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Rapid bacterial identification from clinical specimens by using the MinION™ sequencing device: A pilot study

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ABSTRACT

Aims: DNA sequencing is a powerful tool and less time-consuming for bacterial detection and identification. The aim of this study was to compare the application of the Oxford Nanopore MinION™ sequencing device for direct DNA sequencing from clinical specimens with the routine workup.
Methodology and results: We used conventional bacteriological-based methods to detect and identify bacterial pathogens in 10 clinical specimens. In addition, the 16S metagenomic sequencing was performed by using a MinION™ sequencing device with barcoded primers of a 16S Barcoding kit (Code N° SQK-RAB204, Oxford Nanopore Technologies, UK). The DNA was amplified by PCR using specific 16S primers (27F and 1492R) that contain barcodes and 5' tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters of the 16S Barcoding kit. Data was analyzed with WIMP and EPI2ME to classify and identify species in real-time. Ten clinical specimens were processed for bacterial isolation. A total of 8 urine samples were subjected to culture-dependent methods, successfully identifying the presence of pathogenic bacteria. Out of the total eight urine samples, both methods successfully identified six bacterial pathogens. *Escherichia coli* were identified, and the others were detected as *Salmonella enterica*, *Veillonella parvula* and *Streptococcus anginosus* using MinION™ sequencing. Two urine samples had different results. *Escherichia coli* was detected directly through MinION™ sequencing, bypassing the need for culture results.
Conclusion, significance and impact of study: MinION™ sequencing of 16S rRNA genes could accurately detect diverse bacterial pathogens in clinical specimens. Additionally, the bacterial species classification generated by analyzing 16S rRNA gene sequences can be helpful for rapid identification. The whole procedure takes less than 8 h to complete; same-day diagnosis can be completed.

Keywords: 16S ribosomal RNA, bacterial identification, MinION, nanopores

INTRODUCTION

Infectious diseases caused by bacterial pathogens have a substantial global health impact and could become serious if undiagnosed with several diseases related to the central nervous system, blood infection and respiratory system. These can cause severe issues, including morbidity and increased mortality among patients with compromised immune systems (Bloom and Cadarette, 2019). In fact, the patients may be infected by bacterial contamination in intensive care units, including *Staphylococcus aureus*, *E. coli*, *Klebsiella* spp. (including *Klebsiella pneumoniae*), *Pseudomonas* spp. (including *Pseudomonas aeruginosa*), *Acinetobacter* spp. (including *Acinetobacter baumannii*) (Bhatta *et al*., 2022).

The patient's prognosis strongly depends on early diagnosis and optimized antibiotic therapy. Therefore, identifying the underlying pathogens responsible for the patients is essential. Reducing the time needed to obtain a microbiological diagnosis shortens the duration of broad empirical therapy and its selective pressures (Brouqui *et al*., 2009; Viale *et al*., 2017).

The routine methods for diagnostic microbes were the phenotypic method and the culture-dependent method. It is still considered the 'gold standard' in clinical microbiology for performing antibiotic susceptibility testing and other biochemical tests on significant bacterial isolates. Culture is more sensitive, with detection rates of 80% in the clinical samples of patients with acute disease before the initiation of treatment. The sensitivity of culture varies in case inclusion criteria, patient characteristics, laboratory practices and spectrum of bacterial pathogens. Culture method results are commonly unavailable for at least 24-48 h. It is difficult to detect by culture with

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fastidious, low-growing microorganisms or antibiotic exposure before collecting the samples (Laupland and Valiquette, 2013; Dubourg *et al*., 2018).

The other method for microbial identification in the laboratory is the genotypic method, the molecular diagnostic method. The high throughput DNA sequencing technologies have dramatically expanded the genetic information of all domains of life and microorganisms. The PCR technique can detect pathogens and antibiotic resistance genes in specimens without culture but cannot cover the diversity of organisms, high cost and complexity of clinical microbiology. Culture-independent analysis using next-generation sequencing technology has been advocated as a point-of-care diagnostic tool (Reuter *et al*., 2015; Kobras *et al*., 2021; Verma and Gazara, 2021).

Recently, Oxford Nanopore Technologies released a new third-generation sequencing platform, the MinION™ sequencer, like a USB-attached miniature device, which has shown promise in the ability to sequence long reads with an amount of a hundred thousand base pairs. This sequencer can rapidly detect microorganisms and antibiotic resistance genes in clinical samples. Sequencing platform-specific bioinformatics pipelines were designed and developed in-house to identify bacterial pathogens from the patients. This study aimed to demonstrate the usefulness of the MinION™ approach to identifying bacteria in the clinical sample. The overall purpose of this pilot study is to use the MinION™ sequencer to detect simultaneously the pathogenic bacterial in clinical specimens compared to routine work-up with the conventional methods (Kilianski *et al*., 2015; Brown *et al*., 2017; Goldstein *et al*., 2019; Imai *et al*., 2020; Santos *et al*., 2020).

MATERIALS AND METHODS

Clinical specimens

Eight urine and two sputum samples were selected for this pilot study. The samples were collected by clinicians from visits and hospitalized patients at Hue University Hospital, Hue City, Vietnam. Clinical samples were transported to the microbiological laboratory within 2 h after being collected for microbial analysis. The samples were processed for bacterial isolation and identification by routine microbiological and molecular methods according to the doctor's indication. In addition, aliquots of clinical samples were used for rapid identification by the MinION™ sequencing device.

Bacterial identification

Conventional bacteriological-based methods

The conventional methods used to detect and identify pathogen bacteria in clinical specimens based on traditional culture methods, including enrichment, dilution, plating and isolation of single species colonies for further biochemical tests characterization by using API strip, VITEK® automated system (bioMérieux, France).

Molecular methods

In addition, two sputum samples were performed *Taq*Man™ Real-Time PCR assays to detect *Mycobacterium tuberculosis* by using LightPower ^{iVA} MTB rPCR Kit (Viet A Corporation, HCM, Vietnam).

MinION™ sequencing and identification

DNA extraction from clinical samples

The sputum samples were pre-treated 1:1 volume ratio (v/v) with DTT (Sputafluid, Biolife, Italy) following the manufacturer's instructions (DTT solution 10%, vortexed and left at room temperature for 15 min) (Terranova *et al*., 2018). The urine sediment preparation samples were obtained by centrifuging at 400× *g* for 5 min. The ZymoBIOMICS™ DNA Microprep Kit (Zymo, CA, USA) was used for DNA extraction. Add 250 µL of pre-treated sputum sample or resuspended urine sediment to a ZR Bashing Bead™ Lysis tube (0.5 mm). Add 750 µL ZymoBIOMICS™ Lysis solution to the tube. DNA purification steps were performed according to the manufacturer's instructions and purified DNA was eluted with 20 µL of ZymoBIOMICS™ DNase/RNase free water. Finally, the yield and purity of total DNA were estimated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA).

qPCR for detecting 16S rRNA of bacteria

A quantitative polymerase chain reaction (qPCR) was performed to test the bacterial quantitation through the 16S rRNA gene. The total DNA of *E. coli* (ATCC® 25922™) was used as the control to build the standard curve. The real-time PCR assay was performed by using universal primers, 27F (5'-AGAGT TTGATCCTGGCTCAG-3') and 1492R (5' TACCTTGTTACGACTT-3'), which specifically targets the 16S rRNA genes. Ten ng DNA extractions, 0.4 µM for each primer, 0.1 µM of the probe, 1.25 µL for each primer (10 mM), 10 µL 2× SYBR green Master Mix (Thermo Scientific, MA, USA), 5.5 µL free water were combined in a 20 µL total volume reaction. PCR amplification was profiled as follows initial denaturation at 50 °C for 2 min, 95 °C for 5 min, followed by 35 cycles of 95 °C for 10 sec, 58 °C for 20 sec, 72 °C for 30 sec, then 72 °C for 5 min in Rotor-gene Q MDx - qPCR System (Qiagen, Germany).

MinION™ sequencing 16S rRNA gene

The 16S Barcoding Kit (Code SQK-RAB204, Oxford Nanopore Technologies) was used. A total of 10 ng genomic DNA was used for library preparation and MinION™ sequencing was performed using R9.4 flow cells (FLO-MIN106; Oxford Nanopore Technologies) according to the manufacturer's instructions. MinKNOW software ver. 1.11.3 was used for data acquisition, and EPI2ME cloud application for data analysis (Oxford Nanopore Technologies).

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Bioinformatics analysis

The 'pass' reads were obtained. MinION™ sequence reads (FAST5 data file). Raw data were processed for base calling via Albacore. Then, the data were analyzed by using What's In My Pot WIMP workflow, a quantitative, real-time species identification from metagenomic samples (Oxford Nanopore Technologies).

RESULTS

Species identification by using the culture-dependent method and real-time *Taq*Man PCR assay

Ten clinical specimens were processed for bacterial isolation. A total of eight urine samples were isolated and successfully identified the pathogenic bacteria by culture-dependent methods. There are six bacterial strains that were identified as *E. coli*, *Enterococcus* spp. (Group D), *K. pneumoniae* and *Klebsiella* spp. were detected respectively in each sample. Two sputum specimens were negative with an AFB smear test and real-time *Taq*Man PCR assay.

Optimization of qPCR assays for detection of 16S rRNA gene

The standard curve of bacterial 16S rRNA was performed by qPCR assay. Four samples had a high concentration (>10 ng/µL), five samples with a lower concentration of more than 1 ng/µL and one sample with a concentration of less than 1 ng/µL.

Bacterial identification by Nanopore MinION™ sequencing

The running data show that there were approximately 1450 active nanopores (71%) in flow cells. These results indicated that the MinION™ device run went well since at least 70% of nanopores in the flow cells should be active after launch vibration (Castro-Wallace *et al*., 2017). A high percentage of active pores was observed for the first 4 h. A total of 8.150.000 reads comprising 12,56 Gb, as determined from the MinKNOW software at the end of MinION™ run.

EPI2ME was used to analyze 540 FASTQ- run ID-pass files in this study. EPI2ME platform provides an analysis workflow of nanopore data in real-time. The following analysis workflows: FASTQ 16S QC-Barcoding to determine quality scores or read length distribution and WIMP program to identify bacterial species.

Identifying bacteria pathogens

The WIMP program can rapidly classify and identify bacteria, viruses, fungi or archaeal species. In the total of 2,132,229 reads analyzed, there were 1,884,027 (88.3%) reads classified and 248,202 reads unclassified. Bacteria accounted for 100% of the species identified, which is consistent with culture results. The program processed the results to determine bacterial species within the NCBI taxonomy tree. There were 12 different taxa, but *E. coli* is the most prevalent with the highest cumulative reads (289,387) and others are also present at lower concentrations.

Bacterial identification results from WIMP data were compared to the conventional routine methods (Table 1 and Table 2). Two sputum samples were negative for *M. tuberculosis* in both methods. Identification of bacterial pathogens by using MinION™ sequencer in the oral flora included *V. parvula*, *Streptococcus parasanguinis*, *Streptococcus salivarius* (sample 1) and *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, *V. parvula* (sample 6). *Veillonella parvula* can be associated with diseases such as periodontitis, dental caries and various systemic infections (Marriott *et al.*, 2007). It has also been isolated from women with bacterial vaginosis and associated with hypertension and *P. melaninogenica* (Pietropaoli *et al*., 2019).

Six of a total of eight urine samples and bacteria pathogens were identified in both methods. *E. coli* were identified, and the others were also detected as *S. enterica*, *V. parvula*, *S. anginosus* by using MinION™ sequencing. Two urine samples had different results. *Escherichia. coli* was detected by MinION™ sequencing without culture results in samples 7 and 8.

The MinION™ sequencing can identify the species level of bacteria. *Escherichia coli and Enterococcus* spp. (Group D) were detected by using culture-dependent methods, but these pathogens were determined at the species level as *E. coli*, *Enterococcus hirae*, *Enterococcus faecium* by using the MinION™ sequencer (sample 5). And *K. pneumoniae*, *E. coli* was also identified in sample 7, while the culture method identified the genus level of bacteria as *Klebsiella* spp.

Table 1: Comparing MinION™ sequencing results and the other methods.

Sample	Barcode	Cumulative reads	MinION™ sequence	AFB Ziehl-Neelsen stain	Real-time PCR (*M. tuberculosis*)
1	BC01	12525	*Veillonella parvula*	Negative	Negative
		12340	*Streptococcus parasanguinis*		
		11421	*Streptococcus salivarius*		
2	BC03	44879	*Capnocytophaga gingivalis*	Negative	Negative
		44370	*Prevotella melaninogenica*		
		37102	*Veillonella parvula*		

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Table 2: Comparing the routine bacteria identification method and MinION™ sequencing.

Sample	Barcode	Cumulative reads	MinION™ sequencer	API strip	VITEK®
3	BC04	67749	*Escherichia coli*	*Escherichia coli*	
		10341	*Salmonella enterica*		
4	BC05	81047	*Escherichia coli*	*Escherichia coli*	
		11812	*Salmonella enterica*		
5	BC07	55161	*Enterococcus hirae*	*Escherichia coli*	
		19105	*Enterococcus faecium*	*Enterococcus* spp. (Group D)	
		1620	*Escherichia colic*		
6	BC08	745	*Escherichia coli*	*Escherichia coli*	
		86524	*Veillonella parvula*		
		13527	*Streptococcus anginosus*		
7	BC09	23210	*Escherichia coli*	*Klebsiella* spp.	
		19403	*Klebsiella pneumoniae*		
8	BC10	13498	*Escherichia coli*		*Klebsiella pneumoniae*
		10512	*Klebsiella pneumoniae*		
9	BC11	74943	*Escherichia coli*	*Escherichia coli*	
		10274	*Salmonella enterica*		
10	BC12	9327	*Escherichia coli*	*Escherichia coli*	
		13181	*Veillonella parvula*		

DISCUSSION

Currently, the identification of clinically relevant bacteria largely relies on culture-based techniques. The culture-dependent method was still a “gold standard” for detecting pathogens in the microbiology laboratory due to performing the antibiotic susceptibility testing of significant bacterial isolates and other biochemical tests. However, culture-dependent methods are time-intensive and potentially lead to delayed or incorrect diagnoses. Metagenomic sequencing analysis provides an alternative approach for identifying bacterial pathogens in clinical specimens.

However, laboratories cannot accurately detect antibiotic-resistant or unculturable bacteria in the artificial culture medium. It led to delayed diagnosis (Clarridge *et al*., 1996).

Next-generation sequencing (NGS) and metagenomic sequencing analysis have the potential to dramatically revolutionize the clinical microbiology laboratory by replacing current time-consuming and labor-intensive techniques with a single, all-inclusive diagnostic test. NGS can identify faster clinical pathogens and antibiotic-resistant genes but high cost and complex processes (Chelsie, 2015). Metagenomic sequencing analysis provides an alternative approach for identifying bacterial pathogens in clinical samples (Srinivasan *et al*., 2015; Pietropaoli *et al*., 2019). One of these new approaches is MinION™ sequencing as the third-generation sequencing platform brings many advantages for molecular diagnosis technology containing: sequencing rapid the nucleic acid, allowing DNA modification detection and direct RNA sequencing to be performed, real-time data acquisition and analysis, and the ability to generate long reads (Chaisson and Tesler, 2012; Oikonomopoulos *et al*., 2016; Byrne *et al*., 2017; Simpson *et al*., 2017). MinION™ sequencing can identify microbial by performing the full-length 16S rRNA genes, ITS region and 23S rRNA gene, and the whole genome in a short time (Kilianski *et al*., 2015; Wang *et al*., 2015; Cuscó *et al*., 2018).

This pilot study showed that bacterial pathogens were identified rapidly in 10 clinical samples by using MinION™ sequencing, not dependent on culture methods. Our finding of read per exit status is more than 97%, the mean sequence length reached approximately 1,500 bases and no sample was of a mean quality score less than 9 (except BC12, sample 10). The bacteria occupied the rate of 100%, with no virality. WIMP process results determine bacterial species in the NCBI taxonomy tree.

Four of the ten clinical samples had a total concentration of DNA very low (10 ng/µL). Among the various challenges with implementing NGS for bacterial detection using metagenomics like 16S Barcode, the presence of an overwhelming amount of human DNA is one of the most critical problems to be addressed. The high background of human DNA also affects the sensitivity for pathogens that occur in low abundance in clinical specimens (Hasan *et al*., 2016). However, six low-concentration samples were also identified as the species level of bacteria using MinION™ sequencing, similar to the culture-dependent method. The 16S rRNA gene can be amplified from all bacteria non-specifically without amplifying eukaryotic host DNA or viruses for bacterial species identification. The results indicated the feasibility of MinION™ sequencing for rapid bacteria identification accurately to the species with the low biomass samples.

Two sputum samples were negative for *M. tuberculosis* by conventional smear microscopy with the Ziehl-Neelsen (ZN) stain, real-time *Taq*Man PCR assay and the MinION™ sequencing. MinION™ results also determined the other bacterial pathogens. Real-time PCR might be a more sensitive and reliable method for only

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individual bacteria by gene target. MinION™ devices can sequence the full-length 16S rRNA gene to identify all bacteria (Santos *et al*., 2020).

In our study, we found that there are different identification results between MinION™ sequencing and culture-dependent methods. *Escherichia coli* was identified in 2 un-grown samples by using MinION™ sequencing. *Klebsiella pneumoniae was* identified in both methods (samples 8 and 10). The culture method was a "gold standard" for detecting bacteria, but bacteria identification results were affected by delayed transit and bad storage (Shrestha, 1975). Specimens collected after antibiotic exposure may reduce culture-based bacterial detections (Harris *et al*., 2017). It is also difficult to detect by culture with fastidious or slow-growing microorganisms.

Our study successfully identified the bacterial species *E. hirae* and *E. faecium* (sample 5), as well as *K. pneumoniae* (sample 7). However, the culture-dependent methods could only identify the bacteria at the genus level. The WIMP workflow classifies and identifies species in real-time: as soon as a strand of DNA passes through the pore, it can be base called and analyzed. WIMP makes use of a Centrifuge, which can accurately identify reads when using databases containing multiple highly similar reference genomes, such as different strains of a bacterial species. WIMP processes Centrifuge results to determine the most reliable placement in the taxonomy tree, assigning a score to each taxonomic placement (Brown *et al*., 2017; Imai *et al*., 2020).

The MinION™ sequencing is a new approach with great potential in the rapid identification of bacterial pathogens and antibiotic resistance genes in clinical specimens. Short time, portability, speed and versatility make MinION™ a promising tool for low-resolution, rapid nucleic acid sequencing. Using the 16S rRNA analysis workflow, species identities are returned in almost real-time, making it possible to go from cells to identification in 6-8 h.

CONCLUSION

We sequenced ten clinical samples via the MinION™ portable sequencer. Six of the eight urine samples were matched entirely. Two sputum samples were negative in both methods. This study confirms the effectiveness of the MinION™ sequencing in identifying bacterial pathogens rapidly from the clinical samples through the 16S rRNA gene in a short time.

MinION™ sequencing may be a helpful tool besides the culture method in microbiology laboratories. To use this technology as a supplemental tool in hospitals better, the scientist must have more work experience and research to build up the accuracy workflow, optimize the protocols for preparation of the DNA library and load the clinical sample to MinION™ devices carefully. In addition, the clinician must have good skills in analyzing the data through bioinformatics.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare relevant to this article's content.

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