GENOTYPING - NEW ERA IN DISEASE TYPING

Abstract

Microbial infections have been one of the most dreadful causes behind human fatalities and are also frequently associated with occurrences of endemic and pandemics. It is very important that these pathogens are identified and treated on timely manner to prevent serious consequences and spread in communities. Conventionally microbial culture techniques, Biochemical testing and microscopy are used for pathogen identification. Although very accurate and reliable, these methods frequently fail due to operational reasons and due to fastidious nature of the pathogens. To overcome these challenges, Scientists have developed cutting edge technologies like16srRNA sequencing and Nextgen sequencing.Sequence-based identification, strain typing, and comprehensive isolate fingerprinting can be used effectively for identification of pathogenic microbe associated with health hazard. These modern tools offer great potential in practicing prophylaxis for infection and disease prevention. Nextgen sequencing can handle multiple samples and sequences. The sequence data can be managed using online repositories available as open access tools. Large amount of genomic and proteomic data can be deposited, used and analyzed using informatics tools. Molecular methods like DNA / Genome sequencing, protein purification and sequencing have been a widely used tool for epidemiological fingerprinting of isolates important to public health.

Keywords: *Pathogen, Molecular techniques, Sequencing, prophylaxis, BLAST, PCR, Epidemiology*

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Conventionally many microbes have been invading human systems and have been causing different types of infection. These microbes include all major groups namely bacteria virus, Fungi algae and others. [1]

Today due to advancement in pharmacobiology, discovery of active API , novel drug delivery systems and understanding of disease biology has made cure from microbial infections a big reality. Many different types of treatment options are available to individuals suffering from microbial infections. In order to provide the best therapeutic chance, it is of utmost importance to timely identify the culprit pathogen at the species level and if possible at the strain level. Disease causing pathogen identification helps with accurate diagnosis which in turn facilitates correct treatment options. In the absence of correct identification of the pathogen many times the diagnosis given may be Faulty. The patient suffers for a longer period, he may also be susceptible to acquiring secondary infection by an invading opportunistic pathogen. [2]

These things not only delay the recovery of the patient but are a big threat for human life. Lastly incorrect identification leads to higher chances of incorrect usage of drugs like antibiotics. This further causes the emergence of antibiotic resistance.

Hence pathogen identification is the key to successful therapeutic application.[3,4]

I. TRADITIONAL METHODS FOR PATHOGEN IDENTIFICATION

Traditionally laboratory based cell culture techniques are used for cultivation of pathogens collected from a patent via suitable sampling methods. Blood, urine, sputum samples may be collected from patients. Choice of cell culture system and media selection is a function of preliminary diagnosis which inturn is based on patient history and narration regarding onset of symptoms, duration, severity, mode of acquiring the infection. [5,6]

Patient samples are then cultured and cell culture characteristics / colony characteristics are recorded. A sample table and few culture traits are mentioned below [7,8]

Table1: Examples of Colony Characteristics of E. Coli Isolated from Patients Suffering from Utiare Cultured on Nutrient Agar Medium, Incubated At 37'C / 24 Hours.

Feature	Reference Specifications on Nutrient agar	Reference Specifications on Mac Conkey's agar	Reference Specifications on EMB agar	Reference Specifications on Endo agar
size	2-4 mm	3-4 mm	Pinpoint colonies may form clusters	1-1.5mm
Shape	Circular	Circular	Circular	Circular
Colour	Pale	pink	Green metallic	Dark pink to

				sheen	reddish
Margin	Entire	Entire	Entire	Entire	
Elevation	Low convex	Low convex	Low convex	Low convex	
Opacity	Translucent	Translucent	Opaque	Translucent	
Consistency	Butyrous	Butyrous	Butyrous	Soft	
Gram nature	Gram Negative	Gram Negative	Gram Negative	Gram Negative	
Motility	Motile sluggish)	Motile (sluggish)	Motile (sluggish)	Motile (sluggish)	

Motility Test Performed Using St. NA Butt.

Gram staining done on heat fixed smear using Crystal violet, Iodine, alcohol and Safranin.

Table 2: IMViC test for E.coli isolated from patient suffering from UTI
Legend: += positive test. - = negative test

Indole	(MR) Methyl Red	VP (Voges Proskauer)	Simmon's Citrate slant
+	+	-	-

II. CHALLENGES OF CELL CULTURE TECHNIQUES FOR IDENTIFICATION OF PATHOGEN

Despite the breakthrough of molecular methods, microbial culture techniques like pure culture techniques, Antibiotic sensitivity assays- Disc diffusion, Agar Cup assays, Cross Strip methods remains the cornerstone of routine microbiology as they provide insight culture typing at species ar sometimes even strain level typing, assessment of antibiotic resistance, plasmids status microbial ecology and pathogenicity.

However, a majority of microorganisms in nature are not easily cultivable using standard techniques. Many fastidious microbes grow poorly on common laboratory media, and others are considered uncultivable on axenic media, possibly owing to deficient or partial metabolic pathways. Also usual microbial culturing methods and microscopic identification are time consuming and laborious processes. Microbial cell culture techniques require stringent aseptic handling, and sophisticated Laminar Air Flow cabinets, incubators, autoclaves and reagents. Another hindrance in pathogen identification is the variation in morphology, gram nature, staining properties and other features. Handling microbes also requires training of the technician as regards sample collection, culturing techniques, preservation techniques, staining and correct identification of pathogens. Hazardous organism

handling requires efficient SOPs in place for culturing till disposal of cultured pathogens. [9,10,11]

However, although it is important to improve culture methods for handling fastidious microorganisms, the investigation of unusual infections or outbreaks needs accurate, reliable, reproducible, sensitive and rapid technologies that may help influence the management of patients and epidemiology.

Genome Sequencing like NGS has been an efficient method for rapid identification of pathogens sampled from patients without the hassle of laborious and time consuming culturing.[12,13]

III. BASIC PRINCIPLE OF NGS

Nick McCooke led the pioneer team at Solexa that invented next-generation sequencing, a technology to read DNA at high speed that is nowadays used worldwide and has laid the foundation for precision medicine.

NGS facilitates the interrogation of tens of thousands of genes at one time in multiple samples, as well as discovery and analysis of different types of genomic features in a single sequencing operation, from single nucleotide variants (SNVs), to copy number and structural variants, and even RNA fusions. Next-generation sequencing (NGS) is an advanced molecular technique based on principles of gene sequencing technology. Its salient features are ultra-high throughput, high scalability, and speed. NGS is used to determine the order of nucleotides in entire genomes or targeted regions of DNA or RNA. It can be used to decipher the order of ribonucleotides in conserved regions of 16srRNA coding regions on DNA using specific primers, Taq polymerase and dNTPs as monomers. Main types of NGS include - Whole Genome Sequencing, Whole Exome, and Targeted Panels.[14, 15, 22, 23, 24, 32, 33, 34]

The basic principle of NGS - next-generation sequencing process involves fragmentation of DNA/RNA molecules extracted from unknown samples (EG: bacterial pathogens) into multiple smaller units, subsequent addition of adapters, sequencing the libraries, and reassembling them to form a genomic sequence. [16 ,17, 18]

1. **NGS explained Stepwise:** Patient sample collection (blood, tissue, stool, sputum)
   Extraction of DNA / RNA (For RNA extraction use Reverse Transcriptase to make cDNA copies)
   Genome Amplification
   Next Gen Sequencing
   Data Analysis: NCBI databases / BLAST/ Phylogenetics Tree/ Swissprot/ Uniprot/ IGV/ VISTA/ Bandages

2. **Quality Check for NGS data:** Assessment and Quality check of the raw sequencing data is a crucial step that governs the final outcome. It is also imperative to determine sequence quality and authenticity that in turn feeds all downstream analyses. Raw sequencing data provide a general view on the number and length of reads, presence of contaminating sequences, or any reads with low/ incomplete genome coverage. *FastQC* is

one of the most used applications for performing quality control of sequence read raw data. *Fastp* is an ultra-fast application recently developed for the same purpose. Further for preprocessing of QC checked raw data, trimming of gene sequences, Base corrections, manual filtering, and curating the sequences are performed. Unwanted sequences like adapter are eliminated. [19, 20, 21,]

3. **Preprocessing of QC checked sequence data:** Preprocessing of QC checked sequence data depends on availability of reference data. If the generated sequences are mapped /aligned to a reference genome or transcriptome excavated from databases, then the query sequence can be identified. In case of *de novo* sequences, sequences are aligned into contigs using overlapping regions. This is often done with the in silico assistance of genome processing applications like orientation correction, contig reordering, elimination of repetitive regions and scaffolding. It is important to choose tools without biases for alignment and identification. Mapping tools should be chosen after careful considerations like publication and citation scores.

   Once the genome reads have been mapped and processed, they need to be analyzed in an experiment-specific fashion, what is known as variant analysis. This step can identify single nucleotide polymorphisms (SNPs), indels (an insertion or deletion of bases), inversions, haplotypes, differential gene transcription in the case of RNA-seq and much more. Despite the multitude of tools for genome assembly, alignment and analysis, there is a constant need for new and improved versions to ensure that the sensitivity, accuracy and resolution can match the rapidly advancing NGS techniques.

4. **Visualization of sequence analysis:** If the output sequence is not de novo, reference can be retrieved using either Genome Browser or Integrated Genome Viewer (IGV). Variant Explorer is a good tool if WES or WGS technology is used. VISTA tools help in comparative analysis while visualizing multiple sequences. For organization and building sequences, Bandages and Icarus may be selected. [30]

IV. ADVANTAGES OF NGS

Whole genome sequencing can be performed on as little as 1 ng of DNA that has been extracted from a target tissue or cell samples.

NGS allows screening multiple samples in a cost-effective manner and also promotes detection of multiple variants across targeted areas of the genome—an approach that would be costly and time-consuming using Sanger sequencing.

The development of NGS bench-top sequencers such as the MiSeq (Illumina) and Ion Torrent Personal Genome Sequencer (PGM; Life Technologies) has made genome sequencing compatible with the routine clinical-microbiology workflow. Such a strategy enables, within a few hours, exhaustive access to the genotype, virulence markers and antibiotic-resistance repertoire.[39, 40] Real-time genomics has notably been used to investigate several nosocomial or community-acquired infections. Sherry and colleagues used PGM sequencing of four MDR *E. coli* strains to confirm that the nosocomial outbreak that had occurred in a neonatal unit in Melbourne, Australia, had been caused by a unique clone and to characterize the resistance genes for this outbreak strain. In Germany, Mellmann and

colleagues compared the genomes from two *E. coli* O104:H4 strains from two hemolytic uremic syndrome outbreaks and concluded that the strains had diverged from a common ancestor and that NGS was suitable for the characterization of a pathogen in the early stages of an outbreak. In both cases, genome sequences were obtained in a few days (five and three days, respectively). These findings demonstrated how rapid and precise genomic sequencing, although limited to a few clinical-microbiology laboratories currently, could transform patient management or improve hospital infection control in routine clinical practice.

Although only a few studies to date have described a turnaround time sufficiently short to enable WGS data to influence the course of outbreaks, the increasing number of teams using WGS for epidemiological purposes leaves little doubt as to the likelihood of its systematic use as a first-line tool to track and understand epidemics in the near future.

V. FUTURE OF CLINICAL DIAGNOSIS - NEXT GEN SEQUENCING

When we look at different options available for clinical diagnosis of microbial infections using molecular sequencing technologies, NGS definitely has shown good credibility. It clearly outperforms the conventional and routine Laboratory culturing and Biochemical testing. When compared with other genotyping methods, RT- PCR followed by NGS definitely has proven its worth. This Technique allows bypassing tedious and laborious wet lab procedures, most importantly does not require cultivation of notorious microbes, thus safely escapes chances of laboratory origin outbreaks of Endemic and pandemics type spread of infections with in population. These techniques are safer for technicians undertaking the task of providing identity of the pathogen. Government and industry has also invested for the research in this field in terms of developing precise primers and reagents for amplification of correct target gene and NG based sequencing. Today laboratories can easily carry out sequence analysis using in silico tools since efforts have been put in place towards updating databases, retrieval of data, easy and open access to data at online repositories. NGS is fast, reliable and reproducible. It has demonstrated its potential in identifying members of complex microbiota in metagenomic studies. Research documents talk loudly about the potential of NGS in uncovering the evolutionary relationships amongst new strains and variants of pathogens that further explains mutations. This has made understanding of spread of disease, pattern of epidemiology, and wise choice of therapeutics for combating coinfections, opportunistic pathogens, secondary infections in case of immunocompromised individuals and treatment of patients infected with MDR and XDR variants in an effective manner.

However, many gaps are required to be bridged in order to make Genosequence based diagnosis a routine analysis. The most important criteria is an easy and cost effective access of clinical microbiology laboratories to gene sequencing facilities, and a need for standardized and fully automated sequence interpretation that would ideally be independent of both the sequencing platform and the exact species of microorganism. Also needed is the ability to interpret the genedata to yield correct curated similarity search results, which would enable Molecular technicians and Bioinformaticians, clinicians and public-health epidemiologists to implement effective control measures in real-time and change the course of outbreaks. This implies a constant update and curation of public databases as well as the development of systems-biology-based software that will enable prediction of virulence and antibiotic resistance from genome sequences.

In order to make Genotyping more accessible, trained technicians and laboratories with molecular diagnostic facilities are needed in every area. Government and industries can support in this endeavor. Academicians and researchers may be provided with refresher workshops and hand on training. Need of the hour is introduction of Bioinformatics with special reference to databases, Proteomics, Genomics at undergraduate level and then the techniques of handling sophisticated instruments can be mastered. Bio entrepreneurship in the field of providing novel primers, enzymes, buffers and other reagents offer a lucrative future for young Biotechnologists and Life Science students.[38, 39, 40, 55, 59]

VI. CONCLUSION

The advancement in the field of genome sequencing has changed the field of diagnostics and management of infectious pathogens. It has greatly helped in shedding light on establishing host pathogen relationships, microbial genetic diversity, and evolution of new variants, mutants, and development of antibiotic resistance. Sequencing methods generate genetic maps of selected regions which help in decoding many hidden features and salient functions related to pathogens etiology. NGS are time saving, greener technologies which have slowly paved their way and acquired a place in routine diagnosis and disease management.

In the modern era, NGS has proven advantageous as regards other sequencing methodologies since it helps culture unculturable and fastidious pathogens. NGS technique utilizes a single cell or single bacterial colony as a source of pure genetic material. Amplified bacterial genetic material is then sequenced. The sequence data analysis is done in silicon using open access resources like BLAST at NCBI platform. Similarity Search tools like BLAST and MSA provide ample opportunities in understanding bacterial genome dynamics and help in correct typing of the pathogen till species level. BLAST and MSA will help download sequences in FASTA and other processable formats that can further be used for Protein databases and construction of phylogenetic trees to understand gene functions, mutations and evolutionary relationships amongst conventional pathogen strains and new mutant variants. Thus NGS helps overcome laborious bacterial culturing processes for identification of pathogens, handling of pathogen for longer time, problem of handling and growing fastidious microbes. NGS is an efficient way to cell culture typing in an accurate and reliable way. It helps in early diagnosis, patient management, administration of correct therapy in a timely manner. This also gives a fair chance of establishment of herd immunity in case of notorious infectious pathogen outbreak.

However, single-cell genome sequencing technology also has challenges, which include purity of reagents, primer designs, selection of PCR cycles, genome amplification biases, chimeric DNA rearrangements, database repository and available submitted genome data. Clearly many times there is a need for the improved *de novo* assembly of DNA sequences of previously non-sequenced microorganisms.

In conclusion, the contribution of genome sequence data at open resources, merging of data banks, cost reduction / subsidization for genetic identification tests and expansion of genome sequence laboratory facilities will offer brighter opportunities for harnessing benefits and making it available to cater to the needs of world populations.

VII. ABBREVIATIONS

BLAST	BASIC LOCAL SEQUENCE ALIGNMENT
DNA	DEOXYRIBONUCLEIC ACID
dNTP	DEOXYNUCLEOTIDE TRIPHOSPHATES
FASTA	FAST ALIGN
MLST	MULTI LOCUS SEQUENCE TYPING
MLVA	MULTIPLE LOCUS VARIABLE TANDEM REPEAT ANALYSIS
MR	Methyl Red
MRSA	METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS
MSA	MULTIPLE SEQUENCE ALIGNMENT
MST	MULTIPLE SPACER TYPING
NCBI	NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION
NGS	NEXT GENERATION SEQUENCING
PCR	POLYMERASE CHAIN REACTION
PCR -RFLP	POLYMERASE CHAIN REACTION RESTRICTION FRAGMENT LENGTH POLYMORPHISM
PFGE	PULSE FIELD GEL ELECTROPHORESIS
RT-PCR	REAL TIME POLYMERASE CHAIN REACTION
SNP	SINGLE NUCLEOTIDE POLYMORPHISM
VNTRs	VARIABLE NUMBER TANDEM REPEATS
VP	Voges Proskauer
WGS	WHOLE GENOME SEQUENCING
16srRNA SEQUENCING	16s RIBOSOMAL RIBONUCLEIC ACID SEQUENCING

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