Signature of Gut Microbiome by Conventional and Advanced Analysis Techniques: Advantages and Disadvantages

Sama Rezasoltani [1], Dorrieh Ahmadi Bashirzadeh [1], Ehsan Nazemalhosseini Mojarad [2], Hamid Asadzadeh Aghdaei [2,*], Mohsen Norouzinia [1], Shabnam Shahrokh [1]

1. Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

Gut microbiota is considered as a human organ with its own specific functions and complexity. Development of novel techniques such as shut gun sequencing, metagenomics, and next-generation sequencing (NGS) has enabled bypassing the traditional culture-dependent bias and has significantly expanded our understanding of the composition, diversity, and roles of the gut microbiota in human health and diseases. Although amplicon sequencing characterizes the taxonomic composition of the gut microbiome, it is impossible to cover the direct evidence of the microbial biological functions related to the gut microbial community. Hence, the critical next step for gut microbiome studies is shifting from gene/genome-centric analysis to mechanism-centric techniques by integrating omics data with experimental results. Realizing gut microbial diversity and their bioactive metabolites function will provide insight into the clinical application of gut microbiota in diagnoses and treatments of several diseases. In this review, we focused on explaining the conventional and advanced microbiome analysis techniques regarding gut microbiota investigation with considering the advantages and disadvantages of the platforms.

KEYWORDS:

Gut microbiota, Microbiome analysis techniques, Advantages, Disadvantages

* Corresponding Author:
Hamid Asadzadeh Aghdaei, MD
Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, P.O. Box: 19857-17411 Iran
Telefax: + 98 21 22432517
Email: hamid.assadzadeh@gmail.com

INTRODUCTION

1. Context

Gut microbiota

The new organ, the last human organ, a forgotten organ, or a missing organ, all of these names remark the important role of gut microbiota.[1,2] They can be explained as a community of *bacteria*, *archea*, *viruses*, *fungi*, and *protozoa*. Predominating human gastrointestinal tract microbiota consists of *firmicutes*, *proteobacteria*, *bacteroidetes*, *actinobacteria*, *fusobacteria*, *verromicrobia*, and *cyanobacteria*.[3] An altered gut microbiome composition not only has been shown linked to the gastrointestinal disorders such as colorectal polyps,[4] colorectal cancer (CRC),[5,6] and celiac disease,[7] but also is associated with non-intestinal disorders such as allergy,[8] asthma,[9,10] obesity,[11,12] non-alcoholic fatty liver,[13] cardiovascular diseases,[13,14] and neuropsychiatric diseases.[15-17] These disorders can be attributed to the imbalance of the gut microbiota, which would be referred

to as dysbiosis (dysbacteriosis) or dysregulation of gut microbiota.[16]

Conventional gut microbiome analysis techniques: advantages and disadvantages

A link to the gut microbiome and diseases was first postulated in the 20th century. However, until the 1990s, understanding of the gut microbiome was confined because the microbiological culture was the only way to investigate its composition. Actually, a small fraction of the gut microbiota has been cultured up to now and developments in culture-independent techniques have led to our belief of the complexity of this microenvironment. These techniques have clarified the gut microbiota diversity and composition and have shown that dysbacteriosis and dysregulation of gut microbiota are associated with several diseases. These latest techniques are rapid, accurate, and comfort high throughput to identify and enumerate uncultivable microorganisms.[18] These techniques are based on sequence divergences of the small subunit ribosomal RNA (16S rRNA) that are well conserved between various species of bacteria and provide bacterial species identification and demonstrate gut microbiota diversity. Also, they provide qualitative and quantitative information on bacterial species and gut microbiota alteration in relation to diseases. Examples of these conventional analysis techniques are quantitative real-time polymerase chain reaction (q PCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence in situ hybridization (FISH), 16S rRNA gene sequencing-based on cloning, direct sequencing of 16S rRNA amplicons, shotgun sequencing (table 1).[19,20]

Advance microbiome analysis techniques: advantages and disadvantages

Microbiome shotgun sequencing and metagenomics

Despite applying 16S rRNA technology has significantly improved our knowledge of gut microbiota composition and diversity; it has not illuminated the significant association between microbial patterns and disease initiation or progression. In fact, metagenomics demonstrates the newest growth in gut microbiota composition analysis. It has been widely used in studies including the human microbiome project (HMP).[21-23] This technique is capable of sequencing all the DNA fragments in the sample rather than the sequencing of particular DNA fragments.[24,25] This method is used to analyze the composition and diversity of gut microbiota by data sequencing from the merged genomes of the microbiota. The strength of the technique is that it is strong enough to identify new functional genes. On the other hand, the weakness of the technique is that it cannot provide gene expression profiles and predict how different conditions can regulate it also cannot distinguish DNA of live cells from DNA of dead cells.[26,27]

Whole-genome shotgun metagenomic sequencing is a widely used tool for characterizing the metagenomic content of gut microbiome samples. While whole-genome shotgun metagenomic sequencing data contains gene-level information, it can be an incentive to analyze the millions of microbial genes, which are typically found in microbiome experiments. It is performed by major parallel sequencing of the mixed DNA sample. It implicates random fragmentation of DNA, sequencing of DNA fragments and reconstruction of overlapping sequences to assemble them into a continuous sequence. The advantage of the technique is that information is assembled on the genetic diversity of the gut microbiota. Information on the genetic diversity and composition of the gut microbiota permits correlations to be made between gut microbiota and disease position. Disadvantages of shotgun metagenomics sequencing are that it is costly and also the analysis of a large amount of data is computationally intense and not easily performed in general laboratories.[28,29]

Next-generation sequencing (NGS) in gut microbiota study

NGS technologies in particular targeted amplicon sequencing of the 16S-seq and enabled the identification and quantification of human-resident

Table 1: Conventional gut microbiome analysis techniques considering their strengths and weaknesses

Technique	Explanation	Strength	Weakness
Culture	Cultivation of selected bacteria on selective media	Inexpensive, semi-quantitative method	A small fraction of gut microbiota has been cultured up to now, labor-intensive
Quantitative real-time polymerase chain reaction (q PCR)	Quantification of 16S rRNA. Reaction mixture includes a compound that fluoresces when it binds to double-stranded DNA	Rapid, phylogenetic characterization, quantitative method	PCR bias, incapable of characterizing unknown species
Denaturing gradient gel electrophoresis (DGGE)	Gel separation of 16S rRNA amplicons using denaturant/temperature	Semi-quantitative, bands could be applied for further analysis, rapid	PCR bias, no phylogenetic characterization
Terminal restriction fragment length polymorphism (T-RFLP)	Fluorescently labeled primers are quantified and then restriction enzymes are applied to digest the 16S rRNA amplicon. Digested fragments further separated by gel electrophoresis	Inexpensive, fast, semi-quantitative method	PCR bias, no phylogenetic characterization, low resolution
Fluorescence in situ hybridization (FISH)	Fluorescently labeled oligo-nucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	No PCR bias, Phylogenetic characterization, semi-quantitative method	Related to probe sequences—incapable of characterizing unknown species
16S rRNA gene sequencing based on cloning	Sanger sequencing, capillary electrophoresis, cloning of full-length 16S rRNA amplicon	Phylogenetic characterization, quantitative method	Expensive, PCR bias, laborious, cloning bias
Direct sequencing of 16S rRNA amplicons	Large parallel sequencing of partial 16S rRNA amplicons	Rapid, phylogenetic characterization, quantitative method, capable of identifying unknown bacteria	PCR bias, expensive, laborious
Shotgun sequencing	Massive parallel sequencing of the whole genome	Phylogenetic characterization, quantitative method	Expensive, analysis of data is computationally intense

microorganisms at unprecedented resolution, providing novel insights into the role of the microbiota in health and disease. The ability to rapidly sequence human genomes and to generate genetic, transcriptomic, and epigenetic data and other genome-wide data for a relatively small cost opens up numerous opportunities for translation into the clinic over the next few years.[30,31] NGS as a high throughput procedure has revolutionized the human gut microbiota studies and enabled the exploration of uncultured gut microbial diversity communities as a sufficient and cost-effective technique.[32,33] It consists of the following techniques: 454 GS FLX + (Roche), HiSeq 2000/2500 (lumina), 5500 xl SOLiD (Life Technologies), PacBio RS (Pacific Bioscience), Ion torrent (Life Technologies).[34] Figure 1 depicts NGS technology upon PCR or non-PCR based.

Actually the procedure is amplicon-based profiling and is applied as one of the most widely used techniques for defining gut microbiome diversity. Usually, 16S rRNA for bacteria and archaea as a taxonomically informative gene marker is prevalent for organisms to be studied, targeted, and amplified from the total DNA. The resulting amplicons are sequenced, and downstream bioinformatics analyses are performed to characterize the relative taxonomical abundances in the samples.[34-36] There are many advantages and disadvantages related to applying NGS platforms (table 2).[30]

In a one-run, pyrosequencing sequences 500 million bases with more than 99% accuracy rate.[37] NGS platform compares gene marker profiles across samples in order to identify microbial diversity. Although amplicon sequencing characterizes the taxonomic composition of the gut microbiome, it is impossible to cover the direct evidence of the microbial biological

Table 2: NGS platforms considering the strengths and weaknesses[34]

| Machinery	Chemistry	Strengths	Weakness
454 Gs Flx + (Roche)	Pyrosequencing	Read length is long, sensitive, analysis of multiple samples at the same time, no cloning bias	In hands-genes, expensive, the high error rate in homopolymers, short sequencing reads, extensive, bioinformatics analysis require
HighSeq (2000/25000) (Illumina)	Quantification of 16S rRNA. Reaction mixture includes a compound that fluoresces when it binds to double-stranded DNA	Rapid, phylogenetic characterization, quantitative method	PCR bias, incapable of characterizing unknown species
5500 SOLiD (Life technologies)	Ligation	Low error rate, high throughput	Short length, long process
PacBio RS (Pacific Bioscience)	Real-time sequencing	Easy preparation, cheap, long read length	High error rate, expensive, difficult installation
- Ion torrent (Life Technologies)	Proton detection	Short process, flexible chip reagents	Instrument under development
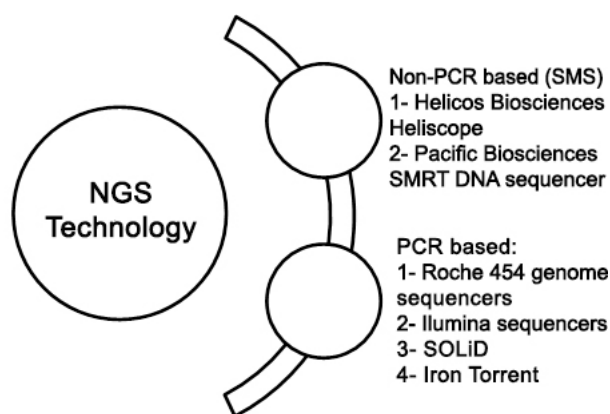
Fig.1: Diagram for different NGS platforms technologies

metabolites and functions, which are related to the gut microbial community. Hence, important next step for gut microbiome studies is shifting from genome-based analysis to mechanism-based techniques by integrating omics data with these results.[35] Alongside, metagenomics, metabolomics, proteomics, transcriptomics, and phenomics data are important for investigating gut microbiome characterization, functions, metabolites, proteins, and RNA.[38,39]

Omics techniques: advantages and disadvantages

By gathering metagenomics, metabolomics, proteomics, transcriptomics, and phenomics data we will reach more realization on the functioning besides diversity of the gut microbiota. The integrative analysis of these omics data will be critical for understanding the host-microbiome interaction mechanisms (table 3).

Data analysis and bioinformatics approaches

The first step for the analysis of the microbiota is to determine the evolutionary association among the microbiota in the gut. Both alignments dependent based and alignment independent based techniques are applied to specify the evolutionary relatedness between them. In the alignment-based analysis, the homologous positions in the gene sequence are identified through a multiple sequence alignment against databases such as Ribosomal Database Project II (RDPII). The most general method for manufacturing alignments is the CLUSTAL online software and databases such as NCBI and multivariate statistical analysis are employed in this way.

One of the advantages of the alignment dependent based method is that it is the most precise approach to permit a detailed map of the phylogenetic relations, but it is not suitable for analysis of large data sets. However alternative methods applied for large datasets can be cost-effective.

Presently, the most used method for alignment independent based technique analysis is principal component analysis.[26]

CONCLUSION

With the newest technologies including microbiome shotgun sequencing, metagenomics, and NGS platforms, a deep understanding of the gut microbiome diversity has been gained, but it is impossible to cover direct evidence of the microbial biological

Table 3: Omics gut microbiome analysis techniques considering the strengths and weaknesses

Techniques: Metaomics	Explanation	Strength	Weakness
Metagenomics	Investigating gut microbiota genomes profiling in high resolution, characterization of genes structures of uncultivated microbiota	Comprehensive sequence data, data of functional contributions of the microbiota, needless to cloning of specific genes	The function of gut microbiota are not generated
Metatranscriptomics	Messenger RNA/cDNA sequencing for high-resolution gene expression profiling, differential microbial gene in expression various physiological/environmental situations	High throughput, high sensitivity, quantification method, characterization of known and unknown gut microbiota, evaluation of microbial interaction	Lack of unique protocol, low stability of microbial mRNA, representatively unknown/ multiple purification steps needed
Metaproteomics	Proteins/Peptides are identified for high resolution protein monitoring and profiling, differential microbial proteins production under various physiological/environmental conditions	Locating and monitoring new functional genes	Lack of unique protocol, unknown proteins in databases, heterogeneous stability
Metabolomics	Metabolites are analyzed for microbial host metabolic profiling	Rapid and easy to perform on every low amount of specimens including faces/ serum/urine, time-efficient, the impact of gut microbiota in health and disease	Lack of unique protocol, unknown metabolites in databases, strict characterization of compound labor-intensive, the combination of host and bacterial molecules

metabolites and functions, which are related to the gut microbial community, along with their role in initiation and progression of diseases. Hence, the critical next step for gut microbiome studies is shifting from genome-based analysis to mechanism-based techniques by integrating omics data with experimental results. Therefore, there is a critical demand to go beyond solely characterizing the gut microbiome composition, systematic modeling, and analysis of the gut microbiome metabolites and function. Generally, understanding gut microbial diversity and their bioactive metabolites function in different types of disorders will provide insight into the clinical application of gut microbiota in diagnoses and treatments of diseases.

ETHICAL APPROVAL
There is nothing to be declared.

CONFLICT OF INTEREST
The authors declare no conflict of interest related to this work.

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