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# A Brief Review on Molecular Diagnostic Tools: Principles, Application and Limitations

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**Abstract:** Although the diagnosis of diseases is still primarily based on conventional methods (staining, culture, biochemical analysis), a series of advanced molecular diagnostic tools are increasingly introduced and incorporated in the workflow of clinical laboratories worldwide. These tools, such as polymerase chain reaction, northern blotting, restriction length polymorphism, flow cytometry, southern blotting, fluorescent in situ hybridization, agarose gel electrophoresis and pyrosequencing that are based on either the properties of nucleic acids (deoxyribonucleic acid and ribonucleic acid) or proteins of the target agents, have improved the efficacy, accuracy and speed of detection and identification of disease causing agents and characterization of the diversity of pathogens. They are mainly involved in medicine, ribonucleic acid analysis, criminal case determination, origin and evolution of species, deoxyribonucleic acid fingerprinting, forensic analysis, immunophenotyping, deoxyribonucleic acid studies, single nucleotide polymorphism, resequencing, tag sequencing and microbial identification. Therefore, advanced molecular diagnostic tools are new class of diagnostic tests that are used for identification of nucleic acids or proteins to test the status of infectious diseases and genetic disorders. But, they have some limitations like expensiveness, high technical need and sensitivity for even minor changes during procedures. So advanced molecular diagnostic tools have a cutting-edge role in the diagnosis of animal and human diseases and they should be implemented in different diagnostic sectors.

**Key words:** Application · Principle · Advantage · Disadvantage · Molecular tools,

## INTRODUCTION

The basis for effective treatment and cure of a patient is the rapid diagnosis of disease and its causative agent that is founded on the analysis of the clinical symptoms coupled with laboratory tests [1]. However, in the face of current problems in laboratory tests of disease, it is apparent that the continued use of older and slower methods of detection techniques is unacceptable. This is because of challenges with the traditional detective techniques that include difficulty in growing of organisms in prepared media or in cell culture, slow or poor growth kinetics *in vitro*, limited sensitivity and specificity, labor and technical expertise requirements [2]. Because of poor diagnostic outcomes of traditional detective techniques and increasing incidence and mortality of humans and animals, the requirement for strict diagnostic approaches became a very urgent issue. As a result, the rapid development of new methods and techniques in the area of molecular biology has gained new insights into the genetic and structural features of a considerable numbers of pathogens [1]. Improvements in antibiotic and antiviral therapy for specific microorganisms will also require development of increasingly more accurate methods of diagnosis. Advanced molecular diagnostic tools are such accurate methods of disease diagnosis [3]. Advanced molecular diagnostics tools are methods for the measurement of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins, or other metabolites to detect certain genotypes, mutations, or biochemical changes that may be associated with certain states of health or disease [4].

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Many different biological techniques fall under the "advanced molecular diagnostics tools" umbrella, which have improved the efficacy, accuracy and speed of detection and identification of disease causing agents. One of the most common techniques is the polymerase chain reaction (PCR), a method of producing large amounts of specifically defined DNA or RNA fragments that can then be used for multiple purposes, including medical, forensic, genetic engineering, environmental analysis, gene therapy, gene tagging and rescuing DNA from extinct life forms [5]. Another widely used techniques are northern blotting, restriction length polymorphism (RFLP), flow cytometry, southern blotting, fluorescent *in situ* hybridization (FISH), agarose gel electrophoresis and pyrosequencing that are involved in RNA analysis, criminal case determination, DNA fingerprinting, forensic analysis, immunophenotyping, DNA studies, single nucleotide polymorphism, resequencing, tag sequencing and microbial identification. However, they have some limitations like expensiveness and high technical need [6]. Therefore, the objective of this paper is to review the principles, applications, advantages and disadvantages of molecular diagnostic tools.

**Polymerase Chain Reaction (PCR):** Polymerase Chain Reaction is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It was developed by Kary Mullis in 1983 [7]. A basic PCR set up requires several components and reagents. These components include: PCR machine, for changing of the temperature; DNA template that contains the DNA region (target) to be amplified; two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target; dNTPs, the building-blocks from which the DNA polymerase synthesizes a new DNA strand; buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase; and *Taq* polymerase or another DNA polymerase with a temperature optimum at around 70°C. *Taq* polymerase, a heat-stable DNA polymerase, is an enzyme originally isolated from the bacterium *Thermusaquaticus*. It is employed in almost all PCR applications [8, 9].

The vast majority of PCR methods use thermal cycling, alternately heating and cooling the PCR sample to a defined series of temperature steps [9]. Typically, a PCR reaction consists of 30 cycles containing initialization, denaturation, annealing and extension/elongation steps [10]. Initialization step consists of heating the reaction to a temperature of 94–96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1–9 min [11]. Denaturation step is the first regular cycling event and consists of heating the reaction to 94–98°C for 20–30 sec. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules [12]. Annealing step requires cooling (to about 50°C) of the reaction mixture to allow primers to select and bind (hybridize) to their complementary positions on the single-stranded DNA template molecules [9]. Extension/elongation step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 75–80°C and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' (five prime) to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand [13].

**Types of Polymerase Chain Reaction:** Polymerase chain reaction is an important tool for use in clinical and diagnostic medicine and research and there is more than just one kind, all with different applications and levels of sensitivity [6].

**Reverse Transcription Polymerase Chain Reaction (RT-PCR):** Reverse transcription polymerase chain reaction is one of many variants of PCR that allows genes to be amplified and cloned as intron free DNA copies by starting with messenger RNA (mRNA) and using reverse transcriptase [9]. This technique is commonly used in molecular biology to detect RNA expression levels. It is often confused with real-time polymerase chain reaction (qPCR), but, they are separate and distinct techniques. While RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. It is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement [14].

**Real-Time Polymerase Chain Reaction (qPCR):** Real-time Polymerase Chain Reaction is the ability to monitor the progress of the PCR as it occurs [9]. In molecular biology,

real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction or kinetic polymerase chain reaction is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, it enables both detection and quantification [15].

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: non-specific fluorescent dyes that intercalate with any double-stranded DNA and sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target [16].

**Multiplex Polymerase Chain Reaction (Multiplex PCR):** Multiplex polymerase chain reaction is defined as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture. It has been applied in many areas of DNA testing including the analysis of deletions, mutations and short tandem repeats (STRs). It is a modification of polymerase chain reaction in order to rapidly detect deletions or duplications in a large gene. This process amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler [17]. Multiplex polymerase chain reaction consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences [18].

**Conventional PCR:** Conventional Polymerase Chain Reaction uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is typically high with a minimum number of 100 to 1000 copies of the target DNA detectable. Analytical specificity can be high, depending on target selection, primer design and assay optimization. Detection methods, such as Southern blotting followed by hybridization probes, can further improve sensitivity and specificity, but are time consuming; require laboratory handling of amplified DNA and interpretation of results [6].

**Applications of PCR:** The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple [9]. It has found widespread application in many areas such as medicine [9], forensic, genetic engineering, environmental analysis, gene therapy, gene tagging and rescuing DNA from extinct life forms [12, 19].

**Medical Application of PCR:** Polymerase chain reaction-based tests have allowed detection of small numbers of disease organisms, in convenient samples. Characterization and detection of infectious disease organisms have been revolutionized by PCR. For example, some disease organisms, such as that for tuberculosis, are difficult to sample from patients and slow to be grown in the laboratory [20].

**Forensic Application of PCR:** DNA fingerprinting is used forensic medicine for identification of criminals and disputed parentages. In its most discriminating form, genetic fingerprinting can uniquely discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene and compared to that from suspects, or from a DNA database of earlier evidence or convicts [19].

**PCR in Environmental Analysis:** It is possible to extract DNA directly from environmental sample, such as soil or water, without bothering to isolate culture living organisms that contain it first [20].

**PCR in Gene Tagging:** Polymerase chain reaction has utilized for developing molecular markers closely linked to specific genes of economic importance. For instance, in tomato, 144 random primers were used to produce 625 PCR products from a set near isogenic lines [12].

**PCR in Gene Therapy:** Polymerase chain reaction proves to be of immense help in monitoring a gene therapy experiments. For example, adenosine deaminase deficiency (ADA) gene was transferred for therapeutic purposes and PCR was used to detect the presence of this gene [19].

**PCR in Rescuing DNA from Extinct Life Forms:** Since any small trace of DNA can be amplified by PCR and cloned or sequenced, some scientists have looked for DNA in fossils [9].

**Advantages of PCR:** Because of its simplicity, PCR is a popular technique with a wide range of applications that depend on essentially three major advantages, which are speed and ease of use, sensitivity and robustness [10, 21].

**Speed and Ease of Use:** Deoxyribonucleic acid cloning by PCR can be performed in a few hours, using relatively unsophisticated equipment. Typically, a PCR reaction consists of 30 cycles containing a denaturation, annealing and extension steps, with an individual cycle typically taking 3–5 min in an automated thermal cycler [10].

**Sensitivity:** Polymerase chain reaction is capable of amplifying sequences from minute amounts of target DNA, even the DNA from a single cell [9].

**Robustness:** Polymerase chain reaction can permit amplification of specific sequences from material in which the DNA is badly degraded or embedded in a medium from which conventional DNA isolation is problematic. As a result, it is again very suitable for molecular anthropology and paleontology studies. It has also been used successfully to amplify DNA from formalin-fixed tissue samples, which has important applications in molecular pathology and genetic linkage studies [21].

**Disadvantages of PCR:** Although PCR has many applications, it has certain limitations [22].

**Need for Target DNA Sequence:** In order to construct specific oligonucleotide primers that permit selective amplification of a particular DNA sequence, some prior sequence information is necessary. This normally means that the DNA region of interest has been partly characterized previously, often following cell-based DNA cloning [23].

**Size and Limiting Amounts of PCR:** Although small segments of DNA can usually be amplified easily by PCR, it becomes increasingly more difficult to obtain efficient amplification as the desired product length increases [9].

**Infidelity of DNA Replication:** Unlike *in vivo*, DNA replication *in vitro* has low fidelity because of proofreading mechanisms. *Taq* DNA polymerase has no associated 3' ? 5' exonuclease to confer a proofreading function and the error rate due to base misincorporation during DNA replication is rather high. This means that, even if the PCR reaction involves amplification of a single DNA sequence, the final product will be a mixture of extremely similar, but not identical DNA sequences [22].

**Northern Blotting:** The northern blot is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample [23], or it is defined as a blotting technique that uses RNA as the target molecule and DNA as a sample [9]. It was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University [24]. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern [23]. So, the technique is called "Northern" simply because it is similar to "Southern", not because it is invented by a person named "Northern" [25]. Northern blot requires gels, especially agarose gel to highlight the 28S ribosomal subunits and probes that are nucleic acids [26]. Generally this technique involves the isolation of RNA molecules from sources of interest and the separation of the isolated RNA by agarose gel electrophersis. The separated RNA in the gel is then transferred to a solid matrix. Then this separated RNA is treated with labelled probe which specifically hybridizes or binds to complementary RNA sequence on the matrix. Unbounded probes are washed off the matrix. The labelled robe, now bound to complementary RNA sequence on the matrix are then read using a suitable detector [25, 12].

**Applications of Northern Blotting:** Northern blotting is not only used in the study and analysis of RNA and gene expression from cells, but also used Ribonuclease protection assay, the RT-PCR, the DNA microarray and in molecular biology research. But it is superseded in most areas by real time PCR and microarray approaches [25].

**Advantages of Northern Blotting:** Advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, measuring of the quality and quantity of RNA on the gel prior to blotting [27]. It is a sensitive and specific technique, which is a choice in the characterization of mRNA expression since it allows visualization of intact mRNA [25].

**Disadvantages of Northern Blotting:** A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through

environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as diethylpyrocarbonate [12]. It measures the accumulation of RNA transcripts, but explains nothing about why the observed accumulation occurs [19]. The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material, ethidium bromide and UV light are all harmful under certain exposures [28].

**Restriction Fragment Polymorphism (RFLP):** Restriction fragment length polymorphism is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. It was developed in 1985 by Sir Alec Jeffreys [27]. Usually there are two types of RFLP analysis: Multi locus polymorphism and Single locus polymorphism. Single locus polymorphism method is more sensitive, easy to interpret and can analyse mixed DNA samples [27]. When performing RFLP, the target DNA is usually subjected to polymerase chain reaction, which produces millions of copies of strands of DNA similar to the original. This amplified DNA is then combined with a set of restriction enzymes, which cleave the DNA in specific locations [9]. After exposure to the restriction enzymes, the two mixtures are transferred to a gel electrophoresis. In gel electrophoresis an electrical current is transmitted through the gel causing the fragments of DNA to migrate through the gel according to their electrophoretic mobility. This distance is roughly proportional to the inverse of the fragment's length. As a result, shorter fragments migrate farther from the origin as they move through the gel [19]. After the gel is run, the DNA is labelled using a radioactive probe and the gel is exposed to x-ray film, which changes colour in the presence of radioactivity. The locations of the fragments of DNA show up on the film as bands. Different samples can be loaded onto the gel in different lanes so that the banding patterns can be compared side-by-side [12, 29].

**Applications of (RFLP):** The applications of RFLP are many. DNA fingerprinting uses the presence of short tandem repeats(STRs) at thirteen different locations on the chromosomes. The lengths of these STRs are detected using RFLP analysis [27]. Analysis of RFLP variation in genomes was vital tool in genome mapping and genetic diseases, such as cystic fibrosis and sickle-cell anaemia [30]. Genetic diversity, genetic relationships, history of domestication, origin and evolution of species, whole genome and comparative mapping, forensic analysis, gene tagging, unlocking valuable genes from wild species, construction of exotic libraries, paternity or criminal cases determination, determination of the disease status of an individual and measuring of recombination rates are analysed by RFLP [19, 27].

**Advantages of (RFLP):** Restriction length polymorphism is highly robust methodology with good transferability between laboratories. It is not based on sequence information and it is well suited for constructing genetic linkage maps [27].

**Disadvantages of (RFLP):** Restriction length polymorphism requires high amounts of DNA, suitable probe library, distribution of probes to collaborating laboratories, different probe/enzyme combinations and low levels of polymorphism in some species. It is costly and time consuming [30].

**Flow Cytometry:** Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. The first fluorescence-based flow cytometry device was developed in 1968 by Wolfgang Gohde [31]. It is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles. The properties measured include a particle's relative size, relative granularity or internal complexity and relative fluorescence intensity [32]. Flow cytometry is made up of three main systems: fluid, optics and electronics. The fluid system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream.The electronics system converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles [31].

**Application of Flow Cytometry:** The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and medicine (especially in transplantation, haematology, tumour immunology and chemotherapy, prenatal

diagnosis, genetics and sperm sorting for sex preselection [33]. It is adaptive to studying many types of biological questions. These can be broken up into several main categories, as immunophenotyping (to determine cell linage, activation status, ability to respond to stimuli and to interact with other cells), functional Assays (to measure intracellular cytokines, phosphorylation, phagocytosis, cellular proliferation and reactive oxygen species) and DNA studies that involves fluorescence associated DNA to determine stages of cell cycle, apoptosis and chromosomal aberrations [32].

**Advantage of Flow Cytometry:** Flow cytometry is used to study heterogeneous populations of cells and to analyse the subpopulations in a few minutes. It has the benefit of highlighting any non-uniform cells [8].

**Disadvantages of Flow Cytometry:** Flow cytometry is more expensive than alternatives such as radioimmunoassay and enzyme-linked immunosorbent assay. And, its sorters are very accurate and purify small or complex subpopulations. But, even a high-speed sorter is not fast enough to achieve the desired results. For instance, a pair of cells is often discarded because the sorter cannot distinguish between cells in time [32].

**Fluorescence *in situ* Hybridization (FISH):** Fluorescence *in situ* hybridization is a cytogenetic technique developed by biomedical researchers in the early 1980s that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complement [9]. Short sequences of single-stranded nucleic acids (such as DNA), called “gene probes,” are designed to match a portion of a gene or metabolic product of the organism or population of interest. A fluorescent dye is attached to the probe so that when the probe binds to target sequences within a cell, it emits fluorescent light that can be observed through a microscope or sorted with flow cytometry. Cells emitting a fluorescent light are called hybridized cells. In flow cytometry, labelled cells are diluted or concentrated depending on the initial cell concentration in the sample so that individual cells pass through a laser beam that detects and counts fluorescently labelled cells. Under ideal conditions, only cells that contain the target gene are recognized by the probe and become fluorescently labelled [34].

**Applications of Fluorescence in situ Hybridization:** Fluorescence *in situ* hybridization has a large number of applications in molecular biology and medical science, including gene mapping, diagnosis of chromosomal abnormalities and studies of cellular structure and function [27]. Fluorescence *in situ* hybridization is often used in clinical studies. Many bacteria do not grow well under laboratory conditions, but FISH can detect directly the presence of the suspect on small samples of patient's tissue. It is also widely used in the field of microbial ecology, to identify microorganisms-species identification. It can also be used to compare the genomes of two biological species, to deduce evolutionary relationships [32]. Virtual karyotyping is another cost-effective, clinically available alternative to FISH panels using thousands to millions of probes on a single array to detect copy number changes [27].

**Advantages of Fluorescence in situ Hybridization:** Fluorescence *in situ* hybridization does not require cultivation of the organisms or any technology-based gene amplification [35]. In contrast to some other electro-Motive Diesels (EMDs), it allows visualization of whole cells that are important to environmental remediation activities[9]. Fluorescence *in situ* hybridization can target several different genes simultaneously, for example, genes associated with specific degrading species of interest and broader microbial groups, such as methane-producing organisms [35].

**Disadvantages of Fluorescence in situ Hybridization:** The FISH method is currently expensive because of the expertise and labor needed for development of validated FISH protocols and direct microscopic counting [20].

**Southern Blotting:** Southern blotting is a technique for transfer of DNA molecules from an electrophoresis gel to a nitrocellulose or nylon membrane and is carried out prior to detection of specific molecules by hybridization probing [36]. It was named after Edward Southern who developed this procedure at Edinburgh University in the 1970s [27].

The goal of the southern technique is to obtain a replica of the gel that retains the original positions of DNA fragments. This is accomplished by transferring, or blotting, the DNA from the gel onto a pieces of transfer medium [37]. Procedures of southern blotting start with cutting of high-molecular-weight DNA strands into smaller fragments by restriction endonucleases.

The DNA fragments are then electrophoresed on an agarose gel to separate them by size. If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel is treated with a base [29]. A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below) the gel [12]. The membrane is then baked in a vacuum or regular oven at 80°C for 2 h or exposed to ultraviolet radiation to permanently attach the transferred DNA to the membrane and exposed to a hybridization probe. After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used [30, 38].

**Applications of Southern Blotting:** The commonest applications of Southern blotting include detection of mutations, DNA fingerprinting and cloning [27]. The method of southern blotting along with RFLP is used to map the genes of an individual. Both of these techniques together map the chromosomes and detect a particular mutation in the genome [8].

**It is also useful in process of fingerprinting:** The evidence which collected from the crime scene is observed with this technique [27]. In regards to genetically modified organisms, southern blotting is used as a definitive test to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism. It is also used for screening the absence or presence of specific sequences, assessing complexity of integration or transgene rearrangements, estimating copy numbers, estimating whether transgenes are inserted at a single or multiple loci and for fingerprinting identity of independent events [39].

Advantages of Southern Blotting: Alternative techniques such as the PCR have superseded blotting in many applications that require the sensitive detection or quantitation of nucleic acid sequences in complex mixtures. Despite this, Southern blotting still has its advantages, for example, in determining the position of sequences in large fragments, detecting chromosomal rearrangements, picking up related sequences with lower homology and determining the number of gene copies [36].

**Disadvantages of Southern Blotting:** Southern blotting is a time consuming, costly, technically demanding and complicated process. It cannot analyse more than one gene and, therefore, detects presence of targets but not interactions or regulations of targets [12].

**Agarose Gel Electrophoresis:** Gel electrophoresis is a method that separates macromolecules on the basis of size, electric charge and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field [37].

Agarose gel electrophoresis is a method used in biochemistry and molecular biology for separation of nucleic acids or protein molecules by passing an electric current through a gel made of agarose. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field [9].

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA [40]. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole [37]. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones. In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired band from a stained gel viewed with a UV transilluminator [41].

**Applications of Agarose Gel Electrophoresis:** Agarose gel electrophoresis is a widely used procedure in various areas of biotechnology. It is used in biology, medicine, forensic and biochemistry by research scientists to distinguish molecules, specifically nucleic acids like DNA or RNA and proteins, of different sizes [42]. It has also applications in estimation of the size of DNA molecules following restriction enzyme digestion, analysis of PCR products and separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer [8].

**Advantage of Agarose Gel Electrophoresis:** Since agarose melts easily in a standard microwave, it is easy to prepare or pour as a gel. Agarose electrophoresis is a visual method of confirming the presence of DNA, RNA, proteins, or a specific size(s) of these, unlike other detection methods, which give the precise quantities but

not their size. It can give very good resolution and separation of large from small molecules as the agarose gel's pore size can be specified by the user and only requires small amounts of sample. The process is fast (minimum of 30 min) and the apparatus is easy to set up and operate [41].

**Disadvantages of Agarose Gel Electrophoresis:** The disadvantages of agarose gel electrophoresis are that gels can melt during electrophoresis, the buffer can become exhausted and different forms of genetic material may run in unpredictable forms [40].

**Pyrosequencing:** Pyrosequencing is a DNA sequencing technology based on the sequencing by synthesis. It a mini-sequencing method that can be automated, that is used for short regions in multiple individuals rather than for sequencing long regions of unexplored DNA [9]. A general procedure of pyrosequencing starts with hybridization of a single PCR amplicon that serves as a template and incubated with the enzymes: DNA polymerase, Adenosine triphosphate (ATP), luciferase, as well as substrates, adenosine 5' phosphosulfate (APS) and luciferin. Then dNTP is added to the reaction in which DNA polymerase catalyses the incorporation into DNA strand, if it is complementary to the base in the template strand [43]. Adenosine triphosphate sulfurylase converts inorganic pyrophosphate (PPi) to ATP in the presence of APS. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. Apyrase, a nucleotype-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is sequentially added [44].It should be noted that deoxyadenosine alfa-thio triphosphate is used as a substitute for the natural dATP since it is efficiently used by the DNA polymerase, but not recognized by the luciferase [9].

**Applications of Pyrosequencing:** Genotyping of Single-Nucleotide Polymorphisms: For analysis of single-nucleotide polymorphisms by pyrosequencing, the 3'-end of a primer is designed to hybridize one or a few bases before the polymorphic position. In a single tube, all the different variations can be determined as the region is sequenced. Furthermore, pyrosequencing enables determination of the phase of SNPs when they are in the vicinity of each other allowing the detection of haplotypes [14].

**Resequencing:** Because pyrosequencing generates an accurate quantification of the mutated nucleotides, the resequencing of PCR-amplified disease associated genes for mutation scanning will be one of the interesting applications. It is currently the fastest method for sequencing a PCR product [44].

**Tag Sequencing:** The sequence order of nucleotides determines the nature of the DNA. Theoretically, eight or nine nucleotides in a row should define a unique sequence for every gene in the human genome [43].

**Analysis of Different Secondary Structures:** Hairpin structures are common features in genomic materials and have been proposed to have regulatory functions in gene transcription and replication. However, analysing these sequences by conventional DNA sequencing usually gives rise to DNA sequence ambiguities seen as run-off or compressions. Pyrosequencing was successfully applied to decipher the sequence of such regions [14].

**Fungal Identification:** Pyrosequencing appears to be a good diagnostic tool for detection and identification of fungal pathogens. It can be utilized in typing of fungi clinically isolated from immune-compromised patients suffering from proven invasive fungal infections. For typing, the DNA has to be amplified by general consensus primers having complementary to a highly conserved region within the 18S rRNA gene allowing the amplification of a broad range of fungal species [45].

**Bacterial Identification:** A DNA primer complementary to the conserved or semiconserved region is usually employed to sequence the variable region. In bacteria, 16s rRNA gene is commonly used to identify different species and strains. By analysing a sequence between 20 and 100 nucleotides on 16S rRNA gene, it is possible to group different bacteria taxonomically and, in many cases to get information about strains [43].

**Advantages of Pyrosequencing:** Pyrosequencing has the potential advantages of accuracy, flexibility, parallel processing and can be easily automated. It is a fast method with real-time read-out that is highly suitable for sequencing short stretches of DNA.

Furthermore, its reagent costs are considerably lower for sequencing short stretches of DNA compared to current available methods [44].

**Disadvantages of Pyrosequencing:** An inherent problem with Pyrosequencing is de novo sequencing of polymorphic regions in heterozygous DNA material. In most cases, it will be possible to detect the polymorphism. However, if the polymorphism is a deletion or insertion of another type, the sequencing reaction can become out of phase, making the interpretation of the subsequent sequence difficult [45].

Another limitation of pyrosequencing are expensiveness, high error rate and the difficulty in determining the number of incorporated nucleotides in homopolymeric regions [14].

## CONCLUSION AND RECOMMENDATIONS

The recent advent of molecular biology has revolutionized the diagnosis of diseases by offering new methods for quicker and more accurate detection, identification and quantification. This rapid advancement in molecular biology has led to the development of a number of molecular diagnostic tools, such as PCR, northern blotting, RFLP, flow cytometry, southern blotting, FISH, agarose gel electrophoresis, and pyrosequencing, which have improved the efficacy, accuracy, and speed of detection and identification of disease causing agents and characterization of the diversity of pathogens. These tools are applied in many areas of applications including RNA analysis, DNA fingerprinting, forensic analysis, immunophenotyping, DNA studies, single nucleotide polymorphism, resequencing, tag sequencing, and microbial identification. Molecular-based diagnostic tools play an increasingly important role in healthcare by improving the opportunities to arrive at early diagnosis and to use appropriate treatment regimen. Therefore, they are developed to overcome the limitations of conventional methods of disease diagnosis that have low sensitivity and specificity. In some cases, it is difficult to grow organisms with prepared media or in cell culture, hence, molecular diagnostic tools are needed. For example, some bacteria do not grow well under laboratory conditions, but FISH can detect directly the presence of the suspect on small samples of patient's tissue. But, accurate procedures are needed for these new technologies as they are sensitive to even a minor change in each respective step of examination.

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