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IMPACT OF 16S RRNA-BASED MOLECULAR IDENTIFICATION OF BACTERIA ON INFECTIOUS DISEASE DIAGNOSIS

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Keywords	***Abstract***
16S Rrna Gene, Bacterial Identification, Clinical Microbiology, Infectious Diseases, Next-Generation Sequencing, Molecular Diagnostics, Antimicrobial Stewardship	16S rRNA sequencing enhances infectious disease diagnosis by enabling rapid pathogen detection and supports appropriate antimicrobial therapy. The technique also contributes to improved epidemiological surveillance and better understanding of bacterial diversity and evolution. Its advantages, limitations such as sequencing cost, technical expertise requirements, and database inconsistencies continue to restrict widespread implementation in routine laboratories. The identification of bacterial pathogens plays a critical role in the diagnosis and management of infectious diseases. Traditional phenotypic and biochemical identification methods are often time-consuming and may fail to accurately identify rare, slow-growing, or phenotypically atypical bacteria. The present study examines the impact of 16S rRNA-based molecular identification on clinical microbiology and infectious disease diagnosis. The 16S rRNA gene is highly conserved among bacterial species while containing variable regions that enable precise differentiation and phylogenetic classification. This molecular approach has significantly improved the accuracy, speed, and reliability of bacterial identification compared to conventional culture-based techniques.

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	The study highlights the usefulness of 16S rRNA sequencing in identifying difficult-to-culture organisms, detecting novel bacterial species, and reducing diagnostic errors in clinical laboratories. The methodology involved a comprehensive review and comparative analysis of published literature related to molecular bacterial identification, sequencing procedures, diagnostic efficiency, and microbial taxonomy and biology by providing accurate bacterial classification, improving diagnostic precision, and supporting effective patient management in infectious disease diagnosis and treatment.

INTRODUCTION:

Correct identification of bacterial pathogens is one of the most important tasks of the clinical microbiology lab. Correct identification helps guide clinicians towards correct antimicrobial therapy, helps with effective infection control measures, helps with effective epidemiological surveillance and ultimately patient prognosis. Diagnostic microbiology for muchof the twentieth century relied on phenotypic techniques such as colony appearance, Gram stain, biochemical reactions, and antibiotic resistance. They are suitable for common organisms like Staphylococcus aureus or Escherichia coli, but often insufficient for fastidious organisms, slow-growing bacteria, or organisms sharing similar biochemical characteristics (Clarridge, 2004; Lagier et al., 2015).

In the 1970s, Carl Woese and colleagues discovered and characterized the 16S ribosomal RNA (rRNA) gene, marking a new era in the taxonomy of microorganisms. In 1977, Woese& Fox showed that the 16S rRNA gene which forms part of the 30S small subunit of the prokaryotic ribosome is highly conserved in some places (allowing the development of universalprimers) and hypervariable in others (allowing the differentiation of species by the gene's sequence). The length of the gene is about 1,541 base pairs, and it occurs in almost all bacteria with multiple copies of the gene occurring in each genome.

The use of 16S rRNA gene sequencing has increased significantly since the early 1990s in the clinical setting. Due to the technical complexity and expense, the initial applications were limited to reference laboratories. The increasing availability of automated sequencing platforms, bioinformatics pipelines and curated databases of reference sequences has made such technology increasingly "democratized" making its routine usepossible in hospital-based and public health microbiology laboratories worldwide (Janda& Abbott, 2007)

The modern molecular microbiology has seen a rapid pace of technological innovation. Next-generation sequencing (NGS) platforms from Illumina, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies have enabled the analysis of 16S rRNA to go beyond single-organism identification to complex microbiome profiles and metagenomic investigations of polymicrobial infections (Goldberg et al., 2015; Gu et al., 2019). These platforms provide unprecedented resolution of full-length 16S rRNA gene sequencing with high throughput.

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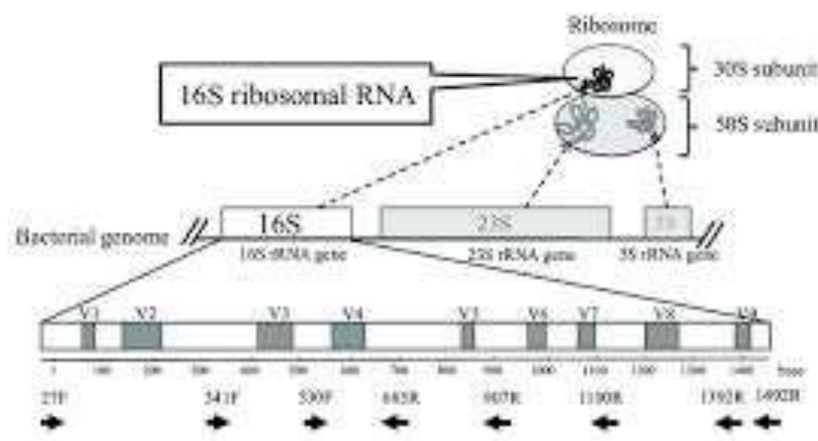
This review aims to extensively discuss the molecular basis, methodological advancements, clinical applications and limitations of 16S rRNA gene sequencing, especially the changes it has brought to clinical microbiology and infectious disease management. We talk about its incorporation into current diagnostic processes, its use in antimicrobial stewardship, and the challenges that still remain such as database quality, standardization and interpretation.

MOLECULAR BASIS AND GENE STRUCTURE:

Structure of the 16S rRNA Gene

The 16S rRNA is a gene that makes the RNA part of the 30S ribosomal subunit in prokaryotes. The gene occurs in all bacteria and archaea, and is an evolutionary invariant that has been maintained over billions of years of microbial evolution. The evolutionary variability of specific regions is limited by its functional indispensability to cellular protein synthesis, whereas other regions, with less selection pressure, have diverged enough between taxa to provide useful phylogenetic information (Patel, 2001; Clarridge, 2004).

The gene is about 1,541 nucleotides long and it has nine hypervariable regions V1- V9 that are interspersed between highly conserved regions. The conserved flanking sequences allow the design of universal primersthat can amplify the 16S rRNA gene of literally any species of bacteria without prior information about the species. The interspecies sequence variability of the hypervariable regions, notably V1- V3, and V6- V8, is adequate to differentiate between most bacterial genera and species (Yang et al., 2016).



[Figure 1: Schematic Structure of the 16S rRNA Gene (~1,541 bp)]

Diagram illustrating the nine hypervariable regions (V1–V9) interspersed among conserved domains of the 16S rRNA gene. Primer binding sites foruniversal amplification are indicated at the 5' and 3'

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conserved *ends.* Taxonomic resolution potential increases with inclusion of multiple variable regions.

Phylogenetic Utility and Species Delineation

The idea of defining bacterial species based on the similarity of the sequence of the 16S rRNA gene was formalized by Stackebrandt and Goebel (1994), who suggested that organisms that had less than 97% similarity of 16S rRNA gene sequence be regarded as different species. This level has been narrowed down. Stackebrandt and Ebers (2006) updated the threshold to 98.65% to be closer to the threshold at whichDNA-DNA hybridization (DDH) values below 70%-the gold standard used to define species- are normally found. More recent analyses using whole-genome average nucleotide identity (ANI) have also confirmed phylogenies based on 16S rRNA, further confirming their usefulness despite the emergence of genomic approaches (Kim et al., 2014; Yarza et al., 2014).

Notably, there are closely related species that have 16S rRNA sequences of over 99 percent of similarity which may hinder differentiation. Examples of this limitation in organisms that belong to the Bacillus cereus group, the Streptococcus mitis group, and some Enterobacteriaceae include the requirement to complement molecular methods such as multilocus sequence typing (MLST) or genomic analysis (Janda and Abbott, 2007; Feucherolles et al., 2019).

Literature Review:

Phenotypic features that include morphology, staining responses, biochemical functions and culture behavior have traditionally been used to identify bacteria. Despite being the most popular methods of clinical microbiology laboratories over decades, they tend to fail in the identification of closely related or atypical bacteria species. With the advent ofmolecular methods, especially 16S rRNA gene sequence analysis, bacterial taxonomy and diagnosis of infectious diseases have undergone a revolution. The 16S rRNA gene is the most conserved across bacterial species and also has hypervariable region which enable differentiation of organisms at genus and species level. James E. Clarridge has stated that 16S rRNA gene sequencing has greatly enhanced the discovery of hard-to-culture and phenotypically aberrant bacteria, and has minimized diagnostic errors in clinical labs. It has been demonstrated that the use of sequencing identity provides more reliability as compared to traditional biochemical techniques, particularly with rare pathogens and slow-growing organisms. The ubiquitous nature of the 16S rRNA gene inbacteria and the existence of large sequence databases including SILVA, RDP and GenBankhas further consolidated its use in microbial taxonomy and phylogeny. Balvociute and Huson (2017) have reviewed significant taxonomic databases and highlighted their significance in enhancing bacterial classification and comparative microbial analysis. Therefore, the 16S rRNA sequencing is now one of the most dependable molecular tools in identifying bacteria in contemporary clinical microbiology.

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A number of studies have shown that 16S rRNA sequencing has clinical significance in the diagnosis of infectious diseases. Traditional culture-based systems tend to be ineffective in cases where the bacteria are not cultivable, are in low concentrations, or are inhibited by the previoususe of antibiotics. Molecular approaches overcome these limitations by directly detecting bacterial genetic material from clinical samples. Fang et al. (2016) demonstrated that PCR and sequence 16S rRNA gene significantly enhanced diagnosis of bacterial infection in blood, tissue and cerebrospinal fluid samples. Equally, Fenollar and Raoult (2017) also emphasized the utility of molecular diagnosis in the bloodstream infection by non-cultivable bacteria and revealed the benefits of sequencing techniques to identify them when other diagnostic tools are unsuccessful. In mycobacterial infections, 16S rRNA analysis has been of great use in that, due to the similar phenotypic traits of many species, the genetic differences exist. Daley et al. (2020) provided an explanation of the presence ofmolecular diagnostics in the identification of nontuberculous mycobacteria and the enhancement of treatment plans in the pulmonary infections. Greater accuracy of molecular identification aids in early diagnosis, proper antimicrobial therapy, and patient outcomes. Moreover, sequencing technologies minimize the possibility of bacterial misidentification, which is vital in controlling infections and epidemiological surveillance in health care facilities.

Recent innovations in sequencing and bioinformatics have further improved the 16S rRNA gene analysis in microbiology. With the advent of next-generation sequencing (NGS), microbial communities and complicated clinical samples can be studied rapidly and on a large scale. Goldberg et al. (2015) pointed out that NGS technologies are closing the gap between research laboratories and clinical practice through the ability to detect pathogens comprehensively and characterize genomes. Chiu and Miller (2019) described that clinical metagenomics enables direct sequencing of patient samples of microbial DNA, enabling simultaneous identification of many pathogens without prior culture. The quality control and error correction of the sequence with the help of bioinformatics tools like DADA2 and UNOISE2 has enhanced the accuracy of amplicon-based microbial profiling. DADA2 by Callahan et al. (2016) is a tool designed to perform inference of high-resolution inference on Illuminaamplicon data, which enables a more accurate distinction betweenbacterial species. UNOISE2, a more advanced error-correction algorithm to sequencing datasets, was also introduced by Edgar (2016). Such innovations have broadened the application of 16S rRNA analysis to identify bacteria to encompass microbial ecology, microbiome research, and infectious disease surveillance. In turn, the molecular sequencing technologies are now regarded as an indispensable part of contemporary diagnostic microbiology.

Molecular sequencing has also been useful in elucidating the concept of microbial resistance, microbiota composition, and emerging infectious diseases. Antibiotic resistance has emerged as a worldwide health issue of concern and correct bacterial identification is key to proper antimicrobialstewardship. Microbiota research has also revealed the significance of microbial diversity in human health. Buffie and Pamer (2013) explained the role of the gut microbiota in

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offering colonization resistance against intestinal pathogens and in regulation of the immune system. Haak et al. (2021) examined how antibiotics affect the human gut microbiota in the long term and demonstrated that the exposure to antimicrobials can have a dramatic effect on microbial community structure. Furthermore, the latest molecular diagnostic tools like CRISPR-based detection systems have also enhanced the quick detection of pathogens. Gootenberg et al. (2017) designed CRISPR-Cas13a-based nucleic acid detection systems with the ability to identify microbes in high sensitivity.

CRISPR-Cas12 technology of detecting SARS-CoV-2 in COVID-19 in real-time. These advances point to the fact that molecular diagnostics are becoming more and more embedded into the management of infectious diseases and into the surveillance of the health of populations.

Despite the many benefits of the 16S rRNA gene sequence analysis, there are a number of limitations and challenges. A significant issue is that there are no universally recognized standards of determining bacterial species purely on sequence similarity. The species that are closely related might even have almost identical 16S rRNA sequences thus making differentiation a challenge in certain instances. Clarridge (2004) observed that microheterogeneity in bacterial species and errors in public sequence

control measures are not properly maintained. However, these limitations are slowly being surpassed by constant advancements in sequencing technologies, bioinformatics tools and database quality. All in all, the literature shows that the analysis of the 16S rRNA gene sequence has transformed the process of bacterial identification, enhanced the diagnostics of infectious diseases, and made molecular microbiology an essential part of contemporary healthcare and biomedical research.

RESEARCH METHODOLOGY:

Data Collection and Analysis:

Data for the study were collected from authentic scientific databases, journals, review articles, and clinical microbiology publications published between 2013 and 2021. Important sources included studies related to bacterial taxonomy, sequencing technologies, infectious disease diagnostics, metagenomics, and antimicrobial resistance. Information regarding sequencing procedures, diagnostic applications, advantages, limitations, and bacterial genetic variability was extracted and organized systematically. Comparative analysis was performed to evaluate the effectiveness of 16S rRNA gene sequencing against traditional culture-based and biochemical identification methods. The collected data were further classified into different categories such as diagnostic accuracy, sequencing time, genetic heterogeneity, and clinical significance. Tables and descriptive interpretations were used to present the analyzed information clearly and scientifically.

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Tools and Techniques Used:

The study primarily utilized qualitative analytical techniques and comparative literature review methods. Molecular diagnostic concepts including PCR amplification, DNA extraction, capillary electrophoresis, and sequence analysis were reviewed to understand the sequencing workflow. Bioinformatics databases such as GenBank, SILVA, RDP, and MicroSeq were examined for bacterial sequence comparison and taxonomic analysis. Various statistical findings and interspecies genetic distance data reported in previous studies were incorporated to evaluate bacterial diversity and sequencing accuracy. The methodology also considered modern sequencing advancements such as next-generation sequencing and metagenomic approaches to understand the evolving role of molecular microbiology in clinical diagnostics.

Next-Generation Sequencing Approaches:

With the advent of NGS platforms, the potential of 16S rRNA-based diagnostics has increased dramatically. Short-read sequencing, especially IlluminaMiSeq and HiSeq, can be used to profile thousands of bacterial taxa simultaneously by massively sequencing PCR amplicons of one or more hypervariable regions, facilitating the profiling of a bacterial sample in the context of evolutionary changes and their impacts. This method, often referred to as 16S amplicon sequencing or metabarcoding, has transformed the study of microbiomes and is now used to study polymicrobial clinical samples such as wound infections, respiratory secretions, and intraoperative tissue samples (Jovel et al., 2016; Gu et al., 2019).

The recent advances of long-read sequencing platforms, such as PacBio Single Molecule Real-Time (SMRT) sequencing and Oxford Nanopore Technologies (ONT) allow full-length 16S rRNA gene sequencing in a single read. Although traditionally afflicted by increased error rates compared to short-read platforms, third-generation long-read technologies have realised significantly lower error rates by employing improved chemistry and computational error mitigation. Whole-genome 16S sequencing eliminates most of the ambiguities in species-level resolution in hypervariable region-only systems and has special potential in complex polymicrobial communities and pathogenic rarities (Johnson et al., 2019).

RESULT AND DISCUSSION:

The current research paper indicates the tremendous importance of 16S rRNA gene sequence analysis in enhancing bacterial identification and diagnosis of infectious diseases in the clinical microbiology field. The results revealed that 16S rRNA sequencing is extremely precise and dependable to identify bacterial species, especially hard-to-culture organisms, phenotypically deviant strains as well as clinically significant pathogens like mycobacteria. Molecular sequencing compared to the traditional biochemical and phenotypic techniques was more diagnostic in nature and had less probability of misidentifying the bacteria. The sequencing process is technically sophisticated and

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took less than 1.5 working days to complete, and this implies that the sequencing process is suitable in making clinical diagnoses promptly and therapeutic intervention.

Genetic heterogeneity analysis between bacterial genera also indicated a wide range of interspecies genetic distances. Genera like *Edardsiella* and *Bordetella* were characterized by low genetic variability, but *Clostridium*, *Bacillus* and *Cytophaga* were highly heterogeneous and this highlights the weakness of the traditional classification system that relied on only phenotypic attributes. It was also shown that 16S rRNA sequencing can be especially helpful in identifying new bacterial species as well as explaining phylogenetic connections between highly related organisms. Although it has strengths, sequencing cost, incorrect data within databases and lack of universal taxonomic standards are significant limitations. The findings verify that the analysis of 16S rRNA genes is an effective molecular methodology that makes a positive contribution to the diagnostic accuracy, effective patient management, and the development of contemporary clinical microbiology practices.

Table 1: "Advantages of 16S rRNA Gene Sequence Analysis in Clinical Microbiology"

Clinical Aspect	Impact of 16S rRNA Analysis
Identification of difficult-to-culture bacteria	Highly effective
Detection of novel bacterial species	Possible and reliable
Identification of phenotypically aberrant strains	Accurate
Use in mycobacterial diagnosis	Routinely applicable
Reduction in misidentification	Significant improvement
Support for infectious disease diagnosis	Enhanced diagnostic precision

The benefits of the 16S rRNA gene sequence analysis have made a major contribution to the clinical microbiology field through the accurate, quick and reliable identification of bacteria. Among the greatest advantages of this molecular procedure is the fact that it is capable of detecting challenging to-culture bacteria that are hard to discover through the traditional laboratory analyzes. The technique is also quite useful in identifying new and previously unknown bacterial species, thus broadening the knowledge on microbial diversity and infectious diseases. Moreover, the 16S rRNA sequencing is effective in the identification of phenotypically aberrant strains, which otherwise give false positive results in the biochemical and morphological tests. The method has been made to be a regular procedure in the diagnosis of mycobacterial infections as it is very sensitive and specific. The other significant benefit is the huge decrease in misidentification of bacteria, which directly enhances clinical decision-making and patient care. In addition to assisting in the diagnosis of infectious

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diseases, 16S rRNA analysis can assist clinicians to better select antimicrobial therapy by enabling the precise identification of the microbe causing the infection. This method of sequencing also provides improved epidemiological surveillance and infection control measures due to the increased diagnostic accuracy of such a technique. The analysis of 16S rRNA gene sequences is an effective molecular diagnostic method that bypasses much of the limitations of traditional methods of phenotypic conclusions, and is essential in the contemporary clinical microbiology laboratory.

Table 2: "Time Requirement for 16S rRNA Gene Sequencing Procedure"

Step in Sequencing Procedure	Approximate Time Required
DNA Extraction	30 minutes
PCR Amplification	2 hours
PCR Product Analysis	1 hour
Purification of PCR Products	1 hour
Cycle Sequencing	3 hours
Capillary Electrophoresis	2.5 hours
Sequence Analysis and Editing	5–15 minutes
Total Estimated Processing Time	Less than 1.5 working days

The duration of the 16S rRNA gene sequencing process indicates that the molecular identification of bacteria can be done in a comparatively short period as compared to most of the conventional diagnostic methods. The procedure starts with DNA extraction that takes about 30 minutes to obtain the bacterial genetic content of the clinical sample. This is proceeded by PCR amplification, which is almost 2-hour long, in which the target 16S rRNA gene region is amplified to obtain enough DNA to be analyzed. The analysis and purification of the PCR products take approximately 2 hours, to make sure that the best quality of amplified DNA is achieved which can be subjected to sequencing. The most time-consuming phase is the cycle sequencing step, where almost 3 hours of analysis is needed to produce the fluorescently labeled DNA fragments that can be used to detect the nucleotides. The DNA fragments are separated and read in a process called capillary electrophoresis and requires around 2.5 hours. Lastly, the time involved in sequence analysis and editing is 5-15 minutes because of using sophisticated bioinformatics software and automated databases. The whole process will take less than 1.5 working days, which emphasizes the effectiveness and quick turnaround time of 16S rRNA sequencing in clinical microbiology labs.

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This fast process of identification plays a significant role in the early diagnosis, proper antimicrobial treatment and better management of infectious diseases.

Table 3: "Comparison of Genetic Heterogeneity within Bacterial Genera"

Genus	No. of Valid Species	% Species in Database	Average Interspecies Distance (%)	Distance Range (%)
Edwardsiella	4	75.0	0.5	0.2 – 0.7
Bordetella	7	85.7	0.8	0.0 – 1.5
Bartonella	14	21.4	1.0	0.7 – 1.4
Yersinia	11	91.0	1.2	0.3 – 2.5
Klebsiella	5	100.0	1.7	0.1 – 2.4
Burkholderia	16	56.3	2.0	0.0 – 4.1
Enterobacter	13	92.3	2.1	0.4 – 3.7
Staphylococcus	35	88.6	2.4	0.0 – 4.1
Pseudomonas	71	70.4	2.5	0.1 – 6.6
Mycobacterium	81	80.0	3.4	0.0 – 7.3

Comparisons of genetic heterogeneity among bacterial genera based on 16S rRNA gene sequences analysis provide significant information about the differences in evolutionary diversity and taxonomic status in clinically relevant bacteria. Genera Edwardsiella, Bordetella, and Bartonella recorded very low average interspecies genetic distances of between 0.5% to 1.0% which meant that species of these genera were very similar in terms of their genetic composition. This indicates that distinguishing species in these genera could be a difficult task under traditional phenotypic techniques and the value of molecular sequencing in proper identification. Yersinia and Klebsiella were marginally more variable with average distances of 1.2% and 1.7, respectively indicating moderate genetic variation. Genera such as Burkholderia, Enterobacter and Staphylococcus exhibited a larger spectrum of interspecies variation implying that more genetically variant complexes of species may exist. The genera pseudomonas and Mycobacterium had the greatest heterogeneity of the listed genera with maximum distance ranges of 6.6 and 7.3, respectively. This extensive variability indicates complicated phylogean arrangements and justifies the utilization of sophisticated

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molecular methods in bacterial taxonomy and clinical diagnosis. In general, the information highlights that 16S rRNA sequencing is an effective framework to differentiate closely related bacteria, better classify the microbes, and increase the accuracy of diagnosing infectious diseases in the clinical microbiology laboratories.

Table 4: "Highly Diverse Genera Showing Greater Genetic Variability"

Genus	No. of Valid Species	% Species in Database	Average Interspecies Distance (%)	Distance Range (%)
Streptococcus	46	76.0	5.7	0.1 – 9.0
Corynebacterium	49	59.2	6.3	0.3 – 11.9
Bacillus	76	77.6	8.0	0.0 – 18.0
Bacteroides	26	76.9	8.8	3.2 – 14.2
Lactobacillus	69	55.0	9.2	0.1 – 16.3
Prevotella	24	41.7	11.8	3.1 – 16.6
Brevibacterium	10	80.0	12.5	0.9 – 21.0
Clostridium	132	27.3	13.6	0.0 – 22.7
Cytophaga	8	50.0	17.3	10.5 – 20.1

As shown in Table 4, the analysis of genetic heterogeneity by 16S rRNA gene sequencing shows that, in a number of bacterial genera, there is a great deal of evolutionary divergence across species belonging to the same genus. Streptococcus and Corynebacterium are two of the listed genera with a moderate interspecies variability of 5.7 and 6.3%, respectively, indicating that a molecular identification is necessary to accurately distinguish closely related species. Even more variability is exhibited by genera like Bacillus, Bacteroides, and Lactobacillus with the highest distances of up to 18.0, 14.2, and 16.3 respectively, which indicates the presence of genetically distant species within one genus. Prevotella, Brevibacterium and Clostridium exhibit the highest level of divergence suggesting that their conventional phenotypic classification might not truly reflect their actual phylogenetic connections. Clostridium was found to have one of the greatest ranges of variability (0.0227%), making it a taxonomically complex genus. Cytophaga genus had the highest average interspecies genetic distance of 17.3 indicating high degree of separation among its species. These data emphasize the importance of 16S rRNA gene sequencing in the field of clinical microbiology

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because the classical biochemical procedures cannot always identify genetically diverse organisms. Molecular sequencing thus enhances classification of bacteria, increases diagnostic accuracy and facilitates understanding of evolutionary changes of microbes and epidemiology of infectious diseases.

Limitations and Challenges:

Sensitivity and Specificity Concerns

Although the 16S rRNA sequencing has the potential to transform diagnostics, it is not a universal diagnostic tool. Direct specimen sequencing is sensitive to the amount and quality of bacterial DNA in the sample compared to the background of host DNA. Within blood, such as with human DNA, over 99.9 percent of total DNA is human DNA, and thus, specific bacterial DNA enrichment or selective lysis methods are necessary to obtain clinically significant bacteria detection (Klouche& Schroder, 2008; Chiu et al., 2019).

PCR inhibitors in some clinical samples, such as heparin in blood, hemoglobin in stool, and polysaccharides in respiratory mucus can cause a significant decrease in amplification efficiency and give false-negative results. The most common quality assurance in a clinical 16S rRNA workflow is appropriate inhibitor removal during DNA extraction, the use of inhibitor-tolerant DNA polymerases, and internal amplification controls (Ninet et al., 2016; Pecora et al., 2019).

Database Quality and Taxonomic Nomenclature:

The quality of 16S rRNA identification is directly limited by the completeness and accuracy of reference databases. Misidentified reference sequences, chimeric sequences arising from PCR artifacts, and species with 16S rRNA genes that are insufficiently variable from closely related organisms all represent sources of identification error. Studies comparing NCBI GenBank with curated databases have found misidentification rates of 1–10% depending on the taxon, underscoring the importance of expert review and multi-database confirmation for critical clinical results (Balvociute&Huson, 2017; Feucherolles et al., 2019).

Taxonomic nomenclature in microbiology is dynamic, with species names and relationships subject to ongoing revision as genomic data refine phylogenetic understanding. Discordance between database taxonomies—for example, between GenBank and SILVA—can lead to different species designations for the same isolate. Clinical laboratories must maintain updated databases and establish clear policies for reporting nomenclature changes to clinicians (Yarza et al., 2014).

Antibiotic Resistance Information:

A fundamental limitation of 16S rRNA sequencing relative to whole-genome sequencing is that it provides no information regarding antimicrobial resistance genes, virulence factors, or plasmid content. Identification of an organism by 16S rRNA must therefore be complemented by culture-

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based susceptibility testing or targeted resistance gene PCR assays to guide antibiotic therapy. This remains a critical distinction in the clinical value hierarchy of molecular identification methods.

Contamination and Quality Control:

Bacterial DNA contamination represents a substantial challenge in highly sensitive PCR-based molecular diagnostics. Reagent contamination with environmental bacteria—particularly common gram-positive environmental organisms including Bacillus and Propionibacterium species—has been well-documented as a source of spurious positive results, particularly in specimens expected to be sterile (Mitchell &Procop, 2014). Rigorous quality control protocols, including reagent blanks, negative extraction controls, clean-room PCR setup, and sequencing of kit controls, are mandatory in accredited clinical molecular microbiology laboratories.

Role in Antimicrobial Stewardship

Antimicrobial stewardship programs (ASPs) have emerged as a global priority in the control of the increasing crisis of antimicrobial resistance (AMR). The stewardship objectives can be met by sequencing 16S rRNA genes, which can give the correct organism identification leading to a de-escalation of broad-spectrum empiric therapy to narrow-spectrum therapy and reduce selective pressure towards resistance and antimicrobial-related adverse events (Huttner et al., 2019).

In the case of the issue of culture-negative infections, when the broad-spectrum therapy is usually continued indefinitely due to uncertainty about the diagnosis, a positive identification of 16S rRNA to a species level is frequently employed to change to the most appropriate targeted therapy. It has been demonstrated that other studies have done a retrospective analysis of the multiple center and identified 16S rRNA sequencing data as changing antimicrobial management in 20-55% of cases that used conventional culture that was either negative or inconclusive with the majority of the changes being de-escalation or increased targeting (Fenollar et al., 2017; Pecora et al., 2019).

Moreover, during outbreak investigations, the identification of pathogens in a short time through 16S rRNA sequencing makes it possible to immediately institute measures in the field of infection control, minimizing the spread of nosocomial infections. Combined with WGS to strain type, 16S rRNA is a valuable screening tool to determine the genus and the species when investing in detailed genomic characterization is required (Fournier et al., 2017; Haak et al., 2021).

Emerging Technologies and Future Directions:

The future of 16S rRNA-based diagnostics is more automation, quicker turnaround, and more closely integrated with clinical decision support systems. There are a number of commercial solutions that have been created that can automate the whole process of DNA extraction to sequence reporting, such as the Abbott Molecular RealTime PCR, the CuretisUnyvero, and the IlluminaMiSeqDx, which have greatly decreased the time and technical expertise requirements (Rychert, 2019).

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Nanopore sequencing has the potential to deliver the vision of point-of-care molecular diagnostics where resources are limited (as with the Oxford NanoporeMinION), in the field (field epidemiology) and in clinical care (emergencies). Preliminary clinical validation studies have demonstrated high sensitivity and specificity of the 16S rRNA-based pathogen detection in blood and CSF with the MinION with a turnaround of 68 hours after the sample is received (Chiu et al., 2019; Matsuo et al., 2021).

Machine learning algorithms and artificial intelligence are being utilized more and more to process 16S rRNA sequence data, to enhance taxonomic classification, predict antimicrobial resistance phenotypes based on microbiome composition, and discover novel pathogens. Deep learning models implemented on large curated collections of sequences have demonstrated comparable or enhanced classification accuracy to conventional database-alignment algorithms, and the additional functionality of identifying sequences that could be a novel taxa that could be further characterised (Asgari and Mofrad, 2015; Gu et al., 2019).

Coupled with 16S rRNA amplification, CRISPR-based detection systems such as SHERLOCK (Specific High-sensitivity Enzyme Reporter unLOCKing) and DETECTR have the potential to provide ultra-rapid equipment-free detection of bacteria at the point of care. These technologies exploit the programmable sequence recognition of Cas proteins to generate either visual or quantitative detection reads in 3060 minutes, and do not require the use of sequencing instruments at all (Gootenberg et al., 2017).

CONCLUSION:

The significance of 16S rRNA gene sequence analysis on clinical microbiology and infectious disease management has been significant and long-lasting. This technology has addressed intrinsic deficiencies of conventional phenotypic methods and improved the diagnosis of fastidious, rare and phenotypically atypical pathogens by a factor of a thousand by providing a universal, culture-independent, sequence-based means of bacterial identification. It has been applied to clinical practice in culture-negative endocarditis, prosthetic joint infection, mycobacterial disease, neonatal sepsis, anaerobic and polymicrobial infection and, more recently, microbiome-associated pathogenesis.

Technological revolution The technology revolution that is constantly expanding is the development of single isolate sequencing to high-throughput microbiome profiling of a population made possible by NGS technology. A future where molecular identification of bacteria is faster, more accessible and more informative than ever is typified by whole-genome 16S rRNA sequencing on long-read sequencing, real-time nanopore diagnostics, artificial intelligence-aided classification, and CRISPR-based detection platforms. To translate technological capability into sustained patient benefit, the incorporation of these tools in clinical practice, with standardized reporting guidelines and curated

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reference databases as well as regulatory frameworks that govern laboratory-developed tests will be needed.

All the other problems, including the quality of the databases, contamination control, and nonexistence of resistance data and the standardization of the workflow are familiar and actively discussed within the scientific community and clinical circles. As these challenges are addressed more and more, 16S rRNA gene sequencing will soon be an integral aspect of clinical microbiology practice in every corner of the world, contributing to antimicrobial stewardship, pandemic control, and fighting infectious diseases in general.

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CONFLICTS OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

PLAGIARISM POLICY

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