H10 was used for *M. smegmatis*, grown at 37 ℃ ,1.2% carbon dioxide incubator for 4 days. *A. niger* was grown on SAB media for a week, at 27.5 ℃ 0.7% carbon dioxide incubator. *Staphylococcus*, *Streptococcus*, and *Neisseria* species were grown at 37 ℃ at 5.0% carbon dioxide incubator. These species are common normal flora in the upper respiratory tract, and were added to the mock patient samples.
McFarland Standards were used to standardize the approximate number of bacteria in the saline suspension. One of the Researchers conducted a blind serial dilution with the absorbance of 0.4, 0.3, 0.2, 0.1, and 0.05 while the other researcher tested. Four samples were created for quality controls two positive and two negative. The other 5 were spiked with a constant absorbance of 0.1 normal flora. While *M. smegmatis* and *A. niger* were added with different variations of absorbance.

DNA Extraction and DNA Quantification

To do mechanical cell disruption before DNA extraction the Freeze-thaw method was performed for all the samples. For DNA extraction QIAamp DNA Mini Kit was used (Figure 1.1).
Quantification of DNA was read with the EPOCH .



DNA Barcoding

DNA extracted samples were than prepared with the 16s-barcoding kit provided by Oxford Nanopore Technologies. This helps to easily identify the genus of the organisms in the samples by the sequencer. Flow cells for the MinION had to be prepared and primed to receive samples. Afterwards 75uL were loaded in to the SpotON sample port, for all 8 samples, on the MinION device and started the sequencing run.

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