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Invited Review Article

METAGENOMICS AND ITS RELEVANCE TO ANIMAL DISEASES AND GUT HEALTH

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Abstract

Metagenomics offers exciting opportunity for diagnosis of infectious diseases. Although, it has a long way to go to find its place in diagnostic laboratories, several researchers have already proved its usefulness in diagnosis of difficult to culture pathogens. Simultaneous and unbiased detection of the microbiological agents is unique hallmark of these methods. The sequence data generated from samples can be used for identification of different classes of microbes, antibiotic resistance genes, mutations in the genes, even reconstructing genomes of the small organisms specially viruses. The two main approaches of metagenomic investigation are phylogenetic marker amplification popularly known as amplicon sequencing and shotgun metagenomics, which implies sequencing the total metagenomic DNA isolated from the samples. Metagenomics applied to the vectors offers possibility to screen all the microbes carried by the vectors and could be an important tool for epidemiological investigations. The review focuses on the approaches and applications of metagenomics, its usefulness and prospects.

Key words: Disease diagnosis, Metagenomics, NGS, Untargeted metagenomics, 16S rRNA amplicon sequencing

Introduction

During last century, several techniques were developed for studying microbes, including physiology, genetics and epidemiology. The identification of the bacterial pathogens in clinical settings is largely dependent on the techniques developed in late eighties. Veterinary diagnostic bacteriology is still practicing physical detection and characterization of causative bacteria under the microscope using techniques developed by Hans Christian Gram and isolation of the bacteria in colonies on petri plates that was initiated by Robert Koch. The microbes have different and specific need for growth on solid media which increases the workflow and skill requirements in the diagnostic microbiology laboratory. Sometimes, the carbon sources in the culture media are not exactly similar to the requirements of the bacterial growth reflecting into success in isolating only limited bacterial members of the community (Nocker *et al.*, 2007).

Pathogen identification based on the traditional approach using morphology, physiology, chemistry, and biochemical characterization generally requires 2 to 5 days. In addition, phenotypic methods fail to identify the

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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. The indirect approaches were thus developed that are independent of the culturing techniques such as, identification of specific proteins and /or nucleic acids. Molecular biology techniques-based assays reduced the pathogen identification time (Castro-Escarpulli *et al*., 2015). Although, these approaches target specific molecules for individual pathogens therefore, it is difficult to detect unsuspected pathogens in the samples and requires multiple assays. The first-generation sequencing technology, Sanger sequencing, along with PCR based tests facilitated molecular identification and characterization of the pathogens and greatly aided in understanding of molecular epidemiology and host pathogen interactions. However, these technologies require prior knowledge of the pathogens and genomic sequences for clonal amplification. 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).

In case of bacterial pathogens, the discrepancy between the cultured diversity and *in situ* existing diversity resulted in adoption of the culture independent techniques for study of the bacterial communities in different niches (Hugenholtz *et al*., 1998; Zoetendal *et al*., 2004). The next generation sequencing techniques have an advantage that they are capable of identifying large number of pathogens simultaneously. The advancements in the Sanger chemistry based nucleic acid sequencing technologies have brought the sequencing-based tests in the diagnostic laboratories. The 16S rRNA gene was being used in identification of the microbes. The 16S rRNA based phylogeny approach was introduced by Carl Woese in 1987 with 12 bacterial phyla with a few culturable representatives in each. Despite the 16S rRNA based lineages not being officially recognized (due to the continuous discovery of sequence signatures belonging to undescribed phyla), presently, the ARB-Silva database lists 67 phyla, including 37 candidate phyla; the Ribosomal Database Project 10, lists 49 phyla, including 20 candidate phyla; and National Centre for Biotechnology Information (NCBI) lists 120 phyla, including 90 candidate phyla. The phyla other than the candidate phyla do not have cultured representatives.

Metagenomics

It was a common belief that the organisms easily cultured from an ecosystem are numerically and functionally significant ones. However, later it was proven that these organisms are rarely dominant (Hugenholtz, 2002). These organisms usually get isolated due to their ability to grow in nutrient rich media at moderate temperature and under routine laboratory conditions. The cultivable organism's proportion constitutes less than 1-10% of the total microbial diversity (Prakash *et al*., 2013). The fact was earlier known as "the great plate count anomaly" but the unculturability could not be proven till the advent of molecular biology tools. Sequencing of the phylogenic marker genes was introduced to identify uncultured microbes in the environment. This approach was used largely to reconstruct phylogenies, comparison of microbial distribution in samples employing sequencing or restriction fragnent lenth polymorphism (RFLP) and quantification of relative abundance of each taxonomic group using hybridization with group-specific probes and primers.

Metagenomics helps to identify the diversity, to study the population structure and to screen and isolate genes of our interest from the members which are yet to be cultured. The 16S rRNA based phylogeny has paved way for faster identification of the pathogens as well as differentiation among the closely associated species based on the full length 16S rRNA gene sequence. The technique was developed for identification of yet to be cultured microbes from the environmental niches. The word 'metagenome' was first used by Handelsman *et al*. (1998) and refers to sequence-based study of collection of all microbial genomes found in a sample. Later, the technique was used extensively for identifying unculturable microbes from different ecosystems as well as directly identification of the functional enzymes from the metagenomic samples. The 16S rRNA based phylogeny, in combination with metagenomics approach, has allowed unbiased comparisons of microbial community members across various biological niche areas. A similar marker gene, 18S rRNA can be used for identification of eukaryotic microbes like unicellular and multicellular parasites. Although, this approach has limitations in resolving the microbial diversity lower taxonomic ranks and the universal primers are not truly universal and does not guarantee the representation of all the microbes in the sample. This approach can identify the species of the microbe but fails to provide information on the subspecies or strain information that is vital for the diagnosis in terms of pathogenicity or antibiotic resistance. This shortcoming has limited the use 16S rRNA based metagenomics into the diagnosis of microbial diseases. A better resolution can be achieved using shotgun metagenomics approach. The term 'shotgun metagenomics' is used to define a methodology of direct sequencing of DNA extracted from a sample without culture or enrichment. This approach is used for clinical samples in the hope of detecting and characterizing pathogens.

Metagenomic approaches

Sequencing the samples for metagenomic diagnosis has two main approaches. The first approach uses amplification of the phylogenic marker genes (preferably the 16/18S rRNA gene) after PCR amplification. The universal presence of the selected phylogenic gene in the organisms facilitates simultaneous detection of several organisms in the sample. The approach is popularly known as amplicon sequencing and a typical workflow is given in Fig. 1. Amplicon sequencing can be used for the samples where the input sample contains tissue or sample matrix that can contribute to the metagenomic DNA. Sample multiplexing; running several samples in a single sequencing run on the next generation sequencer has further reduced the costs and affordability of the technology.

The 16S rRNA gene contains 9 hypervariable regions spanned with constant regions. Parts of the 16S rRNA genes that are not under strong negative selection, the mutations tend to accumulate, and variable regions are formed within the gene. The variable/hypervariable regions are flanked by the conserved regions that constituted the basis of designing the universal primers for simultaneous amplification of variable regions for microbial phylogeny. The amplified 16S rRNA genes from the samples are sequenced using next generation sequencing technologies and the sequence data is used for identification of the microbial composition within the samples. Initially, the term 'next-generation sequencing' was used to describe the high-throughput sequencing chemistry from classical Sanger sequencing. Later, they were recognized as second generation sequencing technologies that were based on nano engineered platforms facilitating the simultaneous sequencing of millions of nucleic acid molecules in one setup and capable of generating several gigabases (Gb) of sequence data that could be used for genome sequencing, variant detections, gene activities and basic understanding of the host pathogen interactions.

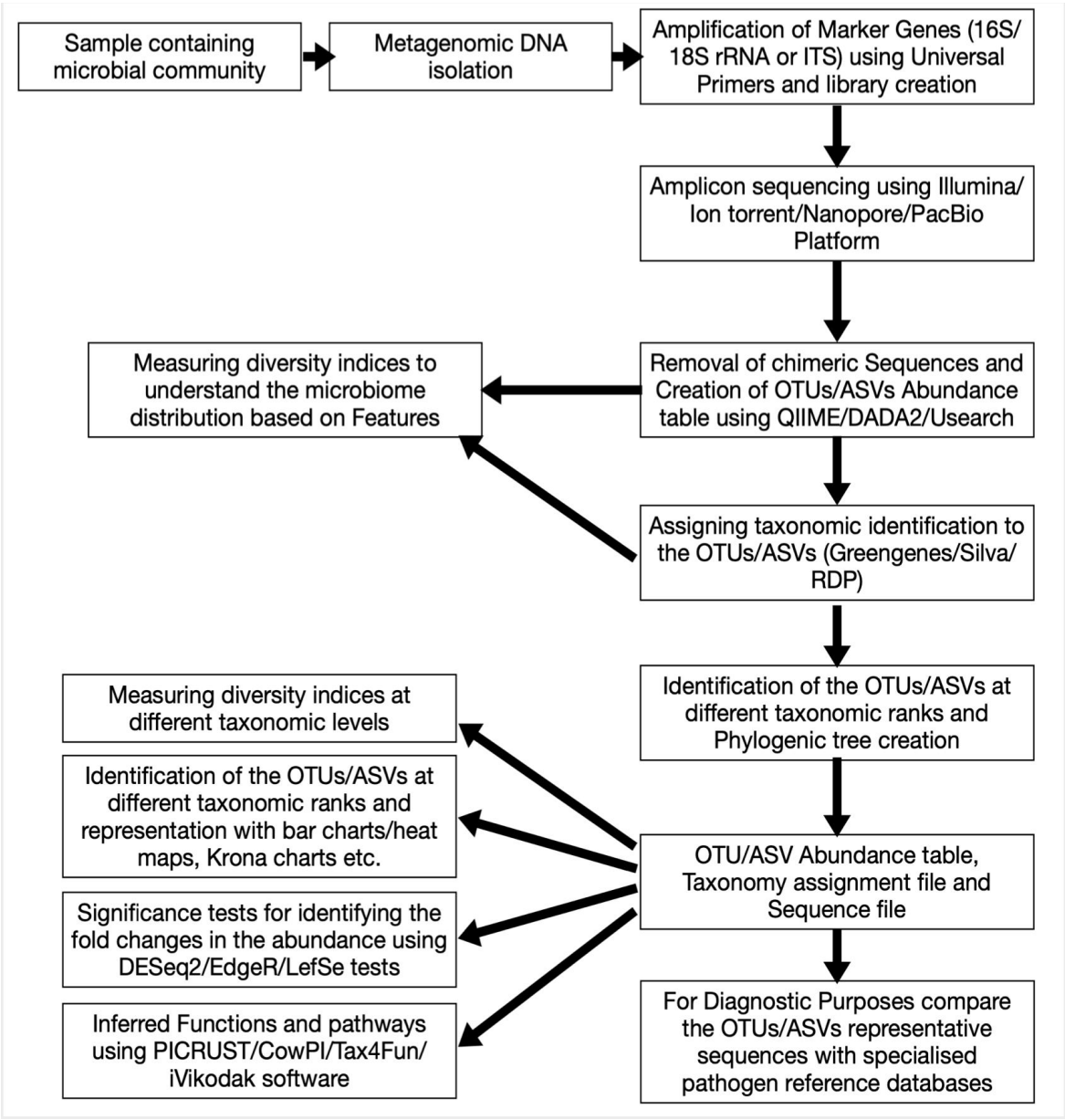

Fig. 1. A typical workflow of targeted amplicon sequencing and bioinformatic analysis

However, the approach could not generate the full length 16S rRNA gene. The second-generation technologies needed clonal amplification of the input DNA molecules to produce detectable signal for sequencing. The third-generation sequencing technologies rely on directly sequencing the nucleic acid molecules directly. The third-generation sequencing technology is still in infancy and suffers from the throughput requirements of open-ended diagnostic utility. Currently, NGS techniques are classified as second- and third- generation sequencing methods.

The second-generation technologies have limitations to sequence the complete 16/18S rRNA genes, which is crucial for taxonomic identification of the microbes up to species and strain level. Further, this approach excludes the viral counterpart which is one of the most

important pathogen classes from animal health as well as zoonotic point of view. Shotgun metagenomics was successfully used to identify all types of microbes from the sequence data (Pallen, 2014). The term shotgun metagenomics refers to sequencing of the total DNA isolated from the sample and is done by using k-mer based taxonomic classifier software. A typical shotgun metagenomics workflow is presented in Fig. 2.

Metagenome and gut health

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. Since the initial colonization immediately after birth these microbes are involved in various functions in rumen as well as intestine (Fig. 3). Recently, the rumen microbes are shown to have association with important economic traits in livestock like feed efficiency (Berry and Crowley, 2012; Shabat *et al*., 2016; Delgado *et al*., 2019; Li *et al*., 2019), enteric methane production (Wallace *et al*., 2015; Roehe *et al*.,

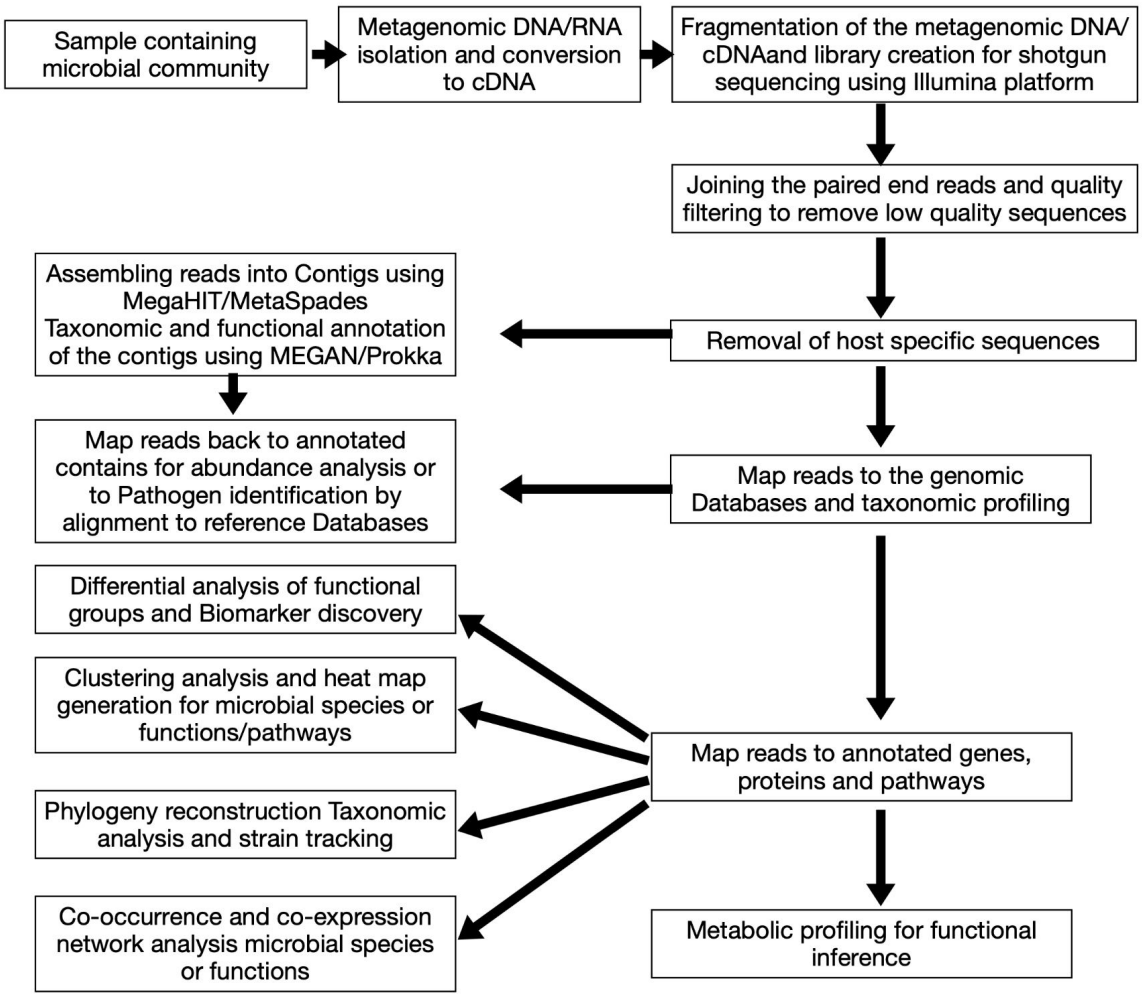

Fig. 2. Typical workflow of shotgun metagenomics used for microbial profiling and disease investigation

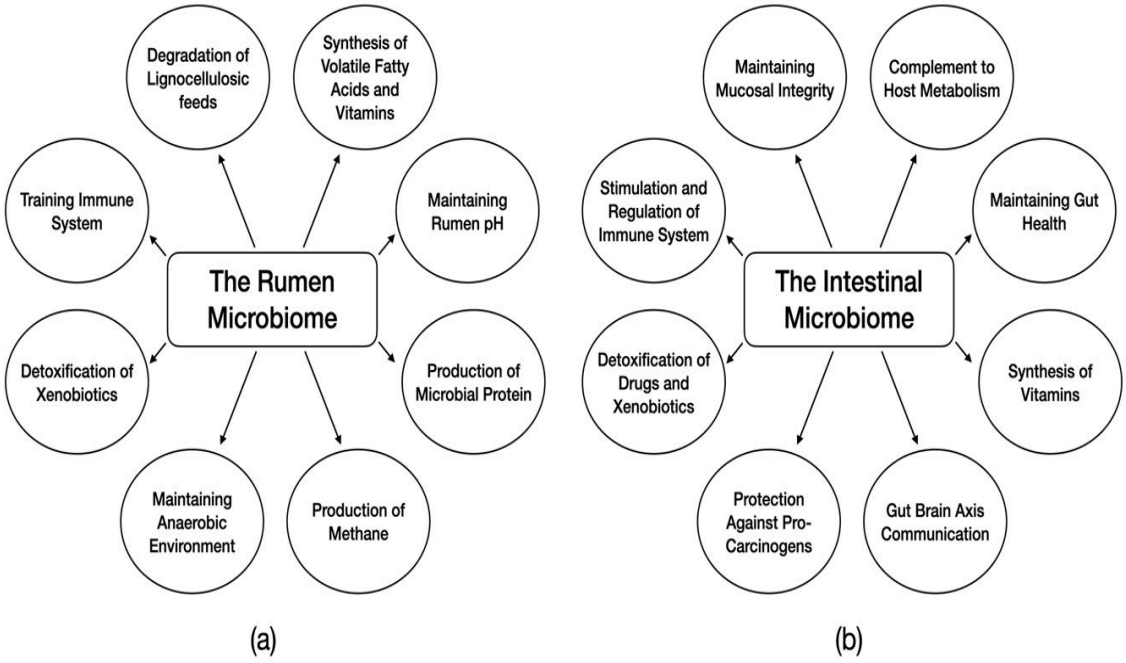

Fig. 3. Functions of rumen microbiome (a) and intestinal microbiome (b) in ruminants

2016; Difford *et al*., 2018), milk composition (Jami *et al*., 2014; Xue *et al*., 2019) and ruminal acidosis (McCann *et al*., 2016). Ruminal acidosis causes dysbiosis in the microbiome composition and ruminal fluid transplantation from healthy counterpart has shown recovery in sheep (Liu *et al*., 2019). The lower tract microbiome is suggested to be involved in maintaining the animal health and interaction with the immune system (Lyte *et al*., 2018).

Limitations of metagenomic diagnosis

Although, metagenomics promises unprecedented opportunity to detect the pathogens in the samples of human, animal and food source, the success of metagenomic exploration depends upon several factors. The first requirement is isolation of the nucleic acids from the samples for sequencing. As the samples are from different composition, the methods for isolation of the nucleic acids are different depending on the sample matrix and contaminating substances. Several laboratories are standardizing methodologies for isolation of high-quality nucleic acids from different clinical samples. Additionally, the data generation requirements are dependent on the amount of input nucleic acids present in the samples. Lower the template higher data needs to be generated from the sequencing platform. The data handling capabilities demand computing infrastructure and specialized software. Expertise in the bioinformatics is required for analysis of the data. And finally, the reference pathogen databases for the investigation are required. Presently the databases and analysis pipelines are being developed at several laboratories, but due to dynamic nature of the databases, the pipelines are also constantly updating, and one need to keep pace with the newer methods continuously.

The targeted amplicon sequencing, being simple and less time consuming, has following advantages. It can be used for identification of multiple pathogens simultaneously in a single test. It targets the specific microbial groups

viz.16S rRNA for bacteria and archaea (Rampini *et al*., 2011; Salipante *et al*., 2013), 18S rRNA for eukaryotes, ITS regions for fungi (Wagner *et al*., 2018) and RNA dependent RNA Polymerase (RdRP) for RNA viruses (Culley *et al*., 2010). It generates relative abundance of the microbial groups in the sample therefore changes in overall microbiome composition can be studied. The technique can capture rare taxa if the depth of sequencing is appropriate. Taxonomical classification is easier, and the reference databases are smaller that can be handled on desktop computers. Curated databases are available for comparison. The approach is suitable for low biomass samples like body fluids as the nucleic acid content is usually low for whole metagenome sequencing. The cost involvement is lower as compared to the whole metagenome sequencing. However, the method has certain disadvantages. It still requires prior knowledge of the microbial group targets and databases availability. If the organism is not present in the reference database used, the potential novel organisms can go undetected. Universal targets sometimes are not truly universal (i.e. the gene may be present, but the chosen primers may not amplify due to mismatches). As the technique requires amplification step, PCR bias can affect the outcome, which could influence the compositional comparisons across the samples. Due to the PCR bias, absolute abundances cannot be estimated correctly. As the second-generation sequencing platforms sequence maximum 500-550 base pairs, it does not cover the full-length marker gene; therefore the identification up to species and further levels cannot be accurately done. Although, the problem can be solved by third generation sequencing technologies, but at present the cost involvement is prohibitively high. The use of larger datasets for comparisons requires computational infrastructure, dedicated data processing pipelines and bioinformatics expertise.

The shotgun metagenomics, also regarded as unbiased approach to estimate and identify the microbial diversity, is better on the following terms. The technique can sequence all the DNA in the sample including bacteria, archaea, viruses and eukaryotes including the parasites and vectors. It is possible to filter the universal marker data from the shotgun metagenomics for using taxonomic identifications. The approach generates total genomic data; therefore, the functional aspects of the microbiomes can be interpreted that can provide opportunity for identifying the virulence factors, antibiotic resistance genes, antimicrobial proteins, functional pathways etc. Further, for both the approaches, online portals are available where data can be uploaded, and results can be visualized or downloaded. Several data analysis pipelines have been developed for handling the clinical metagenome data (Mulcahy-O'Grady and Workentine, 2016). However, as the data generated by shotgun metagenomics is large, in gigabases, it requires more computational power. The assembly of the metagenome is required before using the data for taxonomic or functional assignments. Particularly forviromes, the sample needs enrichment or exclusion of the other microbes that can contribute to the sequence data. High abundance of the host DNA and taxonomically uninformative sequences sometimes present in the data which does not contribute to the outcome and need to be removed using bioinformatics software. Costs are higher as more depth of sequencing is required for complex samples and particularly if the organisms of interest are in low abundance. For example, to detect 10 cells/g sample, probably the metagenomic data needs to be generated to the tune of approximately 400 Giga base pairs (Suttner *et al*., 2020). These requirements can be further depending on the overall complexity of the samples. High bioinformatics technical expertise or human resource is required for data

handling and interpretation as well as large datasets needs a plenty of digital data storage space.

Metagenomics in diagnosis

In situations, where the conventional and molecular tests-based diagnostics fail to identify the causative agents in the samples, the metagenomic approach that is basically culture free and faster, might provide an answer. Today, it is increasingly evident that the differences in host-associated microbial communities can influence the balance between health and disease in conditions not normally thought of as microbial or infectious in origin: for example, inflammatory bowel disease, cancer or obesity. Sometimes, it may not be sufficient to focus diagnostic efforts on single pathogen in clinical samples that is thought to cause disease. Instead, it is now recognized that interactions between organisms in a community can influence disease outcome and, in some cases, it might even be appropriate to treat a whole microbial community as a pathogenic entity. Given the difficulty of culturing most of the viruses, shotgun sequencing to identify and detect human-associated viruses has been tried. The genomes of DNA viruses can be recovered through shotgun sequencing of DNA directly extracted from a sample. To detect RNA viruses, RNA extracted from a sample has to be converted to cDNA (Batty *et al*., 2013). The first use of metagenomics for presumptive diagnosis was reported by Wilson *et al*. (2014) for diagnosis of leptospirosis in the cerebrospinal fluid (CSF) by amplicon sequencing that was not detected in a control sample. The diagnosis was further confirmed by specific molecular tools and serology.

In a first study, fecal metagenomics was used to detect bacterial pathogens (Nakamura *et al*., 2008). 156 Campylobacter sequences were found in a sample taken during a bout of illness but were absent from a convalescent sample from the same individual. The potential of diagnostic metagenomics was demonstrated on stool samples collected during the outbreak of Shiga-toxigenic *E. coli* O104:H4 in Germany during May–June 2011 (Loman *et al*., 2013). The authors could get deep coverage of the outbreak strain genome from several stool metagenomes, Illumina MiSeq bench top sequencer and subsequently using higher-throughput instrument, HiSeq2500, also recovered genome-level coverage of other pathogens like *Campylobacter jejuni*, *Clostridium difficile*, *Salmonella enterica*, that had been detected by routine microbiological investigation in several STEC-negative samples. This study clearly established the proof-of-principle that metagenomics could be used not only to detect, but also to characterize bacterial pathogens within a sample. Next generation sequencing of the pig saliva samples revealed that *Streptococcus* was most abundant genera and *S. suis* was the most abundant species suggesting that the pig saliva is a potent source of *S. suis* infection to piglets and animal handlers (Murase *et al*., 2019). In another study metagenomics was used to identify *S. suis*, a zoonotic pathogen, in a patient whose blood bacterial cultures were negative to post antibiotic therapy (Dai *et al*., 2019). Culture-negative sample of necrotic hepatitis using whole-metagenome shotgun sequencing was used to detect *B. melitensis* (Lazarevic *et al*., 2018). As Chlamydiae require labour-intensive culturing, the metagenomic method was used for characterizing the chlamydial plasmids in samples and a novel species of chlamydia was also reported (Taylor-Brown *et al*., 2017).

Pyrosequencing was used for identifying organisms associated with mastitis, a multi etiological syndrome. The mastitis milk samples were characterized by culture as *Trueperella pyogenes* and *Streptococcus spp*. The mastitis pathogens identified by culture were generally among the most frequent organisms detected

by pyrosequencing. Further, in aerobic culture-negative samples, pyrosequencing identified bacterial pathogens for mastitis, those are known pathogens but so far not been associated with mastitis, and those are currently not known to be pathogens. A possible role of anaerobic pathogens in bovine mastitis is also suggested based on the study (Oikonomou *et al*., 2012). 16S based phylogenomics was also demonstrated to evaluate a sample from various areas of a dairy farm for the presence of bacterial organisms associated with digital dermatitis lesions and successfully detected association of *Treponema* spp. with the lesions and the presence on the hoof trimming equipment (Rock *et al*., 2015). The whole metagenomic investigation of the multi etiologic diseases and syndromes may be useful in identifying the predisposing microbes or indicator microbes for the disease progression. Uterine microbiome profile in the metritis cows revealed shifts in the microbiome composition (dysbiosis), however the causative agents were present in low abundance (Jeon and Galvao, 2018). Metagenomic characterization of the bovine milk microflora from normal and clinical mastitis samples revealed presence of exclusive organisms in affected animals as well as human pathogens were also detected indicating poor hygiene of the milk production (Hoque *et al*., 2019). The same data was used for assessment of resistome profile in the clinical mastitis samples revealing presence of biofilm forming factors in pathogens, antibiotic/drug resistance genes, the information can be useful in selecting appropriate treatment schedule (Hoque *et al*., 2020).

Given the difficulty in culturing the viruses, diagnostic metagenomics can be a promising tool for viral disease diagnosis. Metagenomics was used in diagnosing fetal infections in transplant patients and identified sequences of lymphocytic choriomeningitis virus (Palacios *et al*., 2008). Several recent studies demonstrated the identification of viral agents in the ancient samples. A study of the Tyrolean ice mummy Ötzi genome revealed sequences of *Borrelia burgdorferi*, making it the first known case of Lyme disease (Keller *et al*., 2012). Krause and colleagues recovered *Mycobacterium leprae* genome from the metagenome obtained from a historical dental sample (Schuenemann *et al*., 2013). The first example of post-mortem metagenomic diagnosis of tuberculosis was done from mummified lung tissue of a young woman who died in 1797 (Chan *et al*., 2013). A novel species of Ebola virus (Bundibugyo ebolavirus) was also discovered using this approach from Uganda (Towner *et al*., 2008). In another study, a novel arenavirus responsible for a hospital outbreak of haemorrhagic fever in South Africa was identified (Briese *et al*., 2009).

Metagenomics was applied in veterinary science and diagnosis in recent past to detect several newly emerging or re-emerging diseases. The first virus of high emerging impact was identified by metagenomics is Schmallenberg virus (SBV), belonging to orthobunya virus of the Simbu serogroup (Hoffmann *et al*., 2012). The virus was identified from the blood samples of cattle in Netherlands and Germany showing drop in production and fever, though other diagnostic methods could not detect the cause. Later astroviruses were identified in cattle diagnosed with bovine encephalitis in Europe, influenza D virus in cattle in the United States and France, bat influenza viruses H17N10 and H18N11 in bats in Central and South America, and a novel zoonotic borna virus in variegated squirrels in Germany. From 2009 to 2017 almost 20 animal and zoonotic viral disease agents were diagnosed using metagenomic technique (Hoeper *et al*., 2017). A limited scale metagenomics-based pathogen surveillance study on 6 poultry farms detected the main infectious viruses of poultry, and it analyzed

the subtypes, genotypes and pathogenicity of some detected viruses (Qiu *et al*., 2019). Subsequent to the metagenomic investigation of astroviruses in enteric disorders in humans (Finkbeiner *et al*., 2009), a divergent strain was demonstrated to be associated with the brain tissue of shaking mink syndrome affected minks (Blomström *et al*., 2010). Viral metagenomics can be a valuable tool for molecular epidemiological studies, it was successfully used to detect field rabies viruses from the data generated from the high throughput metagenome sequencing (Orlowska *et al*., 2019). Ticks are important vectors for different tick-borne viruses, viral metagenomics of the vector can be a promising tool to simultaneously identify all the viruses present in a sample, including novel variants of already known viruses or completely new viruses (Damian *et al*., 2020). An exhaustive common livestock species wise list of animal viruses detected using vial metagenomics approach is reviewed by Kwok *et al*. (2020).

Apart from the identification of the disease causative agents, the untargeted metagenomics can be used for identification of the antibiotic resistance (AMR) genes in the samples. Traditional AMR assessment is based on the phenotypic AMR assays, while metagenomic studies discover the genes responsible for AMR (Duarte *et al*., 2020). Recent evidence has indicated that the presence of AMR genes in isolates can be highly corelated with observed phenotypic resistance (Stubberfield *et al*., 2019; Guo *et al*., 2019).

Present challenges to metagenomic approaches

Although, metagenomics promises unprecedented opportunity to detect the pathogens in the samples of human, animal and food source, the success of metagenomic exploration depends upon several factors. The metagenomics in diagnosis has a long way to prove itself a validated tool. Several issues still need to be addressed like, sampling bias and optimum methods for nucleic acid extraction, estimation of required sequencing depth, affordable sequencing platforms, dedicated databases for faster diagnosis, and cost of diagnosis. The first requirement is isolation of the nucleic acids from the samples for sequencing. As the samples are from different composition, the methods for isolation of the nucleic acids are different depending on the sample matrix and contaminating substances. Several laboratories are standardizing methodologies for isolation of high-quality nucleic acids from different clinical samples. The viral RNA isolation from the metagenomic sample is challenging as the molecules are present in very low abundance in the total RNA of the sample and are highly labile. The RNA isolation protocols for each type of samples need to be standardized. Furthermore, the data generation requirements are dependent on the amount of input nucleic acids present in the samples. Lower the abundance of the pathogen in the sample, higher data needs to be generated from the sequencing platform. The data handling capabilities demand computing infrastructure and specialized software. Of course, developing capacity or human resource also needs to be addressed before bringing it to the diagnostic laboratories. Finally, the reference pathogen databases for the investigation are required. Presently the databases and analysis pipelines are being developed at several laboratories, but due to dynamic nature of the databases, the pipelines are also changing, and one need to keep pace with the newer methods continuously.

The prospects of metagenomics are lucrative as it can offer opportunity to detect unsuspected pathogens, simultaneous detection of multiple pathogens of different classes and taxonomic origin, as well as functional information of the pathogens. The

untargeted approach basically does not depend on the prior knowledge of the pathogen and hence allows detection of different classes of pathogens in a single protocol (Nakamura *et al*., 2011). The tool can be valuable in zoonotic and epidemiological studies to understand the disease spread. It is already proven that the diagnostic metagenomics has already played a role in identifying the causes of unknown illnesses and outbreaks. Although there are several challenges in fully implementing the technique for diagnosis, nonetheless, in a decade or two the technique will be method of choice for detecting the hard to culture microbes, unsuspecting agents and apart from the main causative agent, a tool to identify the associated opportunist pathogens in diagnostic microbiology.

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