**Molecular Diagnostics in Microbiology**

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**Abstract:** By developing quicker and more precise methods for diagnosing infectious diseases, molecular and genomic approaches have completely transformed microbiology. Traditional techniques, such as biochemical testing and microbe culture, take much time and could miss strains resistant to antibiotics. On the other hand, molecular and genomic approaches, such as whole-genome sequencing and polymerase chain reaction (PCR)-based procedures offer quick and accurate identification of pathogens, early-stage illnesses, and strains of bacteria that are resistant to bacteria. The potential for targeted medicines, high sensitivity and specificity, and personalized medicine are benefits of these methods. "However, there are obstacles to using molecular and genomic approaches because of their cost, equipment, knowledge, and data analysis. Concerns about the use of genetic data and patient privacy also arise from an ethical and legal standpoint. The extensive use of molecular and genomic techniques, however, is crucial for the future of microbiology since it can help identify types of bacteria that are resistant to antibiotics and improve patient outcomes. The full potential of molecular and genomic approaches in microbiology requires further development, teaching, and investigation of ethical aspects.

**Keywords:** Molecular technique, genomic methods, PCR, microbiology, antibiotic-resistant, data analysis

1. **Introduction**: The leading cause of death and morbidity in the globe is infectious disease. The number of infections brought on by diverse pathogens, such as bacteria, viruses, parasites, and fungi, is always rising. Contributing variables include aging and illnesses including cancer and immunosuppression. Antibiotic, antiviral, antimycotic, and antiparasitic drugs have helped reduce these infectious agents' prevalence. Still, their widespread use has also resulted in the emergence of multi-drug resistant (MDR) pathogens that, if not identified and controlled in time, could spread over wide geographic areas. In terms of diagnosis, standard clinical and microbiological techniques including cultures, serology, and microscopy continue to be the preferred methods and are also reasonably priced.

The standard microbiological cultures, however, are not diagnostic procedures that can reliably identify the infection on their own. Since diagnostic science is the foundation of medical judgment, it is vital to the healthcare system. Diagnostic tests give important information that helps choose the right antibiotics to treat infections, informs personalized treatment plans for various diseases, and provides valuable information on many facets of healthcare, including illness management, prevention, detection, diagnosis, and treatment (1). Molecular diagnostics, immunology, hematology, microbiology, and clinical chemistry are some of the main diagnostic subfields. Due to its ability to provide thorough insights into both diagnosis and treatment approaches, molecular diagnostics has garnered a lot of attention lately. Significant changes have occurred in this sector, revolutionizing healthcare by thoroughly comprehending different illness states (2).

Despite cultivable bacteria, many patients with symptoms and indicators typical of infectious diseases do not exhibit an organism in cultures. Molecular biology techniques are widely used in diagnostic clinical microbiology laboratories for the above reasons. Clinical microbiology laboratories employ various methodologies to assist clinical decision-making about infection diagnosis, treatment, and prognosis. Clinical samples taken from cases are used in the gold standard to detect the pathogen directly. Several techniques are employed, including culture, electron microscopy, and polymerase chain reaction (PCR) for pathogen genome detection. Molecular methods are now used more and more in clinical microbiology diagnoses.

Molecular biology is the branch of biology that studies how genes are translated into RNA, how those RNAs are translated into proteins, and how those proteins affect cellular function. The main characteristics impacting the clinical application of molecular tests will be the subject of this chapter. There are descriptions of the most popular methods. DNA, RNA, and proteins are among the molecular components of cells that may be identified, isolated, and altered thanks to techniques developed by molecular biologists. Our knowledge of the molecular mechanisms behind both normal and abnormal speech and swallowing has improved due to recent developments in basic research, genomics, and proteomics.

Numerous infectious diseases are being diagnosed using molecular and genomic methods [3]. Diagnostic techniques based on polymerase chain reaction are frequently employed to identify viral infections, including influenza, hepatitis, and the human immunodeficiency virus (HIV) [4]. The diagnosis of bacterial infections including gonorrhoea, chlamydia, and tuberculosis can also be done with these techniques [5]. Whole-genome sequencing is another technique that is being used in medical microbiology at a rapid pace. This method involves sequencing all of the DNA present in a microbe sample, allowing for a comprehensive analysis of each bacterium [6]. Whole-genome sequencing can help track the spread of infectious diseases and identify their source when outbreaks happen [7].

Given that the number of basic science papers about voice alone has increased by almost 80% in the last seven years (Benninger, in press), it is more important than ever to comprehend fundamental biological processes to grasp the more specialized literature. The majority of molecular techniques used to identify bacteria rely on some form of DNA analysis, whether it be amplification or sequencing. Simple DNA amplification techniques (PCR, real-time PCR, RAPDPCR) and more intricate procedures based on restriction fragment analysis, targeted gene and whole-genome sequencing, and mass spectrometry are among these techniques. Methods based on distinct protein signatures, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) have also been investigated.

1. **Polymerase Chain Reaction (PCR**): Kary Mullis created PCR in the 1980s and was awarded the Nobel Prize in 1994 (8). Since its description, this method has brought about a true revolution in biological research, bringing basic biological processes into accord in practical fields such as plant and animal genetic enhancements and diagnosis (9). A laboratory technique called polymerase chain reaction, or PCR for short, may quickly create (amplify) millions to billions of copies of a particular DNA sequence so that it can be examined in more detail. Source DNA from a range of tissues and organisms, such as peripheral blood, skin, hair, saliva, and microorganisms, can be used for PCR. For PCR to produce enough copies of DNA for analysis using standard laboratory techniques, just trace amounts of DNA are required. PCR is a sensitive assay because of this.

DNA polymerase, nucleotides, primers, and template DNA are necessary for every PCR assay. The essential enzyme that joins separate nucleotides to create the PCR result is DNA polymerase. The four bases that make up DNA are adenine, thymine, cytosine, and guanine (A, T, C, and G). These are known as nucleotides. These serve as the building pieces that the DNA polymerase uses to produce the final PCR product. The precise DNA product that needs to be amplified is specified by the primers in the process. Short DNA pieces called primers have a specific sequence that complements the target DNA that needs to be found and amplified. These provide the DNA polymerase with an extension point upon which to build. After combining the aforementioned ingredients in a test tube or 96-well plate, they are put in a machine that enables three simple cycles of DNA amplification. In essence, it is a thermal cycler machine. It has a holed heat block that the test tubes or plates containing the PCR reaction mixture are placed into. In distinct, accurate, and pre-programmed steps, the machine raises and lowers the block's temperature (10).

Denaturation, the process by which the two complementary DNA strands of the target DNA separate, is achieved by first heating the reaction solution over their melting points. Then, the temperature is decreased to enable hybridization, sometimes referred to as annealing, in which the particular primers attach to the target DNA segments. Only when primers and target DNA have complementary sequences—for example, A binding to G—do they anneal. The primers can be extended by the DNA polymerase by adding nucleotides to the growing DNA strand when the temperature is increased once more. The quantity of replicated DNA molecules doubles with each iteration of these three procedures.

**PCR components:** Several parts and reagents are needed for a basic PCR setup (11), including:

1. **DNA template:** The sample DNA that has the chosen nucleic acid sequence that requires amplification is known as template DNA. Only DNA plasmid DNA, complementary DNA (cDNA), and genomic DNA (gDNA) can be used as the template. RNAs are used as starting materials for the reverse transcriptase polymerase chain reaction (RT-PCR), however before amplification, they are mostly transformed into complementary DNA (cDNA). It is required that the template DNA have an absorbance ratio of approximately 1.8 and be extremely pure. 30 μg to 50 μg is the optimal amount, however, 0.1 to 200 μg can be utilized.
2. **DNA polymerase:** Taq DNA polymerase, the enzyme extracted from the bacterium Thermus aquaticus, is the most widely used and well-known DNA polymerase used in PCR since its establishment. Taq DNA polymerase is thermally stable and continues to be active after the repeated heating and cooling cycle. It is stable up to 95°C and exhibits the most effective reaction at around 72°C to 78°C incorporating about 60 bases per second. Simply put, DNA polymerases are enzymes that catalyze the synthesis of complementary DNA strands by the sequential assembly of nucleotides by the template strand. For amplification, one to two units of *Taq polymerase* in a 50 L reaction mixture is enough. There are now two other thermostable DNA polymerase enzymes available: *the Pfu enzyme* from *Pyrococcus furiosus* and the *Vent enzyme* from *Thermococcus litoralis*.
3. **Primers:** Short single-stranded oligonucleotide sequences that are complementary to the target nucleic acid sequence in the template DNA are called primers, and they are produced artificially. These are brief sequences that serve as the building blocks for DNA synthesis and range in length from 15 to 30 bases. They anneal in a single-stranded template DNA strand at their complementary positions. This primer's 3' OH-end is subsequently extended by the DNA polymerase enzyme to create a new complementary strand.For a PCR reaction, 10–12 pMol of each primer is usually enough. There are two varieties of PCR primers: forward and reverse primers. The amplification of the antisense strand is caused by the forward primers, which are complementary to the antisense strand (the template strand from 3' to 5' direction). They are also known as 5' primers. Amplification of the sense strand is caused by the reverse primers, which are complementary to the sense strand (the template strand from 5' to 3' direction). They are also known as 3' primers.
4. **Nucleotides (dNTPs or deoxynucleotide triphosphates):** Artificially produced nucleotides known as deoxynucleotide triphosphates (dNTPs) serve as the building blocks of new DNA strands. Deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and deoxycytidine triphosphate (dCTP) are the four distinct dNTPs that are utilized in the PCR. The DNA polymerase enzyme creates a new DNA strand complementary to the template strand by successively adding these four dNTPs to the annealed primer.
5. **PCR Buffers and Other Chemicals:** A buffer system with a pH of 8.0 to 9.5 that is based on Tris and HCl must be used for the entire procedure. The most widely used buffer system is 10X buffer with extra MgCl2. Dimethyl Sulfoxide (DMSO), ammonium sulphate ((NH4)2SO4), polyethylene glycon (PEG), potassium chloride (KCl), magnesium chloride (MgCl2), tetramethyl ammonium chloride, N, N, N-trimethyl glycine, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), 7-deaza-2′-deoxyguanosine 5'-triphosphate, glycerol, formamide, serum albumin, etc. are common ingredients in PCR buffers. The buffer system stops inhibition and the creation of secondary structures while improving the reaction's efficiency and selectivity.
6. **Thermocycler:** The thermocycler, also called the PCR machine, is essentially an electric heating device that controls the temperature as necessary for each PCR step. This device raises the temperature during the denaturation phase, decreases it during the annealing phase, and then raises it once more during the elongation phase. This cycle of temperature increases and decreases is based on the user's pre-programmed setup or instructions before operation.

**Steps of PCR**: Any kind of PCR involves the three fundamental processes of denaturation, annealing, and elongation. The preparation stage at the start and the PCR product analysis stage at the finish are crucial phases in addition to these three main ones. Three steps can be used to summarise the entire process: product analysis, amplification, and pre-preparation.

**Pre-preparation:** Before the actual polymerase chain reaction occurs within the thermocycler, this is the first stage. To amplify the target DNA or RNA segment, a reaction mixture must be prepared and loaded onto a thermocycler that has been preprogrammed. After being extracted from the sample, the DNA or RNA is kept (pre-extracted nucleic acids might be used). Everything is set up, precautions are taken, the area used to prepare the PCR reaction is cleaned, all the reagents are brought to working temperature, the sample is taken out of storage or extracted, the PCR reaction mixture is made, the thermocycler is programmed, and the reaction mixture is loaded onto the thermocycler.

1. **Amplification:** In PCR, it is the primary reaction process that takes place. Denaturation, annealing, and elongation are all part of the amplification stage, and they happen sequentially and cyclically for a predetermined number of cycles that the user has preprogrammed.
2. **Denaturation:** Thermally denatured double-stranded DNA is converted into two single-stranded DNA templates in the first stage of the amplification procedure. About 30 to 90 seconds are spentraising thetemperature to roughly 94°C (90 to 95°C). At this temperature, the weak hydrogen bonds that hold the two DNA strands together are broken by the thermal energy, causing them to split apart.

**dsDNA →   2 ssDNA templates**

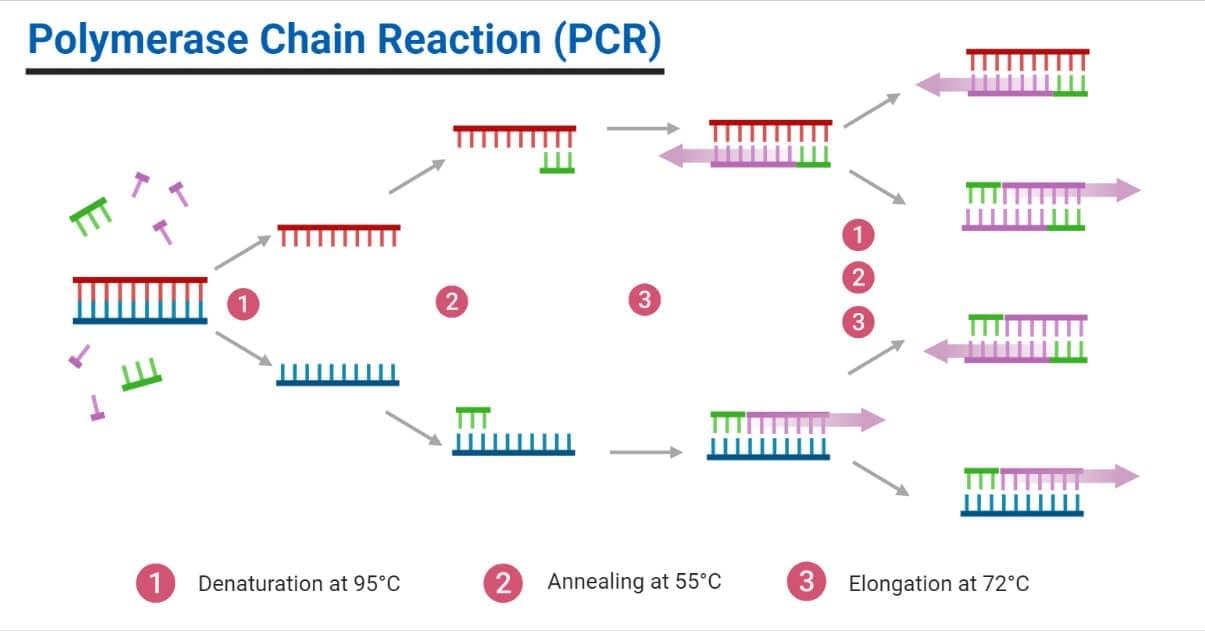
1. **Annealing:** The annealing stage, in which the primer anneals the ssDNA templates at their complementary sites, comes after denaturation. The reverse primer anneals at the complementary site of the sense strand of the template DNA, while the forward primer anneals at the complementary site of the antisense strand. The temperature must be lowered to between 55°C and 70°C for annealing to take place; the annealing temperature varies depending on the primer's GC content. In the majority of PCR procedures, annealing takes only 30 to 60 seconds.

**ssDNA + Forward and reverse primers → ssDNA with annealed primers**

1. **Elongation:** The temperature is increased to 72°C in the last stage of the amplification reaction. This triggers the Taq DNA polymerase enzyme to start creating new DNA strands in the 5' to 3' orientation. The DNA polymerase enzyme transfers nucleotides from the reaction mixture to the annealed primer's 3' OH-end to create a new complementary strand. The length of the sample's nucleic acid sequence and the activity of the DNA polymerase determine how long elongation takes. Typically, elongation occurs at 1 kbps every 0.5-10 minutes. After elongation, a single dsDNA template at the start of the reaction will yield two new dsDNA molecules.

**2 ssDNA with annealed primers + dNTPs → 2 new ds DNAs**

1. **Product Analysis:** This stage occurs after the PCR is finished and involves analyzing the reaction mixture to make sure the intended amplification was accomplished. Agarose gel electrophoresis is typically used to detect amplified DNAs or RNAs. However, with certain PCR types, such as real-time PCR, no further step is necessary.



**Fig 1:** Steps of PCR

**Quantitative PCR:** Beyond just detecting DNA, quantitative real-time, or qRT-PCR, gives information. It shows the amount of a certain gene or DNA that is present in the sample. While the PCR product is being synthesized, qRT-PCR enables real-time detection and quantification.(12). Two popular techniques for identifying and measuring the product are (1) sequence-specific DNA probes made up of fluorescently labeled reports and (2) fluorescent dyes that non-specifically intercalate with double-stranded DNA. These only allow for detection once the probe and its complementary DNA target have hybridized. Reverse transcription, or the conversion of messenger RNA into cDNA, can be used in conjunction with real-time PCR. qPCR is then used to quantify the cDNA (13). End-point PCR, or analysis after the last PCR cycle is finished, has issues that can be avoided by quantifying the desired gene during exponential amplification. Tumor analysis is a perfect example of PCR application. The DNA of proto-oncogenes or tumor suppressor genes can be isolated and amplified using this method. Quantitative PCR can then be used to measure the quantity of the specific gene that was recovered. However, quantitative PCR may measure any combination of proteins, mRNAs, and DNA and analyze individual cells. (14).

**Application of PCR**: PCR is already widely used, and new applications are constantly being developed. The benefits of PCR are numerous. First of all, it is an easy approach to learn and apply, and it yields results quickly (15). A particular product could be produced in millions to billions of copies using this extremely sensitive method for analysis, cloning, and sequencing. Although qRT-PCR has the benefit of being able to quantify the synthesized product, this is also true for qRT-PCR. It is therefore applicable to the analysis of changes in the levels of gene expression in microorganisms, tumors, and other disease states. Even while PCR is a useful method, it has drawbacks. The highly sensitive nature of PCR means that even minute amounts of DNA might contaminate the sample, leading to inaccurate results (15,16). Additionally, some prior sequence data is required to construct primers for PCR. As a result, PCR is limited to determining if a known pathogen or gene is present or absent. An additional drawback is that PCR primers may anneal non-specifically to sequences that are similar to the target DNA but not the same. Additionally, although it happens very infrequently, the DNA polymerase can add erroneous nucleotides to the PCR sequence. The application of PCR is making DNA sequencing significantly simpler, and it is now widely used.

* With its exceptional sensitivity, PCR can amplify minuscule amounts of DNA. Tiny levels of particular bacteria and viruses can therefore be found in tissues using the right primers, which makes PCR a vital tool for medical diagnostics.
* These days, PCR is a great tool for describing DNA samples that are crucial to medicine. RFLPs, for instance, are quickly taking the place of screening for human genetic disorders.
* PCR has become a vital component of forensic medicine because of its high sensitivity. To enable thorough characterization, PCR can even be used to amplify the DNA from a single human hair or a tiny drop of blood found at the scene of a crime.

1. **Real-time PCR:** The polymerase chain reaction (PCR) provides the basis for a molecular biology laboratory technique known as real-time polymerase chain reaction (real-time PCR, or qPCR when applied quantitatively). It tracks a targeted DNA molecule's amplification in real time, during the PCR, as opposed to after the conclusion, as is the case with traditional PCR. Both quantitative and semi-quantitative methods can be applied to real-time PCR. Sequence-specific DNA probes, which are made up of oligonucleotides labeled with a fluorescent reporter and allow detection only after the probe has hybridized with its complementary sequence, and non-specific fluorescent dyes, which intercalate with any double-stranded DNA, are two popular techniques for detecting PCR products in real-time PCR.

According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) criteria, reverse transcription–qPCR should be referred to as RT-qPCR, and quantitative real-time PCR should be abbreviated as qPCR (17). Some publications do not follow the standard that the term "RT-PCR" stands for reverse transcription polymerase chain reaction rather than real-time PCR (18). These days, DNA microarray and quantitative PCR are used to evaluate gene expression. Northern blot, RNase protection test, and differential display were the older techniques used to quantify mRNA abundance.

Northern blotting is frequently used to visualize the amount of a gene's mRNA transcript in a sample, hence estimating the gene's expression level. With this technique, a particular DNA or RNA probe complementary to the target gene is used to probe the purified RNA after it has been separated by agarose gel electrophoresis and moved to a solid matrix (such as a nylon membrane). Despite its continued usage in evaluating gene expression, this method only yields qualitative or semi-quantitative data on mRNA levels and requires comparatively large volumes of RNA (19). DNA integrity, enzyme efficiency, and a host of other variables might lead to estimation mistakes that arise from differences in the quantification technique. As a result, certain standardization systems often referred to as normalization methods have been created. To measure the overall level of gene expression, some have been devised.

However, the most popular ones are designed to measure the particular gene under investigation using a normalizing gene, which is chosen for its nearly constant expression level. Since their roles in fundamental cellular survival typically involve constitutive gene expression, these genes are frequently chosen from housekeeping genes (20,21). This makes it possible for researchers to compare the expression of the genes of interest without knowing the exact amount of expression by reporting a ratio of the genes' expression to that of the chosen normalizer. This makes it possible for researchers to compare the expression of the genes of interest without knowing the exact amount of expression by reporting a ratio of the genes' expression to that of the chosen normalizer.

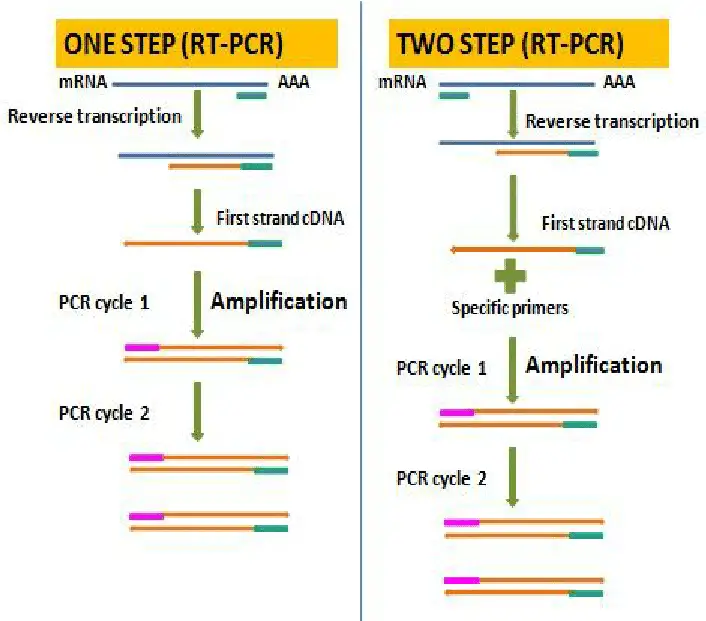
**Steps of RT-PCR:** Both PCR amplification and reverse transcription can be carried out in two steps in a single tube or with two different reactions. In both instances, cDNA is created via reverse transcription of RNA and subsequently utilized as the template for PCR amplification. Primers for cDNA synthesis can be either sequence-specific or non-sequence-specific (a combination of oligo-dT or random hexamers).

1. **Non-sequence-specific primers:**
   * **Random hexamers:**  A mixture of every possible pairing of six nucleotide sequences, known as random hexamers, can haphazardly bind to mRNA and start reverse transcription of the whole RNA pool.
   * **Oligo-dT primers:** The poly-A tail of mRNA molecules is complementary to oligo-dT primers, which enable the synthesis of cDNA only from mRNA molecules.
2. **Sequence-specific primers:**

* Reverse transcription is a target-specific procedure, and sequence-specific primers are the most limited because they are made to bind specifically to mRNA molecules of interest.

**One-step RT-PCR:** Both PCR and cDNA synthesis are carried out in the same reaction vessel using the same reaction buffer. Target-specific amplification and cDNA synthesis are guided by gene-specific primers. Low sample handling, shorter bench times, and closed-tube reactions that lower the possibility of pipetting mistakes and cross-contamination are some of the main benefits of a one-step reaction. The quality and availability of RNA samples influence one-step RT-PCR efficiency. After one-step RT-PCR, the cDNA synthesis product cannot be preserved, so more aliquots of the original RNA sample or samples are needed to repeat reactions or measure the expression of other genes.

**Two-step RT-PC:** In two-step RT-PCR, a mixture of cDNA molecules is produced by cDNA synthesis utilizing random hexamers, oligo-dT primers, and/or gene-specific primers. Certain primers are used for amplifying the resulting cDNAs. cDNA is created in a single reaction in two-step RT-PCR, and a sample of the cDNA is then utilized in a different PCR test. Longer hands-on time, more pipetting manipulations and additional open-tube processes are needed for this, which could increase unpredictability and contamination risk. Quantifying the expression of several genes from a single RNA/cDNA sample or storing the remaining cDNA for later use is possible.

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**Fig 2:** One-step and two-step methods of RT-PCR.

**Application of RT-PCR:** RT-PCR is widely used across many clinical disciplines for example in clinical oncology RT-PCR has been used for the detection and quantification of chromosomal translocations, to monitor minimal residual disease or to show graft versus lymphoma effects (22-24). Other applications include predictive genetic testing and the identification of relevant single nucleotide polymorphisms. Another major area for applications of real-time reverse transcriptase RT-PCR as-says is the quantification of gene expression.The genomes of many clinically significant viruses are made of RNA, and RT-PCR can be used to identify these viruses. RT-PCR has also been used to identify enteroviruses and the West Nile virus, the viral causes of meningitis and meningoencephalitis. The following viruses are being detected using RT-PCR:

1. Dengue virus
2. Hantavirus
3. Human metapneumovirus
4. Severe acute respiratory syndrome (SARS)

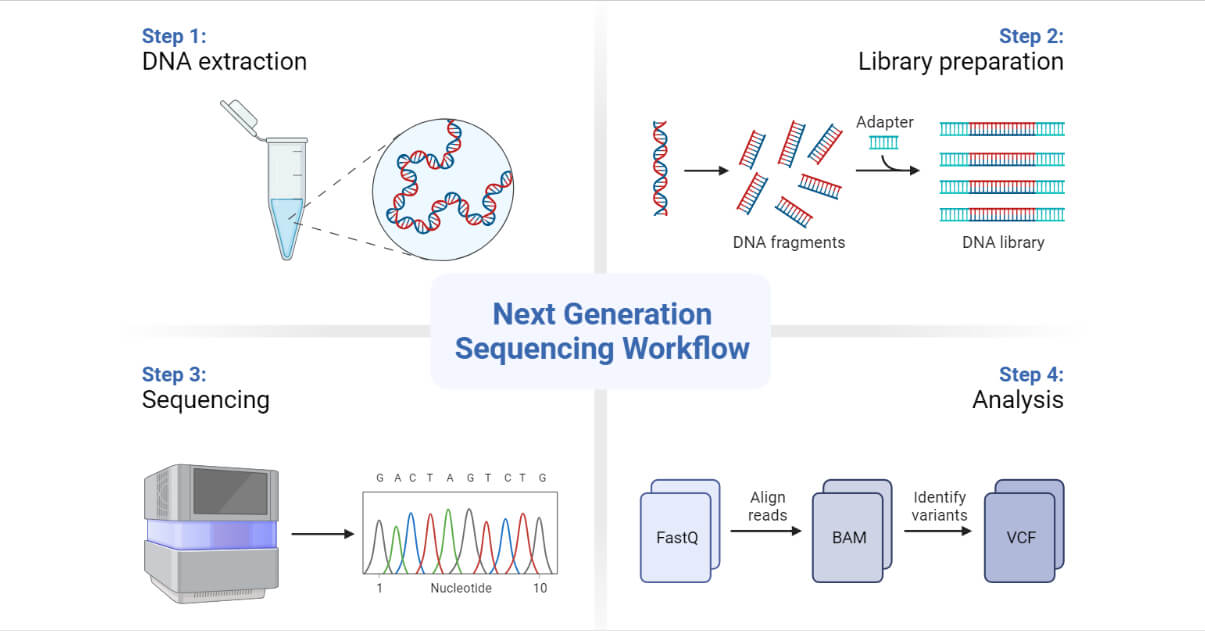
Tests for HIV and HCV viral load, or the quantity of these viruses in a patient's blood, are frequently performed using quantitative RT-PCR assays. By focussing on their rRNA, RT-PCR can also be used to identify other microorganisms, such as bacteria, parasites, and fungi. Since the presence of RNA is more frequently linked to the existence of living organisms, this method is superior to DNA detection.

Like other diagnostics, there are several limitations to RT-PCR methods. RT-PCR is susceptible to inhibition by compounds present in certain biological samples. Because of the necessary use of RNA in an extra enzymatic step, errors can occur. RNA itself is extremely labile compared with DNA, and therefore isolation must be carefully performed to ensure both the integrity of the RNA itself and the removal of contaminating nucleases, genomic DNA, and reverse transcriptase or PCR inhibitors. This can be a problem with any sample source, but clinical samples are of special concern because inconsistencies in sample size, collection, storage, and transport can lead to a variable quality of RNA templates. Conversion of RNA to cDNA during the RT reaction is also subject to variability because multiple reverse transcriptase enzymes with different characteristics exist, and different classes of oligonucleotides can be used to prime RT. Probably the largest present limitation of RT-PCR, however, is not inherent in the technology but rather resides in human error: improper assay development, incorrect data analysis, or unwarranted conclusions. RT-PCR primer sets must be designed and validated by stringent criteria to ensure the specificity and accuracy of the results. For microbiology, false positives or negatives must be considered when designing an assay to detect pathogens. Amplification and melting curves must be visually inspected while independent calculations based on these curves should be double-checked for accuracy. Of course, conclusions based on data derived from RT-PCR are best utilized when the biological context is well-understood

1. **Next-Generation Sequencing:** A new technology called next-generation sequencing (NGS) is utilized to detect variants and mutations in DNA and RNA. Hundreds of thousands of genes or entire genomes can be swiftly sequenced with NGS. The advantages of various sequencing matrices, distinct sequencing chemistries, and bioinformatics technology are all combined in this technology. Largely parallel sequencing of different DNA or RNA sequence lengths, or even the entire genome, is possible with this combination in a comparatively short amount of time (25). After Sanger sequencing, it is an innovative sequencing technique. Numerous significant sequencing steps are involved in NGS. There has been a significant growth in demand for quicker and less expensive sequencing techniques since the first human genome was sequenced. The creation of next-generation sequencing (NGS), or second-generation sequencing techniques, has been fuelled by this need. NGS platforms are capable of massively parallel sequencing, which enables the simultaneous sequencing of millions of DNA fragments from a single sample. Thanks to high-throughput sequencing made possible by massively parallel sequencing technology, a whole genome can be sequenced in less than a day. For low-cost, high-throughput sequencing, several NGS platforms have been developed in the last ten years. The Life Technologies Ion Torrent Personal Genome Machine (PGM) and the Illumina MiSeq are two of the most widely used platforms in research and clinical labs today.

More labs may now access sequencing thanks to the development of this and other NGS platforms, which has led to a sharp rise in clinical diagnoses and research using nucleic acid sequencing.   
For instance, massively parallel sequencing, DNA fragmentation, library preparation, bioinformatics analysis, and variant/mutation annotation and interpretation are all part of DNA NGS.

1. **DNA fragmentation:**  The process of DNA fragmentation divides the target DNA into several short segments, typically ranging from 100 to 300 bp. This can be done in a variety of ways. Enzyme digestion (26), mechanical techniques, and other techniques can all be used to break apart DNA. For instance, DNA can be broken up into short fragments by sonication. Using particular complementary probes of various designs, the brief regions pertinent to the targeted DNA sequences are extracted (27,28). Typically, this technique is called a hybridization capture assay. Amplification using the polymerase chain reaction (PCR) is another method. This technique uses a large number of primer pairs to amplify the targeted DNA segments by PCR. Short portions of the targeted DNA are used in the PCR products. This technique is commonly referred to as amplicon assay (29,30). The next step is to prepare the library using the DNA segments.
2. **Library Preparation:** To give each DNA sample a sample-specific index, such as sample identification, that aids in identifying the patient from whose DNA sequencing was carried out, DNA segments are altered throughout the library preparation process. Additionally, the sequencing adaptors can be introduced to the DNA segments by this procedure. Due to this alteration, all DNA segments can be bound by the sequencing primers, allowing for later massively parallel sequencing.
3. **Sequencing and Imaging:** Nucleotides are added gradually to the developing DNA strand, which is complementary to the template strand, in a process known as sequencing by synthesis. One nucleotide at a time can be incorporated more easily thanks to the sequencing platform. Each of these has a distinct fluorescent or luminous tag attached to it. A camera or sensor picks up the signal that a nucleotide emits during incorporation. The type of nucleotide (adenine [A], thymine [T], cytosine [C], or guanine [G]) determines the signal that is released. It enables real-time sequence determination by the sequencing program. This high-throughput method makes it possible to quickly sequence vast amounts of DNA.
4. **Data Analysis:** The last step in the next-generation sequencing process is data analysis. The first step is the computer conversion of the raw signal data obtained during sequencing into nucleotide sequences. This procedure, called base calling, entails figuring out the matching nucleotides by analyzing the fluorescent or luminescent signals. After being created during the sequencing process, the short sequences, or reads, are either assembled de novo if no reference genome is available or matched to one. When the last stage involves identifying variations between the sequenced DNA and the reference genome, a process known as variant calling, de novo assembly is employed, whereas alignment to a reference genome enables the precise reconstruction of the original DNA sequence. Finding single nucleotide polymorphisms (SNPs), insertions, deletions, and other structural changes is part of this process. After that, annotation gives these variations a biological meaning, which helps us comprehend how they affect gene function and are linked to illnesses.



**Fig 3: Next-generation Sequencing Methodology**

**Applications:** Rapid advancements in numerous biological science-related domains are made possible by the seemingly limitless uses of NGS. Worldwide, infectious diseases continue to be a major contributor to human morbidity and mortality. The therapeutic approach can be aided by the quick and accurate diagnosis of aetiologic microbes. When employing culture technology, the variety of detectable microorganisms is comparatively limited (31,32), including laborious and imprecise identification methods including pathogen isolation, selective culture, and pathological inspection. It can take days or weeks for clinical specimens to get definitive results when it comes to growing harmful bacteria (33). To further encourage the clinical use of NGS, we provide a detailed summary of its applications in the identification of viruses, fungi, and bacteria (Tab.1).

* **Bacteria:** One important and effective technique in infectious disease epidemiology is NGS, which exhibits excellent resolution concerning bacterial genotypes (34). Gram-negative and gram-positive bacteria, anaerobes, and fungi are among the pathogenic microorganisms that appear differently in septic patients under different clinical conditions. About 50% of sepsis patients have culture-negative sepsis, in which the causing organisms are still unknown. Numerous studies have shown that certain microbes are associated with portions of their genomic RNA or DNA. Therefore, NGS of cfRNA or cfDNA in purified plasma can be used to detect pathogens in sepsis samples and provide genetic relatedness information (35).
* **Fungi:** The methods used to examine human fungal communities may have an impact on the analysis and outcomes of fungal detection (36). Given the severity of the fungal disease and the dearth of reliable detection techniques for clinical fungal infections, the application of NGS in fungal diagnostics should be taken into consideration. When it comes to identifying fungal infections, NGS technology has several benefits. First, microbial disorders brought on by hostile environments and slowly proliferating microorganisms, such as fungi, can be treated with NGS technology (37). Furthermore, samples with low fungal burdens benefit from NGS (38). Second, NGS provides more precise fungal species identification and is significantly more specific than alternative methods (39).
* **Viruses:** The use of next-generation sequencing (NGS) to identify viruses has grown in popularity. Furthermore, NGS provides a state-of-the-art instrument for extensive, large-scale genome sequencing of viruses, including coronavirus, hepatitis C virus, and Hantaan virus (HTNV). For the surveillance, tracing, and risk management of viral illnesses, NGS has ushered in a new era of viral genomes. To identify and create preventive strategies for HTNV outbreaks, complete genome sequencing and the isolation of infectious particles are crucial. Twelve HTNVs were isolated from the lung tissues of striped field mice in areas where hemorrhagic fever with renal syndrome (HFRS) is extremely prevalent by Dong Hyun Song et al. HTNV isolates' genomic sequence was obtained using sequence-independent, single-primer amplification (SISPA) NGS. The HTNV S, M, and L segments' nucleotide sequences were covered to 99.4–100%, 97.5–100%, and 95.6–99.8%, respectively, based on the full length of the prototype HTNV 76–118 (40).

**Tab 1: The advantages and disadvantages of sequencing technology in detecting different pathogens**

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| --- | --- | --- | --- | --- |
| **Pathogen** | **Advantage** | **Disadvantage** | **Sequencing approach** | **References** |
| Bacteria | * Simplified bioinformatics. * Amplifies the number of microorganisms present and target. * Reduced price | * Only bacteria identification * Particular microbes. * Can still miss some species due to primer mismatch. * Scarce copies of pathogen sequences may be harder to detect. | t-NGS (16S rRNA) | 41 |
| Fungi | * Reduced price * Better. sensitivity and massive Information. * More speed. * Simplified bioinformatics. * The ability to amplify most of the fungus available. | * Still miss some species due to primer mismatches. * Particularly some subgroups of parasites and fungi. * Scarce copies of pathogen sequences may be harder to detect. | t-NGS (18S rRNA gene/ITS gene sequence). | 42 |
| Viruses | * Higher sensitivity and amount of data allow *de novo* assembly and even read level detected. * Potential to detect the full spectrum of viruses including unknown and unexpected viruses. * Allowing for the thorough identification of minority variants which represent a clear advantage over direct (Sanger) sequencing * Detection of pathogens that do not rely on references or amplification. | * Assembly and characterization of complex/highly repetitive genomic regions * Reconstruction of complete “real” viral haplotype. | WGS; mNGS | 43,44,45 |

1. **Advantages of Molecular Techniques:** Molecular approaches have been used in several fields, including biotechnology, plant genetic improvement, and the food industry, which uses microbes to safely create food (46, 47). In addition to being extremely valuable for investigating fundamental scientific issues, molecular biology techniques can be used for a wide range of issues that impact the human condition as a whole. Applying molecular biology techniques regularly addresses applications such as disease prevention and treatment, the creation of novel protein products, and the modification of plants and animals to achieve desired phenotypic features. These techniques are becoming increasingly prevalent some would even say intrusive in our technologically orientated society due to their broad application. As these methods have advanced, it is now feasible to identify illnesses and genetic mistakes more rapidly and efficiently for upcoming therapies. Furthermore, these molecular methods are also widely used in scientific research and are effective in identifying, genotyping, and cataloging medically significant bacteria and viruses with low genetic material and high specificity (48).

The cell theory, which holds that all organisms are made up of numerous individual cells, has benefited greatly from the application of these techniques to the study of developmental biology because it has made it possible to examine the changes in gene expression that occur as an organism matures. The chromosomal hypothesis of inheritance, which postulates that the chromosomes within the individual cells regulate the physical characteristics of the cells, has been amply supported by the capacity to isolate, alter, and reintroduce particular genes. Elements of the theory of evolution by natural selection, which postulates that complex organisms are descended from more primitive organisms through a process of accumulation of changes in physical traits of cells, have been supported by the isolation and sequence analysis of related genes from multiple species as well as the investigation of the presence of alleles in wild populations.

Several novel techniques, such as DNA fingerprinting, the polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP) analysis, have been made possible by molecular biology and are being used more and more often to identify an individual's genetic characteristics. These analysis techniques are all predicated on the idea that a DNA sample taken from an individual can be used to predict physical traits or to identify the individual from a group because the DNA of an organism contains the genes that code for all of the physical characteristics of a specific individual, and each individual (apart from identical twins) has a unique combination of genes.

1. **Limitations of Molecular Techniques:** Traditional immunoassays and culture procedures can be replaced with molecular diagnostic approaches, which are quicker and more sensitive. However, only a small percentage of analyses have shifted to using rapid diagnostics, despite the undeniable benefits they offer to food safety. Adoption of diagnostic assays based on the pathogen's nucleic acids rather than its phenotypic is still hampered by a few variables. The high number of false positive and false negative outcomes is one important contributing factor. DNA can be found in the environment, in the lab, and even in the tools used to make the reaction mix. This can lead to a false positive for a disease. In contrast to living cell contaminations, which are easily removed from surfaces and laboratory equipment, DNA is more difficult to remove. The presence of inhibitors may result in false negative results. It frequently occurs that the item to be examined is a complex matrix, like salami or cheese, which may contain chemicals that can disrupt the functioning of the enzyme. Thus, a false negative result may result from enzyme inhibition. Consequently, to eliminate inhibitors from certain dietary matrices, ad hoc research on nucleic acid extraction procedures is necessary.

Additionally, to ensure the test's validity, positive controls such as the IPC outlined for the TaqMan PCR must be introduced. Due to competition for the probe and primer annealing, the genomic DNA of the host organism itself can frequently obstruct the detection process, particularly when it comes to processed or ground meat or complex food products. Even with very few host cells, this effect is not insignificant due to the size of vertebrate or plant genomes, which are typically 103–104 times larger than those of bacteria. In many instances, this issue can be resolved by reducing the complexity of DNA, for example, by removing genomic repetitive repeats (47). Generally speaking, bacterial enrichment through culture is a required step due to the abundance of inhibitors or competitors (49,50).

To accomplish enrichment, a stomacher device that mechanically breaks up the food matrix in a culturing broth is usually used. The filtered homogenized sample is next incubated for one or two days at the appropriate growth temperature. An aggressive protocol that may include both chemical and enzymatic reagents is necessary for effective wall lysis in the case of bacteria and fungi with thick cell walls before the extraction of genomic DNA and the amplification reaction.

1. **Conclusion:** The development of new scientific research, faster and more accurate diagnosis, the creation of medications and vaccines, and other processes all depend heavily on the assessment of new developments in molecular biology and biotechnology techniques. As a result, these developments contribute to better public health. The use of molecular and genomic approaches is revolutionizing the field of microbiology. These techniques have improved the speed and accuracy of diagnosing infectious diseases, which can improve patient outcomes. Furthermore, the identification of antibiotic-resistant bacterial strains is made easier by molecular and genomic approaches, which is increasingly important given the growth in antibiotic resistance. Despite implementation challenges, it is anticipated that molecular and genomic approaches will become more widely available and accessible in the future. Medical professionals will need to be trained in the use of these techniques as well as engage in ongoing research and development. Finally, the use of genomic and molecular techniques in medical microbiology has the potential to improve patient outcomes worldwide and transform the way we diagnose and treat infectious diseases.

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