Kari Graham – kag080@shsu.edu

Comparison of Nine Extraction Methods for Bacterial Identification using a Sequencer

Kari A. Graham, BS[1]*; Javier Gomez, MS[2]; Todd P. Primm, PhD[2]; Rachel Houston, PhD[1]

[1]Department of Forensic Science, Sam Houston State University, Huntsville, TX 77340
[2]Department of Biological Sciences, Sam Houston State University, Huntsville, TX 77340

MEMBER THE TEXAS STATE UNIVERSITY SYSTEM

INTRODUCTION

The United States became aware of its vulnerability to a bioterrorism attack after the Anthrax Letter Attack of 2001. This attack revealed the need to implement forensic analysis of microbes to identify the agent in question, prevent another event, and for source attribution. Successful downstream applications depend on an adequate extraction method for bacterial DNA. In the event of an attack, the investigative team may not know what species they are handing, therefore identification of a universal extraction method is of importance. Literature to date is conflicted in what is considered the "best" universal extraction procedure.

The "gold standard" in bacterial species identification is use of the 16S rRNA region of the genome. This 1.5 kb region contains highly conserved sequences as well as 9 variable regions, which are used for species identification. There are also many databases that are dedicated to housing full length sequences of this gene for a number of species.

The advent of third generation sequencing technologies such as the Oxford Nanopore MinION sequencer (ONT, Oxford, UK) could be the answer to identification of unknown species in a timely manner without *apriori* knowledge. The MinION sequencer has been shown to be able to generate long read lengths that span the bacterial 16S rRNA gene and give bacterial identification. Another unique feature of the ONT MinION is that the raw data is analyzed in real time using their Guppy software. However, this platform requires high quantities of very pure DNA with low solvent, salt, and protein contamination compared to other sequencing platforms like the Verogen MiSeq FGx®. Therefore, identification of an extraction method that can handle universally all bacterial types, is relatively cost effective, and be applied to 16S rRNA sequencing on the MinION is critical for microbial forensic analysis.

This study compared the extraction efficiency of 5 commercially available DNA extraction kits as well as two Chelex-100 extraction methods described in literature (1,2), and a boiling method described in literature (3). To assess the universality of each extraction procedure, eight different gram-positive, gram-negative, and acid fast bacterial strains were used. PCR efficiency was also assed to identify any inhibition. This study also investigates the most useful extraction method to use for downstream bacterial identification using the MinION sequencer in forensic situations were species is unknown and needs to be identified rapidly.

MATERIALS AND METHODS

Bacterial Species

E. coli K12, *M. smegmatis* MC²155, *K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 6633, S. aureus ATCC 6538, *F. philomiragia* 25017, *Y. enterocolitica* ATCC 23715, and *B. atrophaeus* ATCC 9372 were collected from the American Type Culture Collection (ATCC, Manassas, VA). Each species was cultured per recommended guidelines and normalized to 10^8 CFU/mL in triplicate. The normalized samples were pelleted, the supernatant decanted, and stored at -80 °C until ready for extraction.

RESULTS AND DISCUSSION

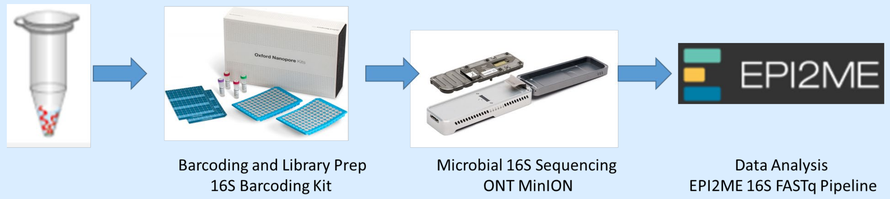

Figure 1: Bacterial identification workflow

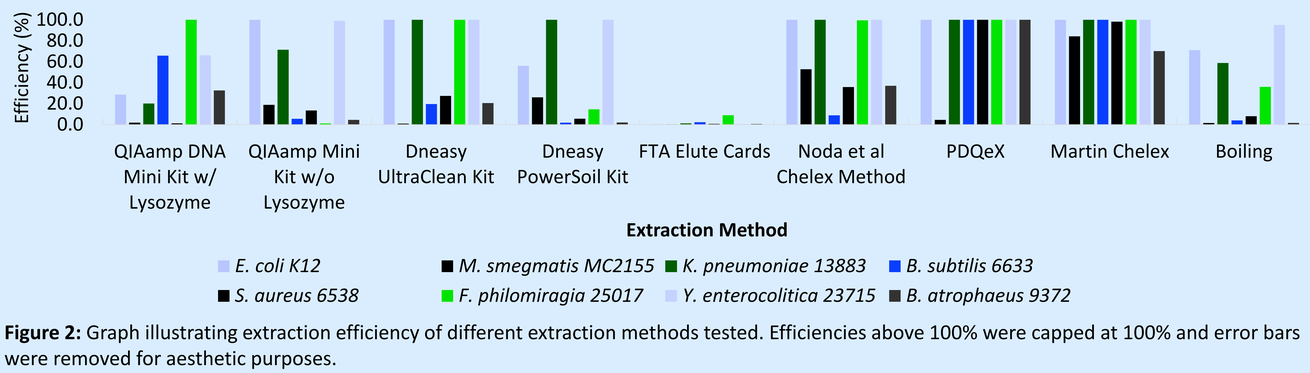

Figure 2: Graph illustrating extraction efficiency of different extraction methods tested. Efficiencies above 100% were capped at 100% and error bars were removed for aesthetic purposes.

- Extraction efficiencies ranged from 0.2% to >100%, and all extraction methods with the exemption of FTA Elute showed comparable results across sample type (**Figure 2**).
  - DNeasy UltraClean, PDQeX Bacteria kit, and both Chelex methods showed the most promise across all bacterial species (**Figure 2**).
- PCR efficiencies ranged from 0.4% to 115% with the FTA Elute method showing the greatest PCR efficiency.

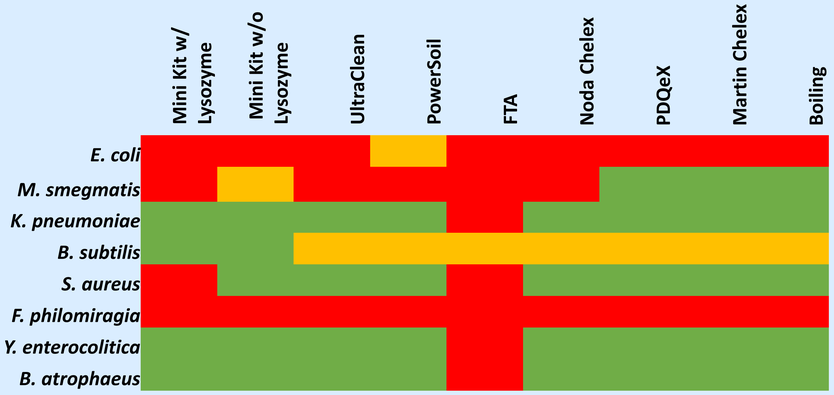

Figure 3: Heat map of the identification results from the EPI2ME software. **Red** – did not identify; **yellow** – genus was identified correctly; **green** – both genus and species identified correctly

- MinION sequencing results showed trends across bacterial species and comparable IDs across most extraction methods (**Figure 3**).
- EPI2ME ID ranged from 84-91% accuracy.
- FTA Elute cards completely failed in identification (**Figure 3**), which was consistent with extraction results (**Figure 2**).
- *E. coli* and *F. philomiriagia* (a surrogate strain for *F. tularensis*) failed to be identified using any one of the extraction methods (**Figure 3**).
- For the species that were identified incorrectly, less than 10% of thousands of reads were aligned to the correct species.

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MATERIALS AND METHODS

Extraction

Five commercial extraction kits and three home-brew extraction methods were performed in triplicate for each bacterial species:

- ✓ QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)
  - Gram negative
  - Gram positive with 20 mg/mL of lysozyme
- ✓ DNeasy PowerSoil Kit (QIAGEN)
- ✓ DNeasy UltraClean Kit (QIAGEN) with 70°C incubation
- ✓ FTA Elute (Maidsone, UK)
  - Samples reconstituted in 40 µL of diH_2O
- ✓ MicroGEM PDQeX Bacteria Kit protocol for colonies and biofilms
  - Gram positive
- ✓ Chelex-100 as per Noda et al. (1)
- ✓ Chelex-100 as per Martin-Platero et al. (2)
- ✓ Boiling method as per Junior et al. (3)

Quantification

Quantification was performed in triplicate using the Qubit™ 2.0 Flurometer with the Qubit™ dsDNA HS Assay Kit (Thermo-Fisher, Waltham, MA).

Library Preparation and Sequencing

Each sample was normalized to 10 ng input DNA. The 16S Barcoding Kit 1-24 was used to prepare the amplicon library for MinION™ (ONT) sequencing per recommended protocol (4). Sequencing was performed using built-in MinKNOW software (find version) on the MinIT instrument connected to a Dell computer for 12 hours.

Data Analysis

Extraction and PCR efficiency data were analyzed using Excel 2016 workbooks. Sequencing FASTq files were analyzed using the EPI2ME (Metrichor, Oxford, UK) automated 16S FASTQ pipeline. The workflow for bacterial species identification is demonstrated in **Figure 1.**

CONCLUSIONS

- Not one "universal" extraction method identified for MinION sequencing
- Multiple extraction methods can potentially be used in bacterial ID other than the recommended method by ONT.
- MinION may need to generate more reads than what was generated during a 12 hour run for 12 samples (approx. 1 M) for positive ID of these species
- Alternatively, universal primers used in the library prep kit may not be adequate for virtually all species
- Bacterial species rather than extraction methods may play a large role in sequencing accuracy but studies assessing error rate should be conducted

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