Advanced International Journal for Research (AIJFR)
E-ISSN: 3048-7641 ● Website: www.aijfr.com ● Email: editor@aijfr.com

Faecal Metagenomics: A Review on Emerging Molecular Techniques Research Advances, Challenges, and Applications

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Abstract

The advent of high throughput next generation sequencing (NGS) technology, it has become possible to study the diversity in microbial community of the gut with extraordinary resolution and accuracy**.** The main aim of this study is faecal metagenomics in wildlife approaches in the wildlife in review collections**.** Recent studies have clearly demonstrated the enormous virus diversity that exists among wild animals. This exemplifies the required expansion of our knowledge of the virus diversity present in wildlife, as well as the potential transmission of these viruses to domestic animals or humans**.** Non-invasive biological samples benefit studies that investigate rare, elusive, endangered, or dangerous species. Integrating genomic techniques that use non-invasive biological sampling with advances in computational approaches can benefit and inform wildlife conservation and management.

1. Introduction

Metagenomics is the study of the collective genomes of microorganisms obtained directly from environmental samples. It is particularly useful for studying microbial communities present in various environments such as soil, water, and the human body. Understanding microbial interactions in these environments are challenging because most microorganisms cannot be cultured using standard laboratory techniques. The gut microbiome is a complex microbial community comprising 100 trillion microorganisms present in the digestive tract (Chen et al., 2013). Because the gut microbiome influences host phenotype, including host health and disease, there has been a large field of research on the gut microbiome and its role in host phenotype and disease (Chen et al., 2013; Chung et al., 2018; Liu et al., 2018; Lkhagva et al., 2021; Nguyen et al., 2017; Nguyen et al., 2019).Metagenomics overcomes the limitations of traditional microbiological methods by using culture-independent approaches. This technique allows researchers to analyse the vast majority of microbes that are unculturable or difficult to grow in laboratory conditions.

Metagenomics employs various genomic technologies and bioinformatics tools to investigate the genetic material of microbial communities in their natural environments. It has greatly enhanced our understanding of microbial diversity, ecology, and functional potential. Unlike traditional microbiological methods that focus on individual organisms, metagenomics emphasizes the study of community genes and their interactions within complex microbial ecosystems. The development of molecular techniques, such as 16S ribosomal RNA (rRNA) sequencing, enabled scientists to study microbial communities without the need for cultivation, thereby laying the foundation for metagenomics. The use of rRNA as a molecular marker was first proposed by Carl Woese in the late 1970s. In 1977, the development of the Sanger sequencing method by Frederick Sanger and his colleagues further facilitated the study of microorganisms. Later, Jo Handelsman and colleagues coined the term "metagenomics" in 1998 and defined it as the study of the collective genomes of microorganisms present in environmental samples. In the early 2000s, the development of high-throughput sequencing technologies enabled the rapid sequencing of entire microbial communities, greatly advancing metagenomic research. In the mid-2000s, different specialized databases and bioinformatics tools were developed to manage the growing volume of metagenomic data. Projects like CAMERA (Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis) and IMG/M (Integrated Microbial Genomes with Microbiomes) provided platforms for storing and sharing metagenomic data. At presently, metagenomics continues to advance along with other omics technologies. The field of metagenomics is rapidly advancing with improvements in sequencing technologies and bioinformatics tools.

Types of metagenomics

Shotgun Metagenomics (Metagenomic Sequencing):

Diverse microbial communities of bacteria, archaea, viruses and single-celled eukaryotes have crucial roles in the environment and in human health. However, microbes are frequently difficult to culture in the laboratory, which can confound cataloguing of members and understanding of how communities' function. Over the past 10 years metagenomic shotgun sequencing has gradually shifted from classical Sanger sequencing technology to next-generation sequencing (NGS). Sanger sequencing, however, is still considered the gold standard for sequencing, because of its low error rate, long read length (> 700 bp) and large insert sizes (e.g. > 30 Kb for fosmids or bacterial artificial chromosomes (BACs). Still, computational approaches to overcome the challenges that affect both assembly-based and mapping-based metagenomic profiling, particularly of high-complexity samples or environments containing organisms with limited similarity to sequenced genomes, are needed. Understanding the functions and characterizing specific strains of these communities offers biotechnological promise in therapeutic discovery and innovative ways to synthesize products using microbial factories and can pinpoint the contributions of microorganisms to planetary, animal and human health. The Shotgun metagenomic sequencing consists of 4 main steps—DNA extraction, library preparation, sequencing, and bioinformatic analysis. The Shotgun sequencing is a laboratory technique for determining the DNA sequence of an organism's genome. The method involves randomly breaking up the genome into small DNA fragments that are sequenced individually. The unlike 16S rRNA sequencing, shotgun metagenomic sequencing can read all genomic DNA in a sample, rather than just one specific region of DNA. For microbiome studies, this means that shotgun sequencing can identify and profile bacteria, fungi, viruses and many other types of microorganisms at the same time. The 16S rRNA gene sequencing is generally limited to identifying bacteria at the genus level (e.g. *Bifidobacteria*). Shotgun metagenomic sequencing on the other hand can identify bacteria and other microorganisms at a species (e.g. *Bifidobacterium longum*) or sometimes even strain level (e.g. *Bifidobacterium longum* 35624) by profiling single nucleotide variants in metagenomic data. Therefore, for broad profiling of bacterial microbiomes, 16S rRNA sequencing would be sufficient. However, if it is necessary to look a little more deeply at the species and strains within your microbiome of interest, shotgun metagenomic sequencing will be more powerful. The analytical capabilities of Although shotgun metagenomic sequencing provides more data than 16S rRNA gene sequencing, the data potential can only be used with the appropriate tools and analysis. 16S rRNA gene sequencing, however, generates simpler data that can be analysed more easily by non-experts. Before deciding on 16S rRNA sequencing vs shotgun metagenomic sequencing, it is important to consider the bioinformatic analysis capabilities available to you. Microbiome Insights have a team of bioinformatic experts that are happy to help with your 16S rRNA or shotgun metagenome sequencing studiesIn metagenomics, shotgun sequencing is done in the same manner as in clonal culture genomics. However, the raw genomic material does not come from a single organism: it comes from a community of microbes, hence the name environmental shotgun sequencing or ESS. Depending on our ability to sample, this DNA may provide only a partial genomic picture of the organisms in the environment, since the genomic material from the more abundant species dominates the sample.

Targeted Metagenomics (Amplicon Sequencing/Metabarcoding):

When sequencing a whole genome, the reads are assembled into progressively longer contiguous sequences or contigs, and finally to the whole genome. Dealing with genomic data, we are used to analysing long stretches of contiguous sequence data. This analysis lets us find not only open reading frames, but also operons, operational transcriptional units, their associated promoter elements, and transcription factor binding sites. Next-Generation Sequencing, coupled with DGGE, is one of the most important and straightforward tools for the identification of genetic diversity and abundance of microbes. To understand the complexity of the microbial community and to interpret their interaction with one another and with regard to their hosts, by utilizing Next generation sequencing technologies represents a possible break through and a significant challenge even in the field of wildlife conservation and captive wildlife management on a global level. In molecular biology and ecology, DNA metabarcoding is a potent tool for analysing biodiversity and species composition in intricate environmental datasets (Evans et al., 2016). To identify and quantify the numerous species present in a sample, it depends on the analysis of particular genetic markers, such as DNA Stions known as "barcodes" or other genetic markers (Deiner et al., 2017). The idea of DNA barcoding was initially suggested at the beginning of the twenty-firstcentury, using short DNA sequences to identify species (Ellis et al., 2010). This method can only be used to analyze single specimens or samples with little diversity.

The capacity of DNA metabarcoding to offer a thorough portrait of biodiversity in a particular habitat is one of its key advantages. Without the need for conventional morphological identification, it permits the simultaneous examination of numerous species from various taxonomic categories, including bacteria, fungi, plants, and animals (Taberlet et al., 2012). This method is especially useful for researching complex ecosystems where numerous species cohabitate or for tracking long-term environmental changes (Gunawardena & Rowan, 2005).

Using a case study on the dietary choices of carabid beetles (Coleoptera: Carabidae) in arable land, we present a comprehensive protocol that describes an entire workflow targeting ITS2 fragments. This workflow utilizes an Illumina HiSeq 2500 system and applies a nested metabarcoding approach to identify the species of weed seeds consumed by individual carabids. We demonstrate a concept that employs bioinformatic tools for targeted amplicon sequencing in a defined order. By analysing the effects of sequencing errors and index hopping on demultiplexing and data trimming, we highlight the importance of clearly describing the software and pipeline used, including their versions, as well as specifying software configurations and threshold settings for each TAS dataset to obtain realistic data output per sample.

Without this information, there is a risk of incorrectly assigning samples or failing to obtain the maximum, or at least a sufficient, number of sequences, which in turn may compromise the results. The concept described here can be used to analyse a large number of samples, enabling the identification of food items at the species-specific level and addressing potential issues that may arise during NGS data processing.

Functional Metagenomics:

Functional metagenomics is a powerful experimental approach for studying gene function, starting from the extracted DNA of mixed microbial populations. A functional approach relies on the construction and screening of metagenomic libraries and physical libraries that contain DNA cloned from environmental metagenomes. This review explores the potential of functional metagenomics to uncover novel enzymes, antibiotics, and therapeutic compounds by analysing environmental DNA (eDNA) without the constraints of traditional culture-based methods. The vast microbial biomass present in diverse ecosystems, ranging from soil to extreme habitats, harbours a wealth of genetic resources that can be harnessed for biotechnological applications. Researchers can utilize modern sequencing techniques and functional screening approaches to identify and characterize genes with advantageous features, such as bioremediation capabilities and industrially relevant enzymes. There are several technically challenging steps in library construction. First, the extracted DNA must be of sufficient length for efficient packaging into lambda phage heads (Parks and Graham, 1997). Extraction usually employs gentle lysis to avoid shearing DNA (Zhou et al., 1996) but even so it may be difficult to achieve large fragment sizes (Kakirde et al., 2010). Extracts are often contaminated with compounds that co purify with DNA,requiring additional purification steps that may lead to sample loss. Common contaminants in soil-derived DNA extracts are humic acids, which may interfere with enzymatic reactions (Tebbe and Vahjen, 1993).

Despite challenges in DNA extraction and host expression systems, integrating functional metagenomics with multi-omics approaches promises to enhance our understanding of microbial interactions and facilitate the development of eco-friendly bioprocesses. This review underscores the importance of functional metagenomics in expanding our repertoire of biomolecules and highlights its potential to significantly impact biotechnology and environmental sustainability. Although excellent commercial products are available for both, in-house vector preparation may still be required when specific expression hosts are to be used in functional screening outside the host range of available commercial vectors (Wexler et al., 2005; Craig et al., 2010; Troeschel et al., 2010; Cheng et al., 2014). The culminating step of library construction is the transduction of E. coli, and although it is possible to generate many thousands of clones with the first attempt, troubleshooting may be required to increase library size.

In functional metagenomics, two types of libraries are commonly prepared by inserting either small or large DNA fragments into vectors However, several challenges and gaps remain. Functional metagenomics, as a transformative approach, holds immense potential for harnessing microbial diversity and discovering novel biomolecules, particularly enzymes, from environmental samples.

Meta transcriptomics

The past few decades have seen significant advancements in sequencing technologies that have transformed how we conduct biological experiments, particularly when it comes to the study of complex microbiomes. The study of microorganisms is a field of great interest due to their environmental (e.g., soil contamination) and biomedical (e.g., parasitic diseases, autism) importance These methods have contributed to many discoveries in the past decade, helping to better characterize microbiomes from environments ranging from the human gut (Qin et al., 2010) to soil (Rondon et al., 2000) to oceans (Venter et al., 2004). Although 16S studies only directly characterize the taxonomic profile of a microbiome, it is a cost-effective option to exhaustively capture biodiversity (measuring the maximal dynamic range of relative abundance) of many samples using minimal sequencing Additionally, together with the development of analysis software, the creation of specific databases (e.g., SILVA or RDP) has boosted the enormous growth of these studies. As the cost of sequencing per sample has continuously decreased, new protocols have also emerged, such as shotgun sequencing, which allows the profiling of all taxonomic domains in a sample.

Using RNA sequencing (RNASeq) to record expressed transcripts within a microbiome at a given point in time under a set of environmental conditions provides a closer look at active members. However, they are not capable of measuring what is actively expressed. Conversely, we advocate that meta transcriptomics is a “new” technology that makes the identification of the mRNAs of a microbial community possible, quantifying gene expression levels and active biological pathways. The gut microbiota is the most studied up to now, as it is known to influence virtually all human cells. In the last 5 years, according to PubMed, more than 4300 articles focusing on the gut microbiota have been published. This remarkable number represents a high percentage of the global publications in the field. As a result, it is now clear that microorganisms residing in the human gut play an important role in the metabolic processes of the host and can therefore be a potential source of new therapeutic strategies and Several studies have demonstrated that functional redundancy exists among related bacterial taxa and, as such, it is an important component of host fitness, as it has been described that functions can be conserved despite perturbations disrupting the balance of bacterial populations. A number of bioinformatic tools have been designed to identify the microorganisms present in a sample. The information generated using high-throughput sequencing is becoming increasingly large and this poses a growing challenge for computational methods, which must minimise processing and memory requirements in order to provide a fast response and avoid overloading computational resources. Therefore, sample pre-processing is an essential step for a proper data analysis workflow.

Growth of metatranscriptomics projects in public repositories, together with associated metadata, over time. Bars plots represent number of metatranscriptomic datasets (i.e. ”runs”) deposited in the NCBI Sequence Read Archive (SRA) on a per annual basis. The pie chart and the stacked bars are colored based on the source/environment (isolation_source) the sample has been isolated from. The lowest bar in grey represents the number of samples in SRA without this pertinent metadata.

Fecal metagenomics

The composition of the gut microbiome changes in response to mild perturbations, such as dietary changes, as well as strong perturbations, including chemotherapy and antibiotic treatments, which can deplete the majority of microbial populations and significantly impact microbiome function. Over the past decades, microbiome research has focused on characterizing these compositional changes and understanding how they influence human and animal health. Beginning of the 21st century has witnessed the strengths of nucleic acid-based tests to identify, characterize and strain type the microbes. Several PCR based tests were developed in past and have been successful in demonstrating the capabilities of identifying the microbial pathogens (Costa et al., 2014).

Meta-transcriptomic next-generation sequencing has transformed virus discovery, dramatically expanding our knowledge of the known virosphere. Nevertheless, the use of meta-transcriptomics for virus discovery faces important challenges. However, accurate identification in non-model species remains challenging due to the frequent absence of host-specific pathogen reference genomes. In this study, we developed a robust computational framework for detecting potentially pathogenic bacteria from metagenomic sequences by mapping them to available reference genomes in databases. Several key parameters affecting the analysis, including mapping algorithm, database configuration, and identification parameters, were analysed to optimize detection sensitivity and specificity.

In this dataset, we compiled gut microbiota compositions based on 16S rRNA gene sequencing of fecal samples, along with associated metadata. Subsets of this comprehensive dataset have been examined in several publications. Typically, a meta transcriptome experiment of the microbiome involves isolation of total RNA from bacteria colonizing the area of interest (eg, gut, skin, and oral cavity).

Mainly Assessment of microbial activity. Identifying functionally active bacteria within a mixed bacterial microbiome may highlight the disease-driving bacteria within a generally inactive microbial pool. Several strategies of determining transcriptionally active bacteria have been described.33,60Gosalbes et al.33 utilized the presence of 16S rRNA transcriptase a way to determine the phylogenetic structure of active bacteria in the gastrointestinal tract (finding the phyla Firmicutes as predominantly active followed by Bacteroidetes). In healthy individuals, characterization of mRNA revealed activation of pathways involved in carbohydrate metabolism, cell component synthesis, and energy production Gut microbiota research is typically conducted through fecal microbiome analysis, but it is important to note that the fecal microbiome does not always represent the entire gut microbiome. Another important example can be seen in the case of the cardiac drug digoxin that can be inactivated by gut microbiome metabolism. Transcriptional profiling revealed that specific strains of the gut bacteria, *Eggerthella lenta*, have a cytochrome-encoding operon that is upregulated by digoxin and is predictive of the cardiac drug inactivation. Using gnotobiotic mice, it was shown that increasing dietary protein could significantly reduce digoxin microbial metabolism and result in increased concentrations of the drug present in serum The largest portion of the host associated microbes are present in the gut of the monogastric animals and in the rumen in case of ruminant animals. The microbes perform various metabolic, physiological and immunological functions (Malmuthuge and Gua, 2016). By virtue of the diverse functional contribution of the gut microbiome, it has been recognized as an organ of the body.

In the fecal sample observation like Pathogen identification based on the traditional approach using morphology, physiology, chemistry, and biochemical characterization generally require 2 to 5 days. In addition, phenotypic methods fail to identify the microorganism up to the species or strain level (Bochner, 2009). In some situations, the pathogens are difficult to visualize under microscope or the organisms are refractory to known culturing methods.

Low-methane cattle show enrichment of bacterial genes encoding membrane-bound hydrogen-uptake hydrogenases for hydrogenotrophic respiration, reductant disposal pathways including propionate and butyrate production, and terminal reductases for nitrate and sulphate reduction, while high-methane cattle show greater abundance of fermentative hydrogen-producing hydrogenases and methanogenesis genes. Similar shifts toward succinate, lactate, and propionate production have been associated with low-methane-emitting cattle and sheep. Conversely, reduced methane production can also reflect the increased activity of hydrogen-consuming bacteria that reduce the electron pool available for methanogens. Indeed, recent studies have suggested ruminants harbor novel lineages of acetogenic bacteria and various hydrogenotrophic respiratory microbes that use electron acceptors such as fumarate, nitrate, and sulphate. These findings suggest that characterizing the genetic complement of hydrogen-cycling genes can reveal the mechanistic basis for alternative hydrogen management strategies, even when active metabolic rates cannot be directly measured.

It’s a case study emerging infectious diseases, particularly those of zoonotic origin arising from wildlife reservoirs, represent a growing threat to global health. Human-driven environmental changes, such as habitat fragmentation, climate change, and urban expansion, have intensified interactions at the wildlife–domestic animal–human interface, thereby facilitating cross-species viral transmission. Despite their epidemiological importance, systematic virological surveillance of wildlife remains challenging. In this case study Faecal samples collected from injured wildlife admitted between September 2020 and September 2021 were analysed to detect both known and novel viruses. This approach builds upon previous PCR-based investigations of the same biological material enhancing a more comprehensive assessment of viral diversity

The method was adopted a shotgun sequencing approach to analyze six sample pools, of which four tested positive for at least one viral target. A diverse range of viral families was identified, including Astroviridae, Circoviridae, Picornaviridae, Adenoviridae, and Retroviridae. These viruses were detected in asymptomatic wildlife admitted to the veterinary hospital, highlighting their potential role as reservoirs of infectious agents.

Conclusion

Metagenomic approaches have proven highly effective in unravelling microbial diversity and functional dynamics across ecosystems, while also providing critical insights into the epidemiology of antimicrobial resistance (AMR). This study highlights that domestic animals exhibit higher AMR abundance and resistive diversity, underscoring the significant role of antimicrobial use in animal production as a major driver of resistance. At the same time, wildlife harbours diverse AMR determinants, including resistance to critically important antimicrobials, indicating its role as a potential reservoir and transmission pathway. Variations in AMR profiles among animal species further reflect the influence of factors such as antimicrobial exposure, habitat, and diet, emphasizing the complex and interconnected nature of resistance dissemination.

However, increasing anthropogenic pressures, habitat degradation, and indiscriminate antimicrobial use pose serious threats to microbial diversity and accelerate the spread of AMR. In conclusion, the integration of metagenomic research with sustainable conservation strategies is essential to protect microbial resources and harness their full potential. Continued research, supported by advanced technologies and responsible environmental management, will play a crucial role in addressing global challenges such as AMR while ensuring long-term ecological and socioeconomic benefits.

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