Pilot study: MinION™-based identification of antibiotic resistance genes from 16S rRNA sequences

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Abstract

Background: The MinION™ is a portable DNA sequencing device that can sequence 16S rRNA genes. 16S rRNA genes are found in all bacteria and can be used to identify bacterial species. By sequencing 16S rRNA genes and analyzing the sequences for antibiotic resistance genes, we can identify bacteria that are resistant to antibiotics. **Materials and methods:** Ten clinical specimens, including two sputum samples and eight urine samples from outpatients and inpatients, were subjected to pathogenic bacteria identification and antibiotic resistance detection using the MinION™ portable device. **Results:** The MinIONTM sequencer successfully identified species levels in ten clinical samples. Based on investigating the 16S rRNA sequencing with AMRA on EPI2ME of Nanopore Technologies, we found eight mutations**. Conclusions:** The MinION sequencer is a valuable tool in medical laboratories for swiftly identifying bacterial species and determining their antibiotic resistance profiles, contributing to efficient antimicrobial resistance diagnostics.

Keywords: *16S rRNA, antibiotic resistance genes, portable DNA sequencing device.*

1. INTRODUCTION

As observed through conventional antimicrobial sensitivity testing, the molecular analysis of genetic mechanisms responsible for specific phenotypic outcomes has become essential to numerous clinical investigations focused on bacterial infections. In certain scenarios where phenotypic results are either time-consuming, inconclusive, or unavailable, molecular analysis can be employed to ascertain the presence of particular genes or point mutations. This approach directly supports the timely implementation of optimal treatment or control strategies. Moreover, molecular characterization serves as a valuable tool in epidemiological studies during outbreaks, especially when phenotypic data lacks the granularity required to manage potential outbreaks involving drug-resistant bacteria [1]. Additionally, the molecular characterization of antimicrobial resistance (AMR) determinants plays a critical role in local, national, and even global surveillance efforts to track AMR trends [2].

Some initiatives have been implemented in Vietnam to address AMR. Vietnam was the first country in the Western Pacific Region to develop a national action plan to combat AMR, which, according to the World Health Organization (WHO), is being implemented. Vietnam also has one of the highest rates of AMR in Asia due, in part, to the overuse of antimicrobial drugs, both in the animal health sector and in humans in hospitals and the community [3].

The MinION™ device is a portable DNA sequencing device that can sequence 16S rRNA genes. 16S rRNA genes are found in all bacteria and can be used to identify bacterial species. By sequencing 16S rRNA genes and analyzing the sequences for antibiotic resistance genes, scientists can identify bacteria that are resistant to antibiotics. The MinION™ device is a powerful tool for detecting antibiotic resistance genes, as it is portable, fast, and accurate. This makes it ideal for use in various settings, including hospitals, clinics, and research laboratories [4,5]. On the other hand, mutations in the 16S ribosomal RNA (rRNA) gene can potentially lead to antibiotic resistance in bacteria. The 16S rRNA gene is a component of the bacterial ribosome, the molecular machine responsible for protein synthesis. Antibiotics often target the bacterial ribosome to inhibit protein synthesis, and mutations in the 16S rRNA gene can alter the structure of the ribosome, making it less susceptible to the effects of certain antibiotics. When a mutation occurs in the 16S rRNA gene, it can change the shape or binding site of the ribosome, making it more difficult for antibiotics to bind to and disrupt the protein synthesis process [6]. The objectives of this pilot study were to examine the data collected by sequencing the full-length 16S rRNA sequences containing mutations that cause antibiotic resistance in pathogenic bacterial strains identified from clinical samples by using the MinION™ device.

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2. MATERIAL AND METHODS

2.1. Materials

Clinical specimens

For this initial study, ten clinical specimens, including two sputum samples and eight urine samples, were taken from both outpatients and inpatients at the Hue University of Medicine and Pharmacy Hospital, Hue City, Vietnam. The clinical specimens were swiftly transported to the microbiological laboratory within a 2-hour following collection microbial analysis. Subsequently, the samples underwent bacterial isolation and identification procedures using standard microbiological and molecular techniques as directed by the attending physician. Additionally, portions of the clinical samples were employed for rapid identification by utilizing the MinION™ sequencing device.

2.2. Methods

The culture-independent approach will be used to directly identify bacteria from patient samples and analyze the 16S rRNA sequence data using the MinION™ device. The sequence data were used for bacteria identification and 16S rRNA point mutation conferring antibiotic resistance (Figure 1).

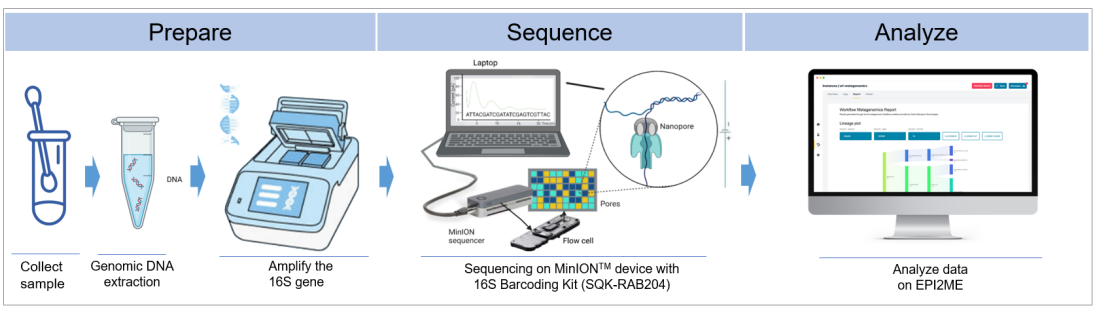

Figure 1. Bacterial 16S rRNA sequencing workflow on MinION™ sequencing device

Genomic DNA extraction

The DNA extraction process involved the utilization of the ZymoBIOMICS™ DNA Microprep Kit (Zymo, CA, USA). Initially, 250 µL of pre-treated samples were introduced into a ZR Bashing Bead™ Lysis tube (0.5 mm), followed by adding 750 µL of ZymoBIOMICS™ Lysis solution to the same tube. Subsequently, DNA purification procedures were carried out per the manufacturer's guidelines. The purified DNA was ultimately eluted using 20 µL of ZymoBIOMICS™ DNase/RNase-free water. Measurements were conducted utilizing the NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA) to assess the yield and purity of the total DNA obtained.

MinION™ sequencing 16S rRNA genes

The 16S Barcoding Kit (Code SQK-RAB204) from Oxford Nanopore Technologies was employed. A total of 10 ng of genomic DNA was used for the library preparation process, and MinION™ sequencing was executed using R9.4 flow cells (FLO-MIN106) as per the manufacturer's recommended protocols. For data acquisition, we utilized MinKNOW software version 1.11.3, while data analysis was performed using the EPI2ME cloud application, both provided by Oxford Nanopore Technologies [7,8].

Sequence 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). The "fastq" format is a 4-line string (text) data format denoting a sequence and its corresponding quality score values. There are different ways of encoding quality in a .fastq file; however, files from Oxford Nanopore Technology sequencing devices use Sanger phred scores. A sequence record comprises 4 lines: line 1: Sequence ID and Sequence description; line 2: Sequence line e.g., ATCGs; line 3: + symbol (can additionally have a description); line 4: Sequence line qualities.

3. RESULTS

3.1. Species identification strategies

In this study, 540 FASTQ run ID-pass files were subjected to analysis using the EPI2ME platform, which offers real-time data analysis capabilities for nanopore sequencing. Two specific analysis workflows were employed: FASTQ 16S QC-Barcoding for assessing quality scores and read length distribution, and the WIMP program for the rapid identification of various species, including bacteria, viruses, fungi, and archaea. Among the 2,132,229 reads analyzed, 1,884,027 reads

(88.3%) were successfully classified, while 248,202 reads remained unclassified. All identified species belonged to the bacterial taxa, aligning with results obtained through traditional culture methods.

The program further delved into the results to ascertain the specific bacterial species within the NCBI taxonomy tree. A total of 12 different taxa were identified, with *E. coli* emerging as the most prevalent species, present in 8 out of 10 samples. Additionally, in eight urine samples, *E. coli* was detected, along with other species such as *Salmonella enterica, Veillonella parvula,* and *Streptococcus anginosus*, identified through MinION™ sequencing (Table 1).

One notable strength of this method lies in its high accuracy. This accuracy is attributed to the use of PCR to amplify the 16S rRNA gene in the specimen, even when bacterial quantities are low. In contrast, traditional identification cultures often struggle to achieve such precision in low-bacteria scenarios.

Table 1. Species identification by MinION™ sequencer device in clinical samples

Sample	Barcode	Sample type	Species identification by MinION™ sequencer
1	BC01	Sputum	*Veillonella parvula* *Streptococcus parasanguinis* *Streptococcus salivarius*
2	BC03	Sputum	*Capnocytophaga gingivalis* *Prevotella melaninogenica* *Veillonella parvula*
3	BC04	Urine	*Escherichia coli* *Salmonella enterica*
4	BC05	Urine	*Escherichia coli* *Salmonella enterica*
5	BC07	Urine	*Enterococcus hirae* *Enterococcus faecium* *Escherichia coli*
6	BC08	Urine	*Escherichia coli* *Veillonella parvula* *Streptococcus anginosus*
7	BC09	Urine	*Escherichia coli* *Klebsiella pneumoniae*
8	BC10	Urine	*Escherichia coli* *Klebsiella pneumoniae*
9	BC11	Urine	*Escherichia coli* *Salmonella enterica*
10	BC12	Urine	*Escherichia coli* *Veillonella parvula*

3.2. 16S rRNA point mutation investigation

For each specimen, species were identified from sequencing reads using either EPI2ME workflows 'Fastq WIMP (What's in my pot?)' and 'Fastq Antimicrobial Resistance' (v3.2.2, https://epi2me.nanoporetech.com/) The WIMP workflow classifies reads using the Centrifuge standard database, whilst the Antimicrobial Resistance workflow uses minimap2 to align full-length of 16S rRNA data to the AMRA CARD

database to identify AMR genes based on rRNA mutation model [9]. The gene clinically Relevant, antibiotics, drug class, and resistance mechanism were obtained from the reports of AMRA CARD (Table 2). Almost all types of gene clinically relevant are point mutations in the domain of the 16S rRNA with the resistance mechanism being antibiotic target alteration.

Table 2. CARD Model: rRNA mutation model

No	Taxon	Gene Clinically Relevant	Antibiotics	Drug Class	Resistance Mechanism
1	*E. coli*	Point mutations in the 3' minor domain of the 16S rRNA	Edeine	Peptide antibiotic	Antibiotic target alteration
2	*E. coli*	Point mutations in the 5' domain of helix 18, in the *rrnB* 16S rRNA gene	Streptomycin	Aminoglycoside	Antibiotic target alteration
3	*E. coli*	Point mutations in the 3' major domain of the *rrsB* 16S rRNA gene	Tetracycline	Tetracycline	Antibiotic target alteration
4	*E. coli*	Point mutations in the 3' minor domain of helix 44, in the *rrsB* 16S rRNA gene	Gentamicin C	Aminoglycoside	Antibiotic target alteration
5	*E. coli*	Point mutations in the 3' minor domain of helix 44, in the *rrsB* 16S rRNA gene	Kanamycin A	Aminoglycoside	Antibiotic target alteration
6	*E. coli*	Point mutations in the 3' minor, 3' major, and central domains in the *rrsC* 16S rRNA gene	Kasugamicin	Aminoglycoside	Antibiotic target alteration
7	*E. coli*	Point mutations in the 3' major domain of the *rrsH* 16S rRNA gene	Spectinomycin	Aminoglycoside	Antibiotic target alteration
8	*S. enterica*	Point mutations in the helix 34 regions of the *rrsD* 16S rRNA gene	Spectinomycin	Aminoglycoside	Antibiotic target alteration

4. DISCUSSIONS

The 16S rRNA gene is widely used for bacterial identification due to its highly conserved regions suitable for universal primers and phylogenetic signals, as well as its highly variant regions that differ across species. This gene is present in almost all bacterial families, providing functional and evolutionary stability, and its sequence length of about 1500-1550 bp is suitable for taxonomical purposes and amplification [10]. The 16S rRNA gene also contains hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. The Ribosomal Database Project (RDP) provides quality-controlled bacterial and archaeal small subunit rRNA alignments and analysis tools, supporting the use of the 16S rRNA gene for bacterial identification [11]. The 16S Barcoding Kit 1-24 enables rapid 16S sequencing for organism identification by narrowing down to a specific region of interest, allowing users to see all the organisms in a sample without sequencing unnecessary genomics. This approach aligns with the use of the 16S rRNA gene for bacterial identification, as it focuses on a specific region of the gene to obtain relevant information without sequencing unnecessary regions of the genome. Nanopore sequencing has been used for the identification of bacteria present in both monomicrobial and polymicrobial samples, resolving microbiological diagnosis. Our study shows that Nanopore sequencing can detect and identify the pathogenic bacteria in low-bacteria urine samples which may not be detected in the culture-independent method. Zhu et al., 2020 applied the nanopore sequencing technique in infectious diseases, including monitoring of emerging infectious

disease outbreaks, identification of pathogen drug resistance, and disease-related microbial community characterization [12].

Nanopore sequencing is now used for real-time detection of antibiotic resistance genes in bacteria. This approach enhances pathogen identification and facilitates the tracking of antibiotic resistance. TNPseq, a novel nanopore sequencing method, aids in identifying bacterial and fungal infections relevant to clinical cases. MinION alone demonstrated high accuracy in detecting antibiotic resistance genes, as evidenced in clinical isolates of *Klebsiella pneumoniae* [13]. Additionally, nanopore sequencing allows for rapid identification of pathogens, plasmids, and antimicrobial resistance genes in bacterial DNA extracted from positive blood cultures [14]. This technology is crucial in addressing antibiotic resistance threats, such as carbapenem-resistant Gram-negative organisms. Rapid nanopore-based DNA sequencing protocols contribute to outbreak investigations and pathogen control [15].

In our pilot study, only the sequences of the 16S rRNA gene were used to investigate AMR genes based on the rRNA mutation model. Eight AMR genes in three classes of antibiotics related to the rRNA mutations: peptide antibiotic, tetracycline, and aminoglycoside (Table 2). Most ribosome-targeting antibiotics interact exclusively with bacterial rRNA. Bacteria have evolved several resistance mechanisms to antibiotics, including through the methylation of specific rRNA nucleotides that prevent the binding of protein synthesis inhibitors to their target sites on the bacterial ribosome. For instance, N1 methylation of A1408 in the bacterial 16S rRNA confers resistance against aminoglycosides [16]. Loss of methylation can also decrease antibiotic sensitivity. A classic example is the lack of methylation at A1518 and A1519 in 16S rRNA by *KsgA*, which confers resistance to kasugamycin [17]. This evidence shows the important role of methylation in regulating the response to antibiotics.

Adenyltransferase enzymes in some bacteria adenylate specific adenine residues in the 16S rRNA, hindering spectinomycin binding to the ribosome and thus impeding protein synthesis inhibition. This modification, a post-transcriptional adjustment, influences ribosome assembly and function in bacteria. The structural mechanism of AadA, a dual-specificity aminoglycoside adenyltransferase, sheds light on how antibiotics like spectinomycin and streptomycin interact with bacterial ribosomes, impacting protein synthesis. This process showcases bacterial resistance mechanisms against ribosomal inhibitors, contributing to our understanding of antibiotic resistance [18]. Deamination of adenine or cytosine residues in the 16S rRNA by bacteria can impact ribosomal structure, reducing susceptibility to spectinomycin binding. This modification alters the RNA sequence, potentially affecting ribosomal function. Notably, adenine deamination can be achieved programmatically using a Cas9–adenine-deaminase fusion in bacteria. The resulting structural changes may influence antibiotic interactions, contributing to bacterial resistance mechanisms. Bacterial deamination of adenine or cytosine in 16S rRNA alters ribosomal structure. Cas9–adenine-deaminase fusion allows programmable adenine deamination in bacteria. This modification reduces susceptibility to spectinomycin binding, impacting antibiotic interactions [19].

Resistance to spectinomycin can arise from mutations in ribosomal proteins that interact with the 16S rRNA. These mutations impact spectinomycin binding or alter the ribosome's overall structure, reducing susceptibility to the antibiotic. Notably, mutations in the spectinomycin binding region of helix 34 of 16S rRNA play a role in this resistance. Additionally, chromosomal mutations in the gene encoding ribosomal protein S12 (*rpsL*) can confer resistance to spectinomycin. These alterations affect the ribosome's response to spectinomycin, contributing to antibiotic resistance. Understanding these molecular mechanisms is crucial for addressing antibiotic resistance challenges [20]. The modifications to the 16S rRNA negatively impact antibiotic efficacy, leading to reduced effectiveness. This occurs because alterations in 16S rRNA can hinder the binding of antibiotics to their target sites, diminishing the drugs' ability to exert their antimicrobial effects. Additionally, mechanisms such as enzymatic modifications of antibiotic targets, as mentioned in the search results, can contribute to antibiotic resistance, rendering the drugs ineffective.

5. CONCLUSION

Through ten clinical samples, bacteria species levels were identified by using the MinION™ sequencer. Eight mutations were found when investigating the 16S rRNA sequencing with AMRA on EPI2ME of Nanopore Technologies. The MinION™ sequencer demonstrated that it is a highly portable device that utilizes nanopore ultra-long read sequencing technology to detect antimicrobial resistance (AMR) rapidly. This device can be effectively used in medical laboratories to identify bacterial species at the genomic level

and assess their antibiotic resistance profiles. The MinION sequencer is a valuable tool in medical laboratories for swiftly identifying bacterial species and determining their antibiotic resistance profiles, contributing to efficient antimicrobial resistance diagnostics.

REFERENCES

[1] Gajic I, Kabic J, Kekic D, Jovicevic M, Milenkovic M, Mitic Culafic D, et al. Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods. Antibiotics 2022;11:427. https://doi.org/10.3390/antibiotics11040427.

[2] Frost I, Van Boeckel TP, Pires J, Craig J, Laxminarayan R. Global geographic trends in antimicrobial resistance: the role of international travel. J Travel Med 2019;26. https://doi.org/10.1093/jtm/taz036.

[3] Torumkuney D, Kundu S, Vu G Van, Nguyen HA, Pham H Van, Kamble P, et al. Country data on AMR in Vietnam in the context of community-acquired respiratory tract infections: links between antibiotic susceptibility, local and international antibiotic prescribing guidelines, access to medicines and clinical outcome. Journal of Antimicrobial Chemotherapy 2022;77:i26–34. https://doi.org/10.1093/JAC/DKAC214.

[4] Santos A, van Aerle R, Barrientos L, Martinez-Urtaza J. Computational methods for 16S metabarcoding studies using Nanopore sequencing data. Comput Struct Biotechnol J 2020;18:296–305. https://doi.org/10.1016/j.csbj.2020.01.005.

[5] Wang Y, Yang Q, Wang Z. The evolution of nanopore sequencing. Front Genet 2015;5. https://doi.org/10.3389/fgene.2014.00449.

[6] De Stasio EA, Moazed D, Noller HF, Dahlberg AE. Mutations in 16S ribosomal RNA disrupt antibiotic--RNA interactions. EMBO J 1989;8:1213–6. https://doi.org/10.1002/J.1460-2075.1989.TB03494.X.

[7] Kai S, Matsuo Y, Nakagawa S, Kryukov K, Matsukawa S, Tanaka H, et al. Rapid bacterial identification by direct PCR amplification of 16S rRNA genes using the MinION™ nanopore sequencer. FEBS Open Bio 2019;9:548–57. https://doi.org/10.1002/2211-5463.12590.

[8] Imai K, Nemoto R, Kodana M, Tarumoto N, Sakai J, Kawamura T, et al. Rapid and Accurate Species Identification of Mitis Group Streptococci Using the MinION Nanopore Sequencer. Front Cell Infect Microbiol 2020;10. https://doi.org/10.3389/fcimb.2020.00011.

[9] Ring N, Low AS, Wee B, Paterson GK, Nuttall T, Gally D, et al. Rapid metagenomic sequencing for diagnosis and antimicrobial sensitivity prediction of canine bacterial infections. Microb Genom 2023;9. https://doi.org/10.1099/mgen.0.001066.

[10] Awad M, Ouda O, El-Refy A, El-Feky FA, Mosa KA, Helmy M. FN-Identify: Novel Restriction Enzymes-Based Method for Bacterial Identification in Absence of Genome Sequencing. Adv Bioinformatics 2015;2015:1–14. https://doi.org/10.1155/2015/303605.

[11] Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141–5. https://doi.org/10.1093/nar/gkn879.

[12] Zhu X, Yan S, Yuan F, Wan S. The Applications of Nanopore Sequencing Technology in Pathogenic Microorganism Detection. Canadian Journal of Infectious Diseases and Medical Microbiology 2020;2020:1–8. https://doi.org/10.1155/2020/6675206.

[13] Zhu X, Yan S, Yuan F, Wan S. The Applications of Nanopore Sequencing Technology in Pathogenic Microorganism Detection. Canadian Journal of Infectious Diseases and Medical Microbiology 2020;2020:1–8. https://doi.org/10.1155/2020/6675206.

[14] Taxt AM, Avershina E, Frye SA, Naseer U, Ahmad R. Rapid identification of pathogens, antibiotic resistance genes and plasmids in blood cultures by nanopore sequencing. Sci Rep 2020;10:7622. https://doi.org/10.1038/s41598-020-64616-x.

[15] Ferreira FA, Helmersen K, Visnovska T, Jørgensen SB, Aamot HV. Rapid nanopore-based DNA sequencing protocol of antibiotic-resistant bacteria for use in surveillance and outbreak investigation. Microb Genom 2021;7. https://doi.org/10.1099/mgen.0.000557.

[16] Kanazawa H, Baba F, Koganei M, Kondo J. A structural basis for the antibiotic resistance conferred by an N1-methylation of A1408 in 16S rRNA. Nucleic Acids Res 2017;45:12529–35. https://doi.org/10.1093/nar/gkx882.

[17] Poldermans B, Van Buul CP, Van Knippenberg PH. Studies on the function of two adjacent N6,N6-dimethyladenosines near the 3' end of 16 S ribosomal RNA of Escherichia coli. II. The effect of the absence of the methyl groups on initiation of protein biosynthesis. J Biol Chem 1979;254:9090–3.

[18] Stern AL, Van der Verren SE, Kanchugal P S, Näsvall J, Gutiérrez-de-Terán H, Selmer M. Structural mechanism of AadA, a dual-specificity aminoglycoside adenylyltransferase from Salmonella enterica. Journal of Biological Chemistry 2018;293:11481–90. https://doi.org/10.1074/jbc.RA118.003989.

[19] Martin-Pascual M, Batianis C, Bruinsma L, Asin-Garcia E, Garcia-Morales L, Weusthuis RA, et al. A navigation guide of synthetic biology tools for Pseudomonas putida. Biotechnol Adv 2021;49:107732. https://doi.org/10.1016/j.biotechadv.2021.107732.

[20] Springer B, Kidan YG, Prammananan T, Ellrott K, Böttger EC, Sander P. Mechanisms of Streptomycin Resistance: Selection of Mutations in the 16S rRNA Gene Conferring Resistance. Antimicrob Agents Chemother 2001;45:2877–84. https://doi.org/10.1128/AAC.45.10.2877-2884.2001.