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Comparison of 16S rRNA sequencing methods for bacterial identification in clinical microbiology laboratories: Sanger sequencing vs. third-generation sequencing

Klinik mikrobiyoloji laboratuvarlarında bakteriyel tanımlama için 16S rRNA dizileme yöntemlerinin karşılaştırılması: Sanger dizilemesi ve üçüncü nesil dizileme

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ABSTRACT

Objective: This study aims to evaluate and compare Sanger sequencing and third-generation 16S rRNA sequencing methods in terms of their ability to accurately identify various bacteria under identical primer sets and specific PCR conditions using four American Type Culture Collection (ATCC) strains. In this context, the capacity of each method was assessed to accurately and comprehensively identify bacteria from pure cultures, where the genus and species of the organisms are confirmed and known.

Methods: In the study, identical primer sets and PCR conditions were used to enable a comparison between the two sequencing methods. While eight primers targeting the 16S rRNA region were used in the Sanger sequencing method, only two primers were used for the same region in the third-generation 16S rRNA sequencing method. The raw data were analyzed using GeneStudio software for Sanger sequencing and an appropriate bioinformatics workflow for the third-generation 16S rRNAsequencing method.

ÖZET

Amaç: Bu çalışma, Sanger dizileme ve üçüncü nesil 16S rRNA sekanslama yöntemlerinin, aynı primer ve belirli PCR koşulları altında dört Amerikan Tip Kültür Koleksiyonu (ATCC) suşu kullanılarak doğru şekilde bakterileri tanımlama yeteneklerini karşılaştırmalı olarak değerlendirmeyi amaçlamaktadır. Bu bağlamda, her bir yöntemin, cins ve türleri önceden doğrulanmış ve bilinen saf kültürlerden bakterileri doğru ve kapsamlı bir şekilde tanımlama kapasitesi değerlendirilmiştir.

Yöntem: Çalışmada, iki farklı sekanslama yönteminin karşılaştırılabilmesi amacıyla aynı primer setleri ve PCR koşulları kullanılmıştır. Sanger dizileme yönteminde 16S rRNA bölgesini kapsayan sekiz primer kullanılırken üçüncü nesil 16S rRNA sekanslama yönteminde aynı bölge için 2 primer kullanılmıştır. Ham veriler, Sanger Dizilemede GeneStudio yazılımında, üçüncü nesil 16S rRNA sekanslama yönteminde ise uygun biyoinformatik iş akışı ile analiz edilmiştir.

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Results: The analysis demonstrated that third-generation 16S rRNA sequencing outperforms Sanger sequencing in achieving comprehensive and efficient profiling of the full 16S rRNA region. Third-generation 16S sequencing reduces primer requirements, minimizes sequence loss in primer-binding regions, and significantly decreases processing time from days to hours, enabling rapid, high-throughput sequencing in a matter of minutes. In contrast, Sanger sequencing provides high single-read accuracy but falls short in terms of speed and efficiency, making it less suitable for broader applications.

Conclusion: This study highlights third-generation 16S sequencing as a faster, more comprehensive, and more effective option for high-resolution microbial investigations. While Sanger sequencing remains a valuable tool for certain scenarios due to its high single-read accuracy, its speed and depth limitations render it inadequate for broader applications. These findings emphasize the advantages of third-generation 16S sequencing in providing scientists with a reliable and thorough approach to characterizing microorganisms for various applications.

Key Words: Third-generation sequencing, 16S sequencing, sanger sequencing, microbial profiling, sequencing method comparison

Bulgular: Analiz sonuçları, üçüncü nesil 16S rRNA sekanslama yönteminin Sanger dizilemeden daha kapsamlı ve verimli bir şekilde tam 16S rRNA bölgesini profilleyebildiğini göstermiştir. Üçüncü nesil 16S sekanslama yöntemi, primer gereksinimlerini azalttığı ve primer bağlanma bölgelerinde dizi kaybını en aza indirdiği gibi, işlem süresini günlerden saatlere düşürerek dakikalar içinde hızlı, yüksek verimli dizileme olanağı tanımaktadır. Buna karşın, Sanger dizilemenin tek okuma doğruluğu yüksek olmasına rağmen, uzun işlem süresi ve düşük verimliliği nedeniyle geniş kapsamlı uygulamalar için daha az uygun olduğu belirlenmiştir.

Sonuç: Bu çalışma, üçüncü nesil 16S sekanslama yönteminin yüksek çözünürlüklü mikrobiyal analizler için daha hızlı, daha kapsamlı ve daha etkili bir seçenek olduğunu ortaya koymaktadır. Sanger dizileme, yüksek tek-okuma doğruluğu sayesinde belirli durumlarda hâlâ değerli bir araç olarak kabul edilse de, yavaşlığı ve sınırlı kapsama alanı nedeniyle daha geniş uygulamalar için yetersiz kalmaktadır. Elde edilen bulgular, üçüncü nesil 16S sekanslama yönteminin mikroorganizmaların karakterizasyonunda güvenilir ve ayrıntılı bir yaklaşım sunduğunu vurgulamakta ve çeşitli mikrobiyal uygulamalarda sağladığı avantajlara dikkat çekmektedir.

Anahtar Kelimeler: Üçüncü nesil sekanslama, 16S sekanslama, sanger sekanslama, mikrobiyal profilleme, sekanslama yöntemlerinin karşılaştırılması

INTRODUCTION

The 16S ribosomal RNA (rRNA) gene, found in all bacterial and archaeal genomes, is an ideal target for bacterial identification (1). This gene encodes the rRNA component of the ribosome, essential for protein synthesis, and contains both variable and conserved regions (2). While the variable regions provide the necessary sequence diversity to distinguish between different species or even strains, the conserved regions enable the design of universal primers that can amplify the 16S rRNA gene across a wide range of bacteria (3). 16S rRNA gene is commonly referred to as a “barcode gene” for bacterial identification due to its ability to classify and identify various bacterial species in ambient or mixed samples (4).

The identification of bacteria begins with morphological characterization, where colony morphology, Gram staining, and cellular structure are analyzed to determine preliminary classifications. This step is followed by biochemical assays, which assess metabolic and enzymatic activity to differentiate

bacterial species based on their phenotypic traits. MALDI-TOF mass spectrometry, which analyzes protein mass spectra, has largely replaced traditional biochemical tests for the precise and rapid identification of microorganisms. By leveraging protein profiling, it compares unique spectra to extensive microbial databases, offering high accuracy and efficiency (5). Molecular techniques such as polymerase chain reaction (PCR) are essential for identifying variants and verifying the identity of bacteria by distinguishing closely related species, identifying genetic variants, and validating the outcomes of biochemical or protein-based techniques through the detection of specific genetic markers or mutations. Consequently, PCR is a vital tool in clinical microbiology, especially when high specificity and sensitivity are required (6).

Advances in sequencing technologies have changed the study of microbial communities, enabling instruments for increasingly comprehensive insights into microbial diversity, composition, and functional potential (7). Sanger sequencing, introduced in 1977, marked a significant milestone in sequencing history and quickly became the most common method for DNA sequencing due to its high base-pair accuracy (8). This method, based on chain-termination principles, comprises stepwise synthesis and detection of individual bases, delivering dependable findings for single-gene targets in isolated samples (9). While highly accurate, Sanger sequencing is fundamentally limited by its lower throughput and extended run times, making it less suited for complex, diverse microbial communities or for cases requiring in-depth metagenomic analysis (10). It is primarily restricted to sequencing individual, cloned genes from isolated organisms, which can be valuable for targeted investigations. However, this approach proves inadequate for broader applications that require profiling multiple species within mixed microbial populations (11).

Unlike conventional short-read based next-generation sequencing techniques, third-generation sequencing is an advanced NGS technology that has significantly enhanced the capacity to generate long-read sequences. While Sanger sequencing can produce reads exceeding 850 bases under optimal conditions, third-generation sequencing offers even longer read lengths, providing advantages in comprehensive microbial profiling (12). Third-generation sequencing systems can provide reads that range thousands to millions of base pairs in length, whereas short-read platforms like Illumina typically produce reads between 100 and 300 base pairs. (13). This extended read length provides significant benefits for resolving complicated genomic sections, assembling genomes, and eliminating ambiguities in sequence interpretation. (14). Third-generation sequencing technologies determine the DNA sequence by monitoring changes in electrical current as the DNA strand spans through a nanopore or detecting fluorescence signals linked with the DNA (15). Third-generation sequencing technology is particularly useful for complex metagenomic, transcriptomic, and genomic studies because it can capture long contiguous sequences in a single read, allowing for full-length gene sequencing, structural variation identification, and more precise mapping of genomic features (16).

Third-generation 16S sequencing, has emerged as a game-changing tool in microbial research, collecting both conserved and hypervariable portions of the 16S rRNA gene. Sanger sequencing frequently needs numerous primers for complete 16S coverage, but third-generation sequencing may sequence large portions of the 16S gene in a single read, minimizing sequence loss at primer sites and requiring only two primers. (17). This feature, combined with third-generation sequencing capacity for high-throughput sequencing targeting full-length 16S genes, enhances resolution and accuracy in microbial profiling. As a result, third-generation sequencing is particularly valuable for metagenomic research and diagnostic applications, where quick and high-resolution microbial identification is crucial (18) (19).

This study aims to evaluate and compare the efficiency, accuracy, and applicability of Sanger sequencing and third-generation 16S rRNA sequencing

for bacterial identification under identical experimental conditions. The hypothesis of this study is that third-generation 16S sequencing offers a more comprehensive and efficient method for bacterial identification compared to Sanger sequencing, due to its ability to sequence the full 16S rRNA region in a single read with minimal primer requirements and reduced sequence loss at primer-binding sites. This study provides critical insights into the effectiveness and suitability of both methods for microbial profiling in clinical and research environments, by evaluating their performance in terms of sequencing depth, error rates, and practical applicability.

MATERIAL and METHOD

Bacterial Isolates

In this study, the bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 25955, and *Escherichia coli* ATCC 25922. Each isolate was cultured by inoculation onto Tryptic Soy Agar (TSA) plates and incubated overnight at 37°C to ensure optimal growth and viability prior to further analysis. These standard reference strains were selected from the American Type Culture Collection (ATCC) to provide consistent and well-characterized microbial samples for reliable comparative analysis.

DNA Extraction

A loopful bacterial colony was transferred to 1 mL of Phosphate Buffered Saline (PBS) and thoroughly homogenized. DNA extraction was performed using the EZ1 Advanced XL system (Qiagen) with the EZ1 DNA Tissue Kit (Qiagen) coupled with EZ1 Advanced XL DNA Bacteria Card, optimized for efficient bacterial DNA recovery. The concentration of extracted bacterial DNA was then measured with the Qubit Flex Fluorometer (Invitrogen) using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen) to ensure precise quantification. The DNA concentrations obtained from bacterial species are as follows: the DNA concentration of *S. aureus* ATCC 25923 was 14.7 ng/µL, *P. aeruginosa* ATCC 27853 had 30.2 ng/µL, *K. pneumoniae* ATCC 25955 had 35.2 ng/µL, and *E. coli* ATCC 25922 showed 18.1 ng/µL.

Amplification of 16S rRNA Region

To achieve broad microbiological representation, the 27F and 1492R primers were used to perform the first amplification of the 16S rRNA region following the given protocol and conditions. For the PCR reaction mix used in 16S rRNA amplification, it consisted of 5 µL Phanta Max Master Mix, 1 µL of 16S Primer Mix (10 µM each), x µL of DNA (10 ng), and water was added to complete the volume to a total of 10 µL. The 16S rRNA amplification protocol involved an initial denaturation step at 95 °C for 30 seconds. The amplification consisted of 35 cycles, each including 20 seconds at 95 °C, 1 minute at 58 °C, and 2 minutes at 72 °C. After amplification, a final extension step was carried out at 72 °C for 10 minutes, followed by holding at 4 °C. This main PCR process produced a consistent 16S amplicon product that was employed as a precursor for both sequencing technologies, standardizing the starting material for downstream comparative analysis of Sanger sequencing and 16S third-generation sequencing. The generated PCR product was the same for both procedures, allowing for direct comparison by limiting variability introduced during sample preparation and evaluating each method's performance on the same amplified target region.

Agarose Gel Electrophoresis

1.5% agarose gel electrophoresis was used to examine the amplified products after the 16S rRNA gene was amplified using PCR. A Thermo Scientific GeneRuler 1 kb Plus DNA Ladder was utilized as the molecular weight marker after DNA fragments were separated by electrophoresis. Successful amplification was confirmed by the amplified 16S rRNA gene's predicted band size of about 1500 base pairs (bp).

Sanger Sequencing

PCR Purification

The Mag PCR Clean-Up Kit was used to purify the PCR products in order to eliminate extra primers, nucleotides, and other impurities. Only high-quality, amplified DNA is used in future sequencing processes thanks to this purification procedure.

Cycle Sequencing Reaction

To amplify the 16S rRNA region, we used eight primers targeting V1-V9 regions within the bacterial 16S rRNA gene: 27F, 355F, 515R, 533F, 787R, 930F, 1391R, and 1492R. Each primer was designed to bind to specific regions within the 16S rRNA gene (20).

The sequences, binding locations, and specificities of each primer are as follows:

- 27F (5'-AGAGTTTGATYMTGGCTCAG-3'): Binds at positions 8–27; targets most bacterial species.
- 355F (5'-ACTCCTACGGGAGGCAGC-3'): Binds at positions 338–355; designed for general bacterial specificity.
- 515R (5'-TTACCGCGGCKGCTGGCAC-3'): Binds at positions 515–533; serves as a universal bacterial primer.
- 533F (5'-GTGCCAGCMGCCGCGGTAA-3'): Binds at positions 515–533; a universal primer for bacterial 16S rRNA amplification.
- 787R (5'-GGACTACCAGGGTATCTAAT-3'): Binds at positions 787–806; specific to most bacterial species.
- 930F (5'-TCAAAKGAATTGACGGGGGC-3'): Binds at positions 911–930; targets a broad range of bacteria.
- 1391R (5'-TGACGGGCGGTGWGTRCA-3'): Binds at positions 1391–1408; a universal bacterial primer.
- 1492R (5'-GGTTACCTTGTTACGACTT-3'): Binds at positions 1510–1492; universal for bacterial amplification.

Utilizing the Applied Biosystems Cycle Sequencing Kit, cycle sequencing reactions were carried out. For every primer, a separate reaction was made, and the final reaction mixture included the following ingredients:

- Primer (5 pmol): 2 µL
- BigDye Terminator (v3.1): 2 µL
- BigDye Buffer (v3.1): 2 µL
- dH2O: 2 µL
- Purified PCR template: 2 µL

Primers were employed at a final concentration of 5 pmol for each sequencing process, and the purified PCR products were used as templates. The reactions were conducted in accordance with the cycle sequencing instructions provided by the manufacturer. Sanger sequencing cycle protocol begins with denaturation at 96 °C for 3 minutes, followed by 25 cycles of 96 °C for 30 seconds, 50 °C for 15 seconds, and 60 °C for 4 minutes. Finally, the samples are held at 4 °C.

Cycle Sequencing Purification

In order to eliminate unincorporated dye terminators, salts, and other impurities, the products were purified using the BigDye XTerminator™ Purification Kit after cycle sequencing.

Sequencing and Data Analysis

The ABI 3500 Genetic Analyzer was used to evaluate the purified cycle sequencing products. Contigs were built after the generated sequencing data was processed using Genestudio software. In order to provide results based on 16S rRNA gene sequence analysis, the final sequence data were submitted to GenBank for taxonomic identification and comparison.

Third-generation 16S rRNA Sequencing Method-Barcoding of PCR Products

Using the unique barcodes in the kit, the 16S rRNA amplicons are barcoded as the initial step in the procedure. This is accomplished by combining the amplicon DNA in PCR tubes with the LongAmp Hot Start Taq 2X Master Mix and the relevant 16S barcodes. Following the PCR reaction mix's preparation, the samples go through a thermal cycling procedure to

bind the barcodes to the amplicon ends. To produce the target PCR products that are prepared for the following steps of the process, this barcoding step is essential.

Barcoded Sample Pooling and Bead Clean-up

Once the 16S PCR amplification is completed, the barcoded samples are pooled. The barcoded PCR products are measured, and equal volumes of each sample are mixed together to form a pooled library. To remove extra primers, nucleotides, and other impurities, the pooled samples are cleaned with AMPure XP beads. This purification phase ensures that the library is free of unwanted contaminants, resulting in clean and concentrated DNA for the next sequencing steps. Following cleanup, the pooled sample is measured to determine the DNA concentration and whether it is of sufficient quality for further processing.

Adapter Ligation

Following the successful pooling and cleaning of the barcoded samples, the next step is to ligate sequencing adapters into the DNA library. This is accomplished by adding the Rapid Adapter (RA) and Adapter Buffer (ADB) to the pooled and purified barcoded samples. The reaction mixture is then incubated at room temperature to allow the adapters to properly ligate to the DNA ends. This adapter ligation is critical for preparing the DNA library for sequencing on the flow cell because the adapters aid in the binding of the DNA to the nanopores during sequencing.

Priming and Loading the Flow Cell

Prior to sequencing, the library must be ready to be loaded onto the Oxford Nanopore Technologies (ONT) GridION instrument's R10.4.1 flow cell. To ensure that the adapters are distributed properly, the produced library is carefully mixed and incubated after adapter ligation. The library is prepared to be moved to the flow cell after the incubation is finished. To ensure effective binding of the DNA molecules during sequencing, the flow cell is primed. After the library has been put onto the flow cell, it is prepared for real-time sequencing, which allows 16S rRNA data to be captured for further examination.

RESULTS

FASTA files for the sequencing data derived from both Sanger and Oxford Nanopore Technologies (ONT) outputs for the following bacterial strains were successfully obtained during the course of this study: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 25955, and *S. aureus* ATCC 25923. This file served as a basis for additional research on the precision, coverage, and caliber of the sequences by thoroughly examining and contrasting the sequencing outcomes for the two platforms (Supplementary File).

When ONT and Sanger sequencing read lengths were compared, ONT sequences showed a definite advantage in generating longer, continuous sequence data that extended till segments denoted by "NNNNNNNNNN." ONT demonstrated a clear advantage by generating near full-length 16S rRNA sequences (~1,500 bp) with only one primer pair. In contrast, Sanger sequencing typically produces shorter reads of approximately 200-300 bp, often covering only partial regions of the 16S gene. This partial coverage in Sanger sequencing may limit taxonomic resolution at the species level, whereas ONT's extended read length provides comprehensive coverage of all hypervariable regions, improving the accuracy and depth of bacterial identification. Moreover, ONT sequencing reduces the need for overlapping read assembly and mitigates the risk of primer-induced biases or sequence dropouts commonly seen in Sanger sequencing. ONT's longer, uninterrupted reads not only allow for more reliable species-level classification but also enhance the efficiency and completeness of microbial profiling from pure cultures.

In comparison to the larger reads from third-generation sequencing, the shorter read lengths produced by the Sanger sequencing method displayed truncation and only partially covered the genome. Eight assembled amplicons were used to generate Sanger reads in our data, which produced a little

quantity of genomic information. These shorter reads provided only a limited picture of the genomic landscape, even though they frequently overlapped with the third-generation sequences. This restriction emphasizes the drawbacks of Sanger sequencing in terms of offering thorough genome coverage, highlighting the benefit of ONT's longer read lengths for applications that need ongoing genomic data.

DISCUSSION

The length differences between the 16S rRNA gene sequences using third-generation sequencing and Sanger sequencing in this work highlight the benefits of third-generation sequencing for acquiring thorough sequence information from the 16S region. The third-generation sequencing method produced lengthy, continuous reads that encompassed a significant amount of the 16S rRNA gene, which has a total length of roughly 1551 base pairs (bp) (21). Interestingly, third-generation sequencing achieved high sequence coverage with a single primer pair, capturing almost the whole 16S gene in a single run (22). Sanger sequencing, on the other hand, yielded shorter sequence reads, requiring eight primers to cover the same region. Due to gaps at primer binding sites, this primer-intensive method not only makes the sequencing technique more complicated but also breaks the continuity of the 16S sequence, which may have an impact on the gene region's completeness. Therefore, the sequence output from Sanger sequencing does not have the continuity that third-generation systems offer, which could affect subsequent analysis like precise taxonomy classification and phylogenetic research. While pointing out the shortcomings of conventional Sanger sequencing for comparable tasks, our results show the value of third-generation sequencing for full-length 16S rRNA gene research, especially for applications needing high-resolution bacterial identification and phylogenetic insight.

Moreover, third-generation sequencing provides an enhanced ability to resolve complex microbial communities by capturing longer sequences with improved fidelity across primer-binding regions, thereby minimizing amplification biases and sequence dropouts. This advantage is particularly relevant in metagenomic studies, where mixed microbial populations require accurate taxonomic resolution. The reduced primer requirements in third-generation sequencing also minimize the risk of sequence dropouts at primer binding sites, a common limitation in Sanger sequencing. Additionally, third-generation sequencing enables the detection of structural variations by sequencing longer fragments that are typically beyond the reach of Sanger sequencing, making it a more powerful tool for bacterial strain differentiation and genomic epidemiology

It is clear that third-generation systems offer many benefits over Sanger sequencing, especially in terms of throughput, speed, and scalability (23). The two approaches' throughput capacities are one of their primary distinctions. Despite being a very accurate technique, Sanger sequencing can only handle large or complex materials effectively since it only processes one DNA fragment at a time. However, third-generation sequencing is more suited for extensive genomic research and metagenomic analysis since it can sequence millions of DNA molecules at once, enabling high-throughput sequencing.

Furthermore, third-generation sequencing only needs two primers to cover a target region, simplifying the procedure and lowering potential sources of mistake, whereas Sanger sequencing often requires up to eight primer pairs, making the process more labor-intensive and prone to difficulties. This enhanced simplicity in primer usage with ONT results in fewer potential biases during sequencing, and the approach also eliminates difficulties like base loss, which can occur at primer binding sites with Sanger. Furthermore, in our study, third-generation sequencing produced analyzable data within approximately 10-15 minutes of run initiation, whereas Sanger sequencing required an average of 8 hours per run, including post-run processing. This speed makes third-generation sequencing a good candidate for rapid sequencing applications requiring immediate findings, like as diagnostic testing or field-based sequencing (24).

Another critical advantage of third-generation sequencing is its ability to analyze low-abundance or difficult-to-culture bacteria, which is a major challenge in clinical microbiology and environmental microbiome studies. Sanger sequencing, while highly accurate, struggles with low-concentration DNA samples, often necessitating extensive culturing steps prior to sequencing. Third-generation sequencing, however, can process complex microbial samples directly, providing more comprehensive and unbiased insights into bacterial communities. This aspect is particularly valuable in clinical settings, where rapid and precise pathogen identification can significantly impact patient outcomes and antimicrobial treatment strategies.

Another key advantage of third-generation sequencing is its library preparation time. While Sanger sequencing can take up to three days to construct a sequencing library, third-generation sequencing decreases this time to three hours, making it a considerably more efficient option for high-throughput sequencing applications. This reduction in preparation time is critical for large-scale investigations, when speed and efficiency are essential. Despite these improvements, Sanger sequencing remains the gold standard for focused investigations because to its high accuracy, particularly when working with smaller, more specialized gene areas.

However, the Sanger method's limitation in missing nucleotides, particularly in regions close to primer binding sites, makes it less suitable for comprehensive sequencing of bigger or more complex materials. Sanger sequencing's accuracy is undeniable in controlled conditions, but third-generation system's capacity for real-time sequencing, fewer primer requirements, and faster, more scalable library preparation make it the superior choice for modern genomic research, especially when large datasets are required quickly.

Despite the clear advantages of third-generation sequencing, it is important to acknowledge its limitations, including higher error rates in homopolymer regions and increased costs associated with initial instrument acquisition. While error correction algorithms and computational tools have significantly improved sequencing accuracy, future advancements in nanopore and single-molecule real-time sequencing technologies will likely address these concerns. The integration of hybrid sequencing approaches, combining third-generation and Sanger sequencing, may provide an optimal balance between read length, accuracy, and cost-effectiveness, particularly for applications requiring ultra-high precision.

In conclusion; Third-generation sequencing surpasses Sanger sequencing in microbial research due to its ability to generate longer, more continuous reads, offering more comprehensive genome coverage with fewer gaps. This is particularly beneficial for sequencing complex microbial genomes that contain repetitive regions and structural variants, which are challenging for short-read methods like Sanger. By improving the quality and efficiency of microbial genome assemblies, third-generation methods reduce the need for extensive sequencing procedures, making them a more cost-effective and dependable option for microbiological studies.

Additionally, third-generation sequencing enhances bacterial identification and classification by capturing full-length 16S rRNA gene sequences with minimal primer interference, providing improved phylogenetic resolution. This capability is especially valuable in areas such as environmental microbiology, clinical diagnostics, and epidemiology, where accurate microbial profiling is crucial for understanding bacterial diversity, monitoring disease outbreaks, and guiding antimicrobial strategies. Although Sanger sequencing remains widely used for targeted gene sequencing, its limitations in read length and throughput reduce its practicality for large-scale metagenomic research. In contrast, third-generation platforms offer high-throughput capacity, faster sequencing, and real-time data generation. As sequencing technology continue to advance—through improved error correction, reduced costs, and enhanced chemistry—third-generation sequencing is poised to become the leading methodology for bacterial genome analysis and taxonomic classification.

Supplementary File

>E.coli_ONT
NNNNNNAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTT-
GCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCG-
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGAC-
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GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGT-
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>E.coli_Sanger
ATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGT-
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GGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT-
GACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT-
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TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG-
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ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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