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MOLECULAR TECHNIQUES FOR MEDICAL MICROBIOLOGY LABORATORIES: FUTURISTIC APPROACH IN DIAGNOSTICS OF INFECTIOUS DISEASES

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ABSTRACT

Diagnostic methods for infectious diseases have stagnated in the last 20–30 years. Conventional diagnostic approaches are not able to fulfill all the desires needed for the effective diagnosis of microbial diseases. Few major advances in clinical diagnostic tools have been achieved after the introduction of PCR, although new techniques are under investigation. Many tools that are used in the "modern" microbiology laboratory are based on very old and labor-intensive technologies. The need to develop new diagnostic tools include more rapid tests without sacrificing sensitivity, reliable, accurate, value-added tests, and point-of-care test. Research has been focused toward development of new alternative methods to improve the diagnosis of microbial diseases. These include molecular-based approaches. This review summarizes some of the new molecular approaches in microbial disease diagnosis.

KEYWORDS: **Microbial disease, molecular diagnostics, LAMP, RT-PCR and pathogens.**

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INTRODUCTION

Microorganisms are minute living things that cannot be seen with naked eye. Some of them are pathogenic while others are non-pathogenic. The first microbes were observed in 1673. Microorganisms were first directly observed by Anton Van Leeuwenhoek (who is considered as father of microbiology) in teeth scrapings, rain water, and peppercorn infusions. Germ theory was proposed by several scientists to understand the relationship between microbes and diseases. Germ theory states that specific microscopic organisms are responsible for causing specific diseases. Major contributions to germ theory or pathogenic theory of medicine were provided by Anton van Leeuwenhoek, Francesco Redi, Rudolf Virchow, Louis Pasteur, Joseph Lister and Robert Koch.

Microbial diseases are sicknesses or ailments that are caused by four different types of microbes in humans. Microbes are only seen by the help of powerful microscope. They are capable of causing diseases in any host if they introduced with an extensive amount. The four different types of microbes include bacteria, virus, protozoa and fungi that cause sicknesses or microbial diseases. Emerging and re-emerging diseases associated with zoonotic, food-borne, water-borne and diseases caused by multi-resistant organisms[1] constitute the major threats in human beings. Detection and identification of the infectious agent causing disease is a highly relevant issue in microbiological diagnostics. Several conventional tools (microscopy, serology, culturing of microbes and sometimes X-rays) are used to diagnose the disease. With the outcome of new pathogens and need to screen large no of clinical samples within time, these conventional techniques are not at par with time and need. Therefore, it is necessary to develop technologies which is more specific, sensitive, accurate and fast as well as can detect very small amount of pathogens in the sample. In the last twenty years, technologies based on nucleic acid amplification techniques (NAATs) have taken an irreversible position in the diagnostic field of infectious diseases. Recent developments in modern diagnostic tools have opened a new era for a vast improvement in parasite detection. Molecular-based approaches such as QT-NASBA[2], LAMP[3] (loop-mediated isothermal amplification), real-time polymerase chain reaction[4], Luminex[5], Microarrays[6], Metagenomics[7] and DNA-based biosensors[8] have shown a high potential for use in disease diagnosis with increased specificity and sensitivity. Molecular diagnostic assays have been assisted in the diagnosis, treatment and epidemiological studies of infectious diseases of microbial origin that affect people worldwide. These tools help in controlling the mortality rate caused by infectious diseases because early detection of an infectious agent can lead to a targeted treatment. Molecular diagnostic methods have been widely accepted in the diagnosis of infectious diseases and also these methods are easy to use, safe, sensitive, reproducible and eventually automated to facilitate the evaluation of large numbers of samples within time.

PREVALENT MICROBIAL DISEASES OF INDIA

Most of the diseases as depicted in the tables 1 are major public health problems leading to high morbidity & mortality resulting in loss of Disability Adjusted Life Years (DALYs) esp. Malaria, diarrhea, pneumonia, TB are quite prevalent in northern part of India including Haryana[9]. New emerging & re-emerging diseases like HIV, NDM-1 (New Delhi metallo-β-lactamase-1) & dengue posing major health problems due to increasing population, migration, urbanization & global climatic changes.

Table 1
List of prevalent disease along with pathogens & sample needed for diagnosis.

Microbial Disease	Causative agent	Sample Type
Diseases caused by bacteria		
Cholera	*Vibrio cholerae*	Stool
Pneumonia	*Streptococcus pneumonia*	Blood
Typhoid	*Salmonella typhi*	Blood
Tetanus	*Clostridium tetani*	Tissue from wound
Diphtheria	*Corynebacterium diphtheria*	Blood
Whooping cough	*Bordetella pertussis*	Blood
Tuberculosis	*Mycobacterium tuberculosis*	Sputum
Diarrhea	*E. coli*	Stool
NDM-1 (Super bug)	*E. coli* and *Klebsiella pneumoniae*	Blood
Trachoma	*Chlamydia trachomatis*	Conjunctival scrap
Gonorrhea	*Neisseria gonorrhoeae*	Blood, urethral swab
Septicaemia	*Streptococcus*, *clostridium*, *staphylococcus*, *salmonella* spp.	Blood
Syphilis	*Treponema pallidum*	Blood
Bacillus dysentery	*Shigella dysenteriae*	Stool
Diseases caused by virus		
Influenza	*Orthomyxovirus*	Throat swab
Chickenpox	*Varicella zoster*	Vesicle fluid and scrap
Measles	*Rubeola virus*	Blood
Rabies	*Lyssa virus*	CSF, blood
Mumps	*Paramyxovirus*	Blood
AIDS	*Human Immunodeficiency Virus*	Blood
Hepatitis B and E	*Orthohepadnavirus* and *Hepavirus*	Blood
Japanese Encephalitis	*Flavivirus*	Blood
Dengue	*Flavivirus*	Blood
Chikungunya	*Chik virus*	Blood
Diseases caused by protozoa		
Malaria	*Plasmodium falciparum*	Blood
Amebic dysentery	*Entamoeba histolytica*	Stool
Giardiasis	*Giardia intestinalis*	Stool
Diseases caused by fungi		
RTI	*Candida albicans*	Urethral swab
Ringworm	*Microsporum gypseum*	Skin scrap
Thrush	*Candida albicans*	Skin scrap

CONVENTIONAL DIAGNOSTIC METHODS USED IN LABORATORY

In daily routine, pathogens can be detected by several ways in clinical microbiological laboratory. Strategic approach is required for diagnosis of microbial diseases, since the etiological agent can be of bacterial, viral, fungal or protozoan origin. Traditional methods for microbial diagnosis of clinical samples will be performed by microscopy[10] and culture[11] techniques. If the pathogen is non-culturable and fastidious micro-organism, indirect detection method such as serology (ELISA) can be used for the identification of causative agent[12]. This approach is totally based on the pathogen-specific antibodies present in the patient's serum. However, a convalescent serum sample is required in order to get a reliable result. Conventional diagnostic methods in clinical microbiology are inexpensive tools but require more time to give results. Interpretation of the culture results requires expert technical skill. Rapid diagnosis of pathogens (within the same day), needed for the bulk screening of humans infected with a multi-resistant micro-organism, is beyond the power of conventional tools used in the clinical microbiological approaches. Other drawback of conventional methods are low sensitivity, seropositive, microbial growth is slow, sub typing is mandatory and microscopy gives false positive results.

MOLECULAR METHODS USED FOR DETECTION OF PATHOGENS

Molecular diagnostic tools are more appropriate for diagnosis of infectious agents that are difficult to detect, identify or test for susceptibility in a timely fashion with conventional methods. Strategies concerning the use of molecular diagnostic techniques for the disease diagnosis are need of the time. Identification of the infectious agents causing diseases is essential to provide an accurate diagnosis. To meet all these needs, innovative technologies have been developed that detect single pathogen, multiple syndrome related pathogens and genotypic drug resistance pathogens. The use of molecular biology techniques, such as nucleic acid amplification, has the potential to provide revolutionary changes in the diagnosis of infecting pathogens. The main idea behind the development of molecular diagnostic tools is that every organism contains some unique, specific DNA sequences. Molecular diagnostics make these species specific DNA visible. In clinical microbiology, we will be able to detect smaller amount of DNA or RNA of pathogens that is currently possible with molecular tools. This reduces the time needed for the identification and determination of the antimicrobial susceptibility of slow-growing pathogens. The diagnosis of pathogens which can not be culture, now become possible with molecular diagnostics. Most molecular assays mainly depend on three basic components, including nucleic acid extraction[13], amplification/analysis[14] and detection of an amplified product[15]. Some of the latest molecular diagnostic techniques are discussed below.

1. QUANTITATIVE NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION

QT-NASBA[2] is based on the single-stranded RNA sequences amplication without the interference of DNA and can be applied for the diagnosis of several human diseases caused by microbes, such as human immunodeficiency virus[16] and malaria[17]. This technology is highly sensitive and specific and is able to detect very low level of target in clinical samples[17]. Moreover, pathogen viability can be checked by the detection of specific RNA molecules which serve as a suitable marker for pathogen[18]. In QT-NASBA, primers and probes are selected on the basis of the sequence of the small subunit of rRNA gene. Quantification is achieved by co-amplification between WT (Wild type)-RNA and a fixed amount of Q (Quantification)-RNA as inhibitor which is modified *in vitro* by site-directed mutagenesis. NASBA is followed by Electrochemiluminescence (ECL) detection,

where amplicons are hybridized to a ruthenium-labeled generic probe and the capture probes were bound to streptavidin-coated magnetic beads. Competitive amplification of WT-RNA and Q-RNA molecules in one reaction using the same primers results in quantification of the target species. WT-RNA and Q-RNA signal is provided by Electrochemiluminescence for each sample and the ratio between these two signals is directly correlated to the number of pathogens in a sample. Due to the competition for the same amplification primers, generation of low signal for WT-RNA and a high signal for Q-RNA determine low pathogen concentration in the sample, whereas a high pathogen concentration generates a high signal for WT-RNA and a low signal for Q-RNA. This method is used as diagnostic tool for malaria, tuberculosis etc.

2. REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

Advancement in diagnostic tools changes the routine microbial diagnosis significantly over the last two decades. Sensitive detection of microbes before the implementation of the polymerase chain reaction (PCR) methods was difficult to diagnose by conventional diagnostic protocols such as microscopy, culture and serology. Conventional PCR is now rapidly replaced by an improved version in many diagnostic laboratories. RT-PCR is the modified version of conventional PCR. In real-time PCR, the quantification of the target sequence concentration can be achieved through the use of various fluorescent dies/ probes, such as Sybergreen, Taqman probes, fluorescence resonance energy transfer (FRET), and Scorpion primers[4]. The concentration is measured through comparison with standard curves. This eliminates the gel electrophoresis step needed to visualize the amplicons which greatly reduces the risk of contamination and the introduction of false-positive results. RT-PCR can be used to obtain high-throughput analysis of different sequences in one single-closed tube reaction when multiplexed[19]. By using multiplexed RT-PCR, Shokoples et al.[20] were able to detect four human *Plasmodium* species (*P*. *falciparum*, *vivax*, *malariae*, and *ovale*) in a single reaction tube with a very low parasitized sample. Disease like measles, mumps, dengue, chikungunya, influenza, hepatitis, AIDS, malaria, tuberculosis etc. are diagnosed by using RT-PCR. For research purposes, Real-time PCR shown to be a valuable tool in routine clinical laboratories by the implementation of numerous applications of this powerful method.

3. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Loop-mediated isothermal amplification (LAMP) is a unique amplification method, and is able to discriminate between a single nucleotide difference[3]. LAMP is highly specific and sensitive. The working of LAMP depends on the use of six different primers specifically designed to recognize eight distinct regions on a target gene, amplification occurs only when, if all primers bind and form a product[21]. Recently, parasitologists have developed a LAMP approach for the diagnosis of several parasitic diseases including the human parasites *Entamoeba*[22], *Plasmodium*[23] etc. Han et al.[24] used a LAMP assay based on the 18S rRNA gene for the detection of the four human *Plasmodium* species. LAMP assay is more sensitivity as nested PCR but have a greater specificity than nested PCR. Both yield similar results but LAMP results can be obtained in a faster time because LAMP is carried out at a constant temperature (usually in the range of 60–65°C). This unique feature of LAMP results in higher yields and also eliminates the requirement to buy a thermal cycler. This shortens the reaction time by eliminating the time required during thermal changes. Their results are compared with other methods and found consistent with these studies and demonstrated that by using the LAMP assay, an improved specificity and sensitivity with rapidity can be obtained. LAMP is used to detect the sample infected with

pathogens causing malaria, amebic dysentery etc.

4. LUMINEX XMAP TECHNOLOGY

Luminex is a bead-based xMAP (multianalyte profiling) technology, a combination of system that involves flow cytometry, fluorescent beads (microspheres), lasers and digital signal processing. This technology has the capability of measuring different analytes simultaneously in a single sample[25]. This assay enables the detection of specific analytes by capturing from a given sample. The antigens, antibodies or oligonucleotides are used in the assay as probes and these probes can be covalently bound with the microsphere beads. Up to 100 microspheres are available each emitting unique fluorescent signals after the excitation with laser therefore allowing the identification of different targets in the sample[26]. Over the years, Luminex platform is used to develop several DNA tests that have been used for the identification and genotyping of bacteria, viruses and fungi such as *Escherichia coli*[27], *Mycobacterium*[28], *Salmonella*[27] and *Candida*[29] spp. Currently, Luminex technology is used to identify multiple organisms or different genotypes of one particular organism in the same reaction by using a very low volume. This approach is useful in the study of antigenic diversity and the diagnosis of parasitic diseases[28]. Bacterial, fungal and protozoal diseases are mainly diagnosed by Luminex xMAP technology.

5. MICROARRAYS

The microarrays technique's working generally based on the Southern hybridization. Nucleic acid hybridization between labeled targets in the test sample and probes on the array enables the detection of multiple gene targets in a single experiment[6]. The microarrays offered possibility in miniaturizing many different pathogen specific probes on a single support which enhances their sensitivity and specificity. In the future, the opportunity provided by microarrays making them the technique of choice for pathogen diagnosis. Microarrays are used to detect influenza, malaria, tuberculosis, trachoma pathogens.

6. METAGENOMICS

Two major developments in Metagenomics and associated meta-strategies make them forefront of biology- 1) enhanced capability of sequencing large meta-datasets in many centers are provided greatly by the deployment of next generation DNA sequencing technologies. The main idea behind this technology is to provided new opportunities for developing sequencing projects at large scale which was a difficult task several years ago and 2) increasing interest towards the importance of complex microbial communities in mammalian biology and human health and disease. The U.S. National Institutes of Health[30] approved human microbiome project (HMP) in May 2007 as one of two major components of Road Map version 1.5. The needs of this project have resulted in generating the interest and focus towards the genome centers for applying parallel DNA sequencing techniques to human biology at larger scale which was not done previously. The entire population of microbes that colonize the human body including the oral cavity, nasopharynx, respiratory tract, gastrointestinal tract, genitourinary tract and human skin are referred as Human microbiome. Different microorganisms including bacteria, viruses and fungi (mostly yeasts) constitute the microbiome.

The discipline of metagenomics[7] is a new and increasingly sophisticated field for the culture-independent genomic analysis of all the micro-organisms. Metagenomics is concerned with the direct isolation of pathogen's DNA, followed by cloning (such as Escherichia coli) of the complete genomes of the entire microbial population[31]. The resulting DNA library is then used for the functional and sequence analysis. Metagenomics is used to obtain a medically meaningful microbial identification, which is important for genus- or species-level classification. Genera and

species are distinguished by the data obtained from sequencing of 16S rRNA at levels of 95% and 97% pair-wise sequence identities, respectively[32]. Metagenomics approaches may be directed at examining microbial composition or the broader issue of tackling phylogenomic diversity of highly complex microbial populations. Sequencing of universal and conserved targets, such as 16S rRNA genes, is the basic approach of Meta-genomics to identify microbes in a complex community. By amplification of selected target regions within 16S rRNA genes, microbes (specifically bacteria and archaea) can be identified by the effective combination of conserved sequence and intervening variable sequences that facilitate genus and species identification. Viral and bacterial diseases are diagnosed by this technique.

7. DNA BIOSENSORS

Over the past twenty year's, the practice of DNA sequence detection has become more emerging field in the microbial disease diagnosis. This is based on the two factors- 1) amount of DNA sequence information from humans and other organisms and 2) the advances in the technology that enable us to develop new tools needed for the monitoring of bio-recognition between bio-receptor and analyte. DNA Biosensor[8] technology gives a rapid, simple, specific, sensitive and economic method for the detection of specific DNA sequence (i.e. pathogen). During the sensing of nucleic acids, transducer surface is immobilized with single-stranded (ss) oligonucleotide probes forming a recognition layer on transducer that binds its complementary (target) DNA sequence to form a hybrid. The hybridization between probe and target DNA is recognized as a measurable signal (light, current, frequency) by the transducer and passed to the processor to provide a readable output. Both bacterial and viral diseases can be diagnosed by DNA biosensors.

ADVANTAGES OF MOLECULAR DIAGNOSTIC TOOLS

Molecular diagnostic tools offer a rapid, specific and more sensitive alternative approach to traditional microscopy, immunoassays and culture techniques. Both viable and non viable pathogens can be detected by DNA based assays. Molecular-biological techniques are ideal for research laboratories. These techniques can detect very low level of pathogen in the sample where conventional methods show low efficiency.

DISADVANTAGES OF MOLECULAR DIAGNOSTIC METHODS

Although providing considerable potential in the field of microbial disease diagnostics, the routine use of molecular diagnostic tools is hindered by a number of indefinable problems. 1) However, these methods provide positive results which helps in little quantitative determination of the infection level but do not indicate that whether the pathogen is replicating or causing disease in the species tested. 2) The molecular diagnosis tests are highly specific but the ability of many viruses to rapidly change its genetic structure can not detect a virus that has altered its genetic profile. 3) In most cases, the detection of pathogen using molecular diagnostic techniques requires an appropriate knowledge of the target sequence.

CONCLUSION

Molecular diagnostic techniques have a significant role in clinical microbiology, although their adoption will never replace conventional methodologies because they are considered as the cornerstone of modern microbiological methods. Inadequacy of the conventional approaches leads to the use of such molecular diagnostic assays and can be implemented in specialized laboratories to enhance laboratory diagnostic efficiency, where the use of such assays will be mainly confined to diagnosis, identification and

genotyping. Use of these methods in bacteriology has occurred at a much slower rate than in clinical virology. The inadequacy of conventional virology has accelerated the adoption of molecular approaches. There is a need to develop more improved diagnostic technologies which should be more rapid, specific, simple and affordable.

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