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# Review on Polymerase Chain Reaction and its Diagnostic Merit Over Conventional Techniques in Animal Disease

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**Abstract:** The polymerase chain reaction (PCR) is a nucleic acid-based technique that enables the rapid and sensitive detection of specific micro-organisms. It is the best-known and most successfully implemented diagnostic molecular technology to date. It can detect slow-growing, difficult-to-cultivate, or uncultivatable microorganisms and can be used in situations in which clinical microbiology diagnostic procedures are inadequate, time-consuming, difficult, expensive, or hazardous to laboratory staff. Inherent technical limitations of PCR are present, but they are reduced in laboratories that use standardized protocols, conduct rigid validation protocols and adhere to appropriate quality-control procedures. Improvements in PCR, especially probe-based real-time PCR, have broadened its diagnostic capabilities in clinical infectious diseases to complement and even surpass traditional methods in some situations. Furthermore, real-time PCR is capable of quantitation, allowing discrimination of clinically relevant infections characterized by pathogen replication and high pathogen loads from chronic latent infections. Automation of all components of PCR is now possible, which will decrease the risk of generating false-positive results due to contamination. The vast majority of PCR methods use thermal cycling, *i.e.*, alternately heating and cooling the PCR sample to a defined series of temperature steps. PCR have stages, procedures, principles and techniques with its variations for the application in selective DNA isolation as well as amplification and quantification of DNA. Traditionally, strategies for identifying most microbial pathogens involve isolation on selective agar media or cell cultures and the use of phenotypical tests but these techniques are usually slow and laborious. Diagnosis of viral diseases should be a major target for PCR application because laboratory tests for identification of viruses are either slow, expensive or insensitive. In relation to bacterial diseases, PCR can be used for the rapid detection of those pathogens whose *in vitro* cultivation is difficult, time-consuming or unavailable. Parasitic infestations will probably be the last field of veterinary clinical diagnosis to incorporate PCR techniques, partly because of the relative scarcity of important parasitic diseases in the main countries where PCR research is being developed. It has not yet found applications in routine microbiological analysis of veterinary clinical samples. In general, PCR will most likely become the standard diagnostic test in situations where either the micro-organism level is low, differentiation between morphologically identical organisms is required, or whether the immune response to the infection is uninformative.

**Key words:** Polymerase Chain Reaction · Diagnosis · Virus · Bacteria · Parasites · Fungus

## INTRODUCION

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of apiece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence developed in 1983 by Kary Mullis [1]. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include the identification of genetic fingerprints (Used in forensic sciences and paternity testing) and the detection and diagnosis of infectious diseases. Primers (Short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (After which the method is named) are key components to enable selective and

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repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified [2]. PCR can be extensively modified to perform a wide array of genetic manipulations. Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus* [2, 3]. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (Also called DNA primers) which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, *i.e.*, alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA [3].

To control the incidence of infectious diseases in animals, early diagnosis plays a major role. In comparison to serological diagnosis, molecular diagnosis using nucleic acids are quicker and help in taking earlier decision to control and prevent the disease in animal population. Nucleic acid amplification help in making multiple copies of a desired sequence of a gene initially present at very low concentration there by confirming the presence of microorganism for which that gene sequence is specific [3]. During 1970 ´S, desired quantities of DNA were synthesized in bacteria. After 1985 PCR was used at a large scale for DNA amplification. The polymerase chain reaction (PCR) is now most widely used method for DNA amplification for detection and identification of infectious diseases and genetic disorders. PCR amplifies desired quantities of DNA using in vitro amplification process [4]. Therefore, the objectives of this review paper are:

- To highlight about Polymerease chain reaction applications, procedure and principle, stages and techniques with its variations
- To over view diagnosis of animal diseases by using PCR and its diagnostic merits over conventional techniques.

**General Account about Polymerase Chain Reaction**

**Brief History of Polymerase Chain Reaction:** A 1971 paper in the Journal of Molecular Biology by Kleppe and co-workers first described a method using an enzymatic assay to replicate a short DNA template with primers *in vitro* [5]. However, this early manifestation of the basic PCR principle did not receive much attention and the invention of the polymerase chain reaction in 1983 is generally credited to Kary Mullis [6]. At the core of the PCR method is the use of a suitable DNA polymerase able to withstand the high temperatures of >90 °C (194 °F) required for separation of the two DNA strands in the DNA double helix after each replication cycle. The DNA polymerases initially employed for in vitro experiments presaging PCR were unable to withstand these high temperatures [4]. So the early procedures for DNA replication were very inefficient and time consuming and required large amounts of DNA polymerase and continuous handling throughout the process [3].

The discovery in 1976 of Taq polymerase a DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus*, which naturally lives in hot (50 to 80 °C (122 to 176 °F) environments [2] such as hot springs paved the way for dramatic improvements of the PCR method. The DNA polymerase isolated from *T. aquaticus* is stable at high temperatures remaining active even after DNA denaturation [7] thus obviating the need to add new DNA polymerase after each cycle. This allowed an automated thermocycler-based process for DNA amplification. When Mullis developed the PCR in 1983, he was working in Emeryville, California for Cetus Corporation, one of the first biotechnology companies. There, he was responsible for synthesizing short chains of DNA. Mullis has written that he conceived of PCR while cruising along the Pacific Coast Highway one night in his car [8]. He was playing in his mind with a new way of analyzing changes (Mutations) in DNA when he realized that he had instead invented a method of amplifying any DNA region through repeated cycles of duplication driven by DNA polymerase. In Scientific American, Mullis summarized the procedure: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents and a source of heat. He was awarded the Nobel Prize in Chemistry in 1993 for his invention [9] seven years after he and his colleagues at Cetus first put his proposal to practice. However, some controversies have remained about the intellectual and practical contributions of other scientists to Mullis' work and whether he had been the sole inventor of the PCR principle. The basic principle of replicating a

piece of DNA using two primers was described by Gobind Khorana in 1971[6] but Progress was limited by primer synthesis and polymerase purification issues Later on Kary Mullis properly exploited this process of amplification and invented PCR in 1983.

**Polymerase Chain Reaction Principles and Procedures**

***PCR Principle:*** The principle of the PCR relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction mixture for DNA melting and enzymatic replication of the DNA. Primers (Short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. A basic PCR set up requires several components and reagents. These components include: *DNA template* that contains the DNA region (Target) to be amplified [10].

Two *primers* that are complementary to the 3' (Three prime) ends of each of the sense and anti-sense strand of the DNA target [3]. There are different elements that are needed for the complete synthesis of a given DNA. These are: *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70 °C, *Deoxynucleotide triphosphates* (dNTPs) and 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.

*Divalent cations,* magnesium or manganese ions; generally $Mg^{2+}$ is used, but $Mn^{2+}$ can be utilized for PCR-mediated DNA mutagenesis, as higher $Mn^{2+}$ concentration increases the error rate during DNA synthesis, monovalent cation potassium ions [9, 10].

The PCR is commonly carried out in a reaction volume of 10-200 ìl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermo-cyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube [10, 11].

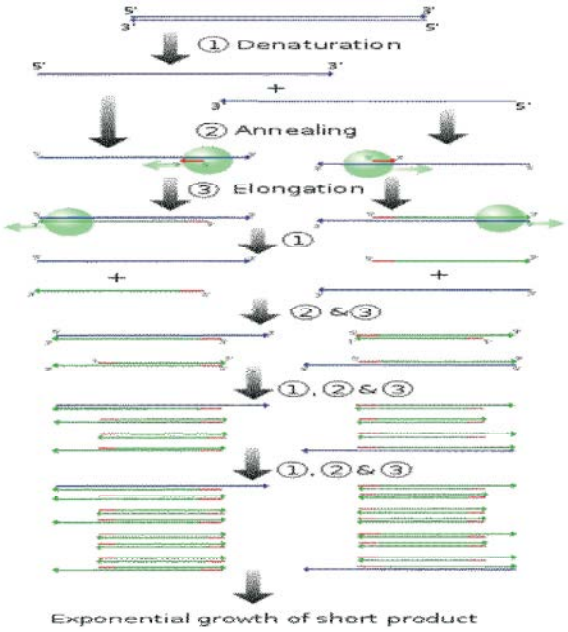


Fig. 1: Schematic drawing of the PCR cycle. Source: Mark [2012]

***PCR Procedure*:** Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Fig. 1). The cycling is often preceded by a single temperature step (Called hold) at a high temperature (>90°C) and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction and the melting temperature (Tm) of the primer [2].

As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified [12].

Denaturing at 94-96 °C. (2) Annealing at ~65 °C (3) Elongation at 72 °C. Four cycles are shown here. The blue lines represent the DNA template to which primers (Red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (Green lines), which themselves are used as templates as PCR progresses [3, 12].

*Initialization step:* This 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

minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR [7, 12].

***Denaturation Step*:** This step is the first regular cycling event and consists of heating the reaction to 94-98 °C for 20-30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules [4, 7, 12].

*Annealing step:* The reaction temperature is lowered to 50-65 °C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis [3, 4, 6, 12].

***Extension/elongation Step*:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80 °C [13] 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' 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. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, *i.e.*, if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (Geometric) amplification of the specific DNA fragment [7].

***Final Elongation*:** This single step is occasionally performed at a temperature of 70-74 °C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended [2-4, 6].

***Final Hold*:** This step at 4-15 °C for an indefinite time may be employed for short-term storage of the reaction. To check whether the PCR generated the anticipated DNA fragment (Also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (A molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. PCR is used to amplify a specific region of a DNA strand (The DNA target). Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb) although some techniques allow for amplification of fragments up to 40 kb in size [14].

**Polymerase Chain Reaction Stages:** The PCR process can be divided into three stages: *Exponential amplification:* At every cycle, the amount of product is doubled (Assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA need to be present. *Leveling off stage:* The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting. *Plateau*: No more products accumulate due to exhaustion of reagents and enzyme [2].

**PCR Optimization:** A number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plastic ware and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR [15].

**PCR Techniques and Its Variation:** *Allele-specific PCR*: a diagnostic or cloning technique based on single-nucleotide polymorphisms (SNPs) (Single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template

and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence [16].

**Asymmetric PCR:** Preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (Arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required [17]. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction [18].

**Hot-Start PCR:** A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the melting temperature (e.g., 95°C) before adding the polymerase [19]. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody [20] or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature [19].

**Inverse PCR:** is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence [21].

**Ligation-Mediated PCR:** Uses small DNA linkers legated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA foot printing [22].

**Miniprimer PCR:** Uses a thermostable polymerase that can extend from short primers ("Smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions and is used to amplify conserved DNA sequences, such as the 16S (Or eukaryotic 18S) rRNA gene [23].

**Multiplex-PCR:** Consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis [24].

**Nested PCR:** Increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments, but it requires more detailed knowledge of the target sequences [24].

**Quantitative PCR (Q-PCR):** Used to measure the quantity of a PCR product (Commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. *Quantitative real-time PCR* has a very high degree of precision. QRT-PCR methods use fluorescent dyes, such as Sybr Green, Eva Green or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (*R*eal *T*ime PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR often used in conjunction with Q-PCR [11].

**Reverse Transcription PCR (RT-PCR):** For amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene

is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (Corresponding to the transcription start site) is typically identified by RACE-PCR (*Rapid Amplification of cDNA Ends*) [25].

**Thermal Asymmetric Interlaced PCR (TAIL-PCR):** For isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence [26].

**Touchdown PCR (*Step-Down PCR*):** A variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the $T_m$ of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer $T_m$. The higher temperatures give greater specificity for primer binding and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles [27].

**Universal Fast Walking:** for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (Using only one gene-specific primer and one general primer - which can lead to artefactual 'Noise') by virtue of a mechanism involving lariat structure formation [28]. Streamlined derivatives of UFW are LaNe RAGE (Lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe [29].

**Application of PCR**

**Selective DNA Isolation:** PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material [11].

**Amplification and Quantification of DNA:** Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar [17]. Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification [17, 30].

**Criteria for Comparison of PCR and Conventional Methods:** There are different criteria for comparison between the PCR and conventional methods for their validation as a diagnostic tool. Criteria for Assay Development and Validation: definition of the intended purpose(s), optimization, standardization, repeatability, analytical sensitivity, analytical specificity, thresholds (cut-offs diagnostic sensitivity, diagnostic specificity, reproducibility and fitness for intended purpose [31].

**Limitations and Advantage of PCR:** PCR has several advantages. It is a simple technique to understand and use and it produces results rapidly [32]. It is a very sensitive technique that allows rapid amplification of a specific segment of DNA. It makes billions of copies of a specific DNA fragment or gene, which allows detection and identification of gene sequences using visual techniques based on size and charge. Modified versions of PCR have allowed quantitative measurements of gene expression with techniques called real-time PCR. However, PCR also has limitations. The DNA polymerase used in the PCR reaction is prone to errors and can lead to mutations in the fragment generated. Because it is highly sensitive, any form of contamination of the sample by even trace amounts of DNA can produce misleading results [32, 33]. In addition, to design primers for PCR, some prior sequence data are needed. Therefore, PCR can be used only to identify the presence or absence of a known pathogen or gene. Another limitation is that the primers used for PCR can anneal nonspecifically to sequences that are similar, but not completely identical, to target DNA. Moreover, incorrect nucleotides can be incorporated into the PCR sequence by the DNA polymerase, albeit at a very low rate [34].

**Polymerase Chain Reaction in Diagnosis of Animal Diseases:** PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses. The primers used need to be specific to the targeted sequences in the DNA of a virus. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give veterinarian a significant lead time in treatment. The amount of virus ("Viral load") in a diseased animal can also be quantified by PCR-based DNA quantitation techniques [30].

**Polymerase Chain Reaction in Veterinary Microbiology:** The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes [30, 35].

**Viruses:** Detection of foot-and-mouth disease virus by rRT-PCR- Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed livestock (Cattle, sheep, pigs and goats). RT-PCR assays can play an important role for the rapid and sensitive detection of FMDV in a wide range of clinical sample types. Recent development of rRT-PCR methodology employing a fluorescently labelled probe to detect PCR amplicons has allowed the diagnostic potential of molecular assays to be realised. These assays are highly sensitive and obviate tube opening after amplification thereby reducing the potential for cross-contamination of test samples by post-PCR products. In order to increase assay throughput and minimise operator errors, rRT-PCR assays for FMDV can be automated using robots for nucleic acid extraction and liquid handling equipment to set-up the reaction mixes [36]. Vesicular epithelium is the sample of choice to collect from affected animals since it is rich in virus during the acute phase of infection [37].

*Bovine viral diarrhoea virus (BVDV)* BVDV is causing considerable economic losses in cattle throughout the world. PCR can readily detect BVDV [38] and PCR analysis of bulk tank milk samples has provided a rapid and sensitive method to screen herds for the presence of the virus. Additionally, it is possible to discriminate among different BVDV strains using PCR [39].

*Detection of bluetongue virus by rRT-PCR* Bluetongue is an arthropod-borne viral infection of ruminants caused by bluetongue virus (BTV) only sheep are clinically affected while cattle are usually asymptomatic reservoirs. PCR-based procedures have been developed for the diagnosis of BTV [40]. Whole blood seems to be the most convenient clinical sample for PCR detection of BTV infection in sheep [41]. In 2006 an rRT-PCR assay was developed using a conserved region in RNA segment 5 of BTV-2 and BTV-4 [42].

*Bovine herpes virus-1 virus (BHV-1)* BHV-1 causes infectious bovine rhinotracheitis (IBR), an economically important disease of cattle characterized by acute respiratory infection and reproductive problems. Virus detection is often requested for the laboratory diagnosis of most cases of respiratory and reproductive problems in cattle. Several reports have described the PCR of different BHV-1 genes from tissue cultures [43] and bovine semen [44]. Other ruminants viruses for which PCR protocols have been successfully developed include bovine leukaemia virus [45] bovine corona virus [46] rotavirus [47] and Maedi-visna virus [48].

**Detection of Classical Swine Fever Virus by RRT-PCR:** Classical swine fever (CSF) causes major economic losses, especially in countries with an industrialised pig production system and is therefore a disease notifiable to the Office International des Epizooties [49]. PCR has been accepted by the European Union as an official method for confirming this disease [50]. Leifer *et al*. [51] developed a multiplex rRT-PCR assay for the simultaneous detection and differentiation of CSFV field strains and the C-strain vaccine virus.

Intensive breeding of poultry means that high populations often live in confined spaces. Under such conditions, the entry of a virulent virus can cause high mortality and big economical losses. Rapid diagnostic tests are needed to minimize the consequences of viral outbreaks in these environments [52].

**Detection of Avian Influenza Virus by rRT-PCR:** The risk of zoonotic Avian influenza (AI) infections in humans also remains a concern for public health authorities [53]. In terms of AI diagnosis, rRT-PCR provides rapidity, sensitivity and specificity for diagnosis directly from clinical specimens. The matrix (M) gene is highly conserved for all sixteen Haemagglutinin (H) subtypes from all geographical regions and is an ideal target for global generic AIV detection. M gene rRTPCR has been described and validated in testing clinical specimens obtained from live bird markets (LBMs) in the USA and from experimental infection studies [54].

**Detection of Newcastle disease virus by rRT-PCR-:** Newcastle disease virus -specific rRT PCR have utilised intercalating dyes such as SYBR-green [55]. Use of hybridization probes improved assay reliability: not only by offering a higher specificity, but also by raising the analytical sensitivity of detection of NDV–RNA by this method Wise *et al.* [56].

**Equine Viral Arteritis (EVA):** When a stallion is identified as EAV positive, the first priority is to ascertain whether virus is being shed in their semen before the animal is allowed to cover mares. PCR may be used to detect the presence of the virus in the semen [57].

*Equine herpes virus* (EHV)-PCR has applied to detect EHV 1 and 4 in aborted equine fetuses [58] and in nasopharyngeal swab specimens from horses with respiratory or neurological disease [59]. Other equine viral diseases which have been diagnosed by PCR include equine infectious anaemia [60] and African horse sickness [61].

Rabies is still one of the most life threatening zoonosis in some regions of the world. Obviously, fast and accurate detection of infected animals is of vital importance. Research results have shown that PCR can play a remarkable role in the rapid, sensitive and specific detection of the rabies virus and the technique should spread among the reference laboratories located in regions at risk [35, 40].

**BACTERIA:** Staphylococcus: Several staphylococci, mainly *Staphylococcus aureus* strains, cause acute and chronic mastitis and can lead to gangrenous mastitis. Rapid detection of staphylococci, including those killed by heat treatment, in suspected food could prevent food borne staphylococcal gastroenteritis and differentiation of *S. aureus* strains has been achieved by DNA amplification fingerprinting [62].

**Listeria Monocytogenes:** Because PCR is able to detect low numbers of bacteria; it may be a tool for increasing the sensitivity of listeria detection in CSF of ruminants [63]. It is also important to detect asymptomatic carriers because of the zoonotic nature of the infection. Jaton *et al.* [64] developed a sensitive nested PCR assay for the detection of *L. monocytogenes* in human CSF. Additionally, PCR has confirmed its usefulness to detect specific strains in the epidemiological investigations of listeriosis [65].

**Anthrax:** PCR amplification of some *Bacillus anthracis* genes has already been reported, allowing the detection of even a single spore of *B. anthracis*[66]. Henderson *et al.* [67] examined the variation among isolates of *B. anthracis* using restriction patterns and PCR found that the *Bacillus anthracis* profiles were unique when compared with those of closely related species, including *B. cereus, B. thuringiensis* and *B. mycoides.* Their results showed that isolates of *Bacillus anthracis* are almost completely homogeneous and distinct from other members of the *B. cereus* group [66, 67].

**Clostridium Botulinium:** Botulism is a severe foodborne disease caused by *Clostridium botulinum* and Botulinal neurotoxins A, B, C, D, E and F have all been implicated as causes of human and/or animal disease. PCR has a great potential for the identification of botulism neurotoxin producing strains and clearly demonstrated that PCR methods should be used for the development of highly sensitive and specific assays for organisms harboring botulism neurotoxin genes [68].

**Escherichia Coli:** Enterotoxigenic *Eschenlchia coli* (ETEC) -is a major cause of diarrhoea in neonatal and post weaned calves, lambs and piglets. Identification of *E. coli* strains from cows with clinical mastitis can be accomplished by PCR amplification using repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences. Such procedure has revealed that *E. coli* strains isolated from repeated episodes of clinical mastitis in the same cow have similar genotypes. Because verocytotoxin genes can be detected by PCR, this technique has become useful to determine the prevalence and clinical significance of EHEC isolated from cattle herds with and without calf diarrhea [69].

**Salmonella:** Amplification of salmonella genes offers a specific and direct means of detection [70]. Booster PCR methods for the genus-specific detection of salmonellas in equine and chicken faeces have been developed [71] with detection possible within 10-12hr from the time of submission of samples.

*Ovine foot rot* is a highly contagious, economically serious disease of sheep with worldwide distribution, especially in temperate farming areas. Although foot rot results from a mixed bacterial infection, *Dichelobacter nodosus* has been shown to be the essential pathogen for the initiation and establishment of the disease. The use of

PCR based on specific regions of 16S rRNA constitutes a competent assay for foot rot [72]. PCR assays employing virulent and benign-specific primers are capable of specific and sensitive differentiation of strains causing virulent, intermediate or benign foot rot [27].

**Brucella:** Bacteria of the genus *Brucella* are well-known as intracellular pathogens that cause animal and human infections. Rapid and sensitive PCR detection and also differentiation of brucella with or without extraction of DNA has been accomplished [73].

**Mycobacteria:** The bovine tuberculosis organism, *Mycobacterium bovis,* is very closely related genetically to *Mycobacterium tuberculosis* [74] itself the cause of most human tuberculosis. In some situations, tuberculosis in cattle and other farmed animals can be frequently caused by the less well known members of the *M. tuberculosis complex*, *Mycobacterium caprae* [75] and *Mycobacterium africanum* [76] and this can also be linked with disease in humans [77]. Occasional cases in cattle have also been caused by *Mycobacterium microti* [78] and the seal bacillus, *Mycobacterium pinnipedii*. While distinction between these tuberculosis species by PCR amplification and DNA sequencing of selected genes, insertions/deletions or single nucleotide polymorphisms [79] is important because they each raise different epidemiological considerations in terms of animal and human disease. In cases where mycobacterial infections are found not to be due to members of the M. tuberculosis complex, it is also becoming increasingly common for organisms to be speciated 1by PCR amplification [80]. The 16S RNA gene is now widely used for this purpose [81].

*Mycobacterium paratuberculosis* causes Johne's disease, a commonly diagnosed disease of sheep, goats and other ruminants. The organisms can be detected by PCR from intestinal and lymph node tissue of infected animals [79].

*Mycoplasmas* are known to produce a wide spectrum of animal diseases. Cattle infected with *Mycoplasma mycoides* subsp, *mycoides* infection can either remain apparently healthy or develop contagious bovine pleuropneumonia (CBPP), a disease characterized by respiratory problems [80]. Bashiruddin *et al.* [80] described the use of PCR to detect specific DNA in clinical material and isolates from outbreaks of CBPP in cattle and buffaloes in Italy.

PCR can identify the aetiological agent within 2 days of extraction of clinical material and the specificity of the PCR test to distinguish *Mycoplasma mycoides* subsp, *mycoides* from other subspecies was confirmed. Unfortunately, efforts have been hampered by difficulties in differentiating *M. hyopneumoniae* from cross reacting *Mycoplasma flocculare* and *Mycoplasma hyorhinis* [79, 80]. Stemke *et al.* [81] developed a method for differentiation of those three species on the basis of amplification of a 16S rRNA gene sequence.

**Parasites:** Parasitic infestations will probably be the last field of veterinary clinical diagnosis to incorporate PCR techniques, partly because of the relative scarcity of important parasitic diseases in the main countries where PCR research is being developed [82].

*Leishmania* is a group of infestations of the viscera, skin and mucous membranes caused by protozoa of the genus *Leishmania.* Multicopy 16S rRNA has been the basis of some PCR assays that specifically detects *Leishmania* sp. [83]. Kinetoplast DNA (kDNA) is a target of interest because both maxi-and mini-circles are present in each cell in multiple copies. PCR has been used to detect leishmanias in conjunctival biopsies [84] showing that a number of cases of ocular inflammation can be attributed to this parasite.

**Trypanosoma:** In some tropical countries, the protozoan parasites of the genus *Trypanosoma* are responsible for life-threatening diseases in animals and humans and PCR is now being used to evaluate the vectorial ability of *Glossina longipalpis* in Western Africa [85].

*Echinococcus* is a disease in animals and humans caused by larval stages of different cestode species of the genus *Echinococeus,* especially *Echinococcus granulosus* and *Echinococcus multilocularis.* The application of PCR to detect echinococci can allow the identification of biopsy material obtained from liver lesions of unknown aetiology and the demonstration of adult-stage parasite tissue or eggs in samples derived from faeces, small intestines or anal swabs of definitive carnivore hosts [85-87].

Dictyocaulus are common parasites of ruminants and to a lesser extent, horses. Random amplified polymorphic DNA (RAPD) PCR has proved to be a valuable tool to examine genome differences among *Dictyocaulus* species from cattle, sheep and fallow deer [85, 89].

**Protozoa:** Neospora caninum and Toxoplasma gondii are cyst-forming coccidian parasites of veterinary clinical relevance primarily because of the neurological symptoms they cause. The two parasites are also well known for causing congenital infections associated with abortion and/or severe damage of the fetus. In humans, only T. gondii has been demonstrated so far, showing the same range of neuropathological and congenital damage [90].

In the last few years, diagnosis of neosporosis and toxoplasmosis was much improved by the development of PCR tests which allow a fast and methodically highly sensitive identification of the parasite through the amplification and subsequent demonstration, of parasite-specific DNA sequences (90, 91, 92, 93). The present report describes the elaboration of a reliable and specific DNA hybridization immunoassay (DIA) on the basis of a previously descibed method (8), which, in contrast to a conventional DNA analysis by gel electrophoresis, provides unambiguity in the detection of N. caninum-and T. gondii-specific diagnostic amplification products. This technical achievement may considerably simplify the routine application of PCR procedures in diagnosis of the two coccidian parasites [94].

**Fungus**

**Candida Albicans and *Aspergillus Fumigatus:*** Both conventional DNA-based methods and exact DNA-based methods have provided useful insights into the epidemiology and population structure of C. albicans and non albicans with other yeast species. The major drawback of the conventional methods lies in their lack of standardization, reducing their potential for interlaboratory comparisons and therefore global population studies; but these methods are very suitable to investigate epidemiological trends at a local level [31, 95]. Exact DNA-based methods, including PCR based methods, MLP and MLST, have emerged as very efficient typing tools. The main advantage of these methods is that generated data are unambiguous and highly reproducible and can be stored in databases offering an unprecedented degree of portability and accessibility to all interested users. Such techniques are much more appropriate for global epidemiology. At present, MLST is the only typing method that has a public database and represents the most powerful approach for phylogenetics of C. albicans, whereas MLP analysis needs further standardization [95]. Invasive aspergillosis is a leading cause of mortality in immunocompromised hosts [96]. Central nervous system (CNS) aspergillosis has been diagnosed with increasing frequency over the past decade [97, 98]; parenchymal abscesses represent the majority of these cases, with true meningitis being rare. There is evidence that PCR can detect Aspergillus DNA in patient samples in the absence of GM antigenaemia [99]. Early detection of Aspergillus fumigatus in patient cerebrospinal fluid (CSF) has been reported using the Platelia Aspergillus EIA kit for GM and by nested PCR [100]. There is still a need for tools that enable earlier diagnosis of infection and that will allow optimal treatment with the prospect of improving the outcome of CNS aspergillosis. Murine model of cerebral aspergillosis to examine the potential of real-time PCR as a tool to track dissemination of A. fumigatus during infection and to determine the suitability of minute volumes of CSF and blood as diagnostic samples for cerebral aspergillosis were carried out properly [101].

**Comparison of Polymerase Chain Reaction with Other Conventional Methods as Their Diagnostic Tools:** Traditionally, strategies for identifying most microbial pathogens involve isolation on selective agar media or cell cultures and the use of phenotypical tests but these techniques are usually slow and laborious. The important cost that animal infectious diseases can have on national economies has therefore stimulated the search for faster, more sensitive and more specific methods to identify microbial pathogens. Many useful nucleic acid probes and immunological assays have been developed for diagnostic purposes, but these techniques also have some deficiencies [89]. The emergence of PCR, however, offers the potential to improve the laboratory-based diagnosis of pathogens [12, 27, 89]. The outstanding research effort focused on this technique, together with the remarkable development of molecular biology have minimized the deficiencies and allowed its increased general use as a diagnostic tool. PCR has advantages as a diagnostic tool in conventional microbiology, particularly in the detection of difficult to cultivate microorganisms, a slow growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models or under special situations in which conventional methods are expensive or hazardous [12, 22, 81].

Due to the stability of DNA, nucleic acid based detection methods can be also used when inhibitory substances, such as antimicrobials or formalin, are present. Therefore, through the use of molecular

techniques has been able to identify different pathogens, to elucidate its epidemiology, to achieve standardization of diagnostic methods and to establish strategies of prevention and control of diseases, advancing in sanitary regulations in different countries [88, 89].

Molecular diagnosis could be the most appropriate technique for the species identification of mastitis pathogens that are difficult to detect and identify by conventional methods. PCR-based diagnostics may offer significant advantages over other diagnostics for its speed and its sensitivity when used for mastitis pathogens detection [92].

The big advantage of the molecular techniques is that isolation of infectious Newcastle disease viruses (NDV) and subsequent egg passage and/or cell line passages are not needed for diagnostic purposes. Therefore, detection of genetic material specific for NDV directly from a wide range of specimens, *i.e.* blood, faeces, tissues and from different avian species speeds up the diagnostic process significantly. Another advantage of molecular techniques is the possibility of differential diagnosis by multiplex-RT-PCRs between pathogens causing similar clinical signs, *i.e.* avian influenza and ND. RT PCR can be achieved by universal primers that amplify targeted regions of NDV and thus detect its presence [93]. Aradaib *el al*.[102] compared the value of PCR with virus isolation for the detection of epizootic hemorrhagic disease virus (EHDV) in clinical samples taken from naturally infected deer and concluded that PCR assays for EDHV can provide a diagnostic alternative superior to the current cumbersome and time consuming virus isolation procedures.

When compared with virus isolation and other classic techniques, PCR is the method of choice for diagnosis of many poultry viruses including Marek's disease virus, reticuloendotheliosis virus [103] avian leucosis virus [104] infectious bronchitis virus [105] Newcastle disease virus [106] lymphoproliferative disease virus [107] and infectious bursal disease virus [108]. Sensitive studies using reference strains of BVDV from persistently infected carriers have shown that reverse transcription (RT)-PCR has greater sensitivity than other tests, including enzyme-linked immunosorbent assay (ELISA); unfortunately, cost currently makes this technique unsuitable for large-scale testing but it should be valuable as a confirmatory test in cases where ELISA results are in the 'suspicious range' or where the viral titre is low, such as in batches of foetal bovine serum [109].

The identification of the closely related members of the *Mycobacterium tuberculosis* complex (MTC) has remained a challenging task in diagnostic laboratories [104]. MTC includes a variety of closely related mycobacteria, namely, *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. africanum* and *M. microti*. A panel of classical tests based on microbiological features such as growth rate and phenotypic and biochemical characteristics has conventionally been utilized to distinguish members of MTC [110]. However, these tests are slow, cumbersome, unreliable and time-consuming. The high degree of variability among these tests warrants the development of molecular biological tools for identification of MTC members. In this regard, multiple gene targets have been used to date to detect and differentiate genetically identical species, such as *M. tuberculosis* and *M. bovis*. The gene targets include *pncA* Barouni *et al.* [111] *gyrB* Chimara *et al*. [112] *oxyR* Sreevatsan *et al.* [113] and *katG* Haas *et al.* [114]. Richter *et al.* [115] has targeted multiple loci and genes to differentiate *M. tuberculosis* from *M. bovis*. However, to date no single accepted protocol(s) that can unambiguously differentiate all members of the MTC is available. Identification of the etiological agent belonging to MTC is important for determination of the origin and reservoirs of infection and also for implementation of appropriate public health measures. A PCR-restriction fragment length polymorphism (RFLP) method utilizing the *hupB* gene, encoding a histone-like protein of *M. tuberculosis*, as a target for detection and identification of *M. tuberculosis* and *M. bovis* from other members of the MTC and non-tuberculous mycobacterial and non-mycobacterial species [116]. RFLP patterns using PCR amplified DNA is an excellent method for bacterial typing and has already been used for the identification of the bacterial strains involved in human food borne outbreaks [117]. Histological examinations enable rapid decisions to be made on suspect carcasses during meat inspection. However, agents other than *M. bovis* can induce similar lesions and additionally, the microscopic detection of acid-fast organisms can only detect bacteria in great concentrations. Laboratory culture of *M. bovis* is sensitive but requires viable bacteria and the growth of this organism may take 6-8 weeks. Species identification procedures extend the reporting time even further. Tests based on PCR have been shown to be very promising for mycobacterial detection in clinical samples [118]. Apart from its use in direct diagnostic clinical assays, PCR is now playing an important role in

many laboratories to determine or to confirm species identification after culture. In some cases, this is simply replacement of a confirmatory DNA probe test, by a PCR test, Sun *et al.* [119] to confirm that a cultured organism belongs to the M. tuberculosis complex. Increasingly though, PCR is being used for distinguishing species of the M. tuberculosis complex, either M. tuberculosis from *M. bovis* Nassar *et al.* [120] or a more sophisticated analysis, often by multiplex PCR, to identify a range of mycobacterial species [121]. The tuberculin test is limited to the detection of infected animals, whereas from the viewpoint of describing the origin, link and reservoirs of infection, identification of mycobacterial species in bovines has its merits. Traditionally, culture followed by a panel of biochemical tests has been used for speciation of mycobacteria. This has inherent disadvantages, as most mycobacteria of clinical importance are slow growers and hence are difficult to isolate and cultivate. The time required for primary isolation ranges from 4 to 6 weeks in the case of solid culture media and 10 to 15 days by radiometric and other automated systems. Moreover, the paucibacillary nature of clinical samples also accounts for the low efficiency of isolation of pathogenic mycobacteria [122]. In addition to difficulties in primary isolation of mycobacterial pathogens, particularly *M. bovis*, there have been reports of difficulty in differentiating closely related mycobacterial species. Therefore, there is a need to develop molecular biological tools like PCR-based assays for reliable, early detection and speciation of mycobacteria in clinical samples and, as a consequence, for determination of the disease burden caused by diverse pathogenic mycobacteria [123].

The sequencing of the enterotoxins and fimbrial genes has made possible the application of nucleic acid-based methods for their detection [124]. These methods have the advantage that they are readily applicable to a large number of isolates, in contrast to classic methods such as agglutination, infant mouse, ligated swine intestine and cell culture assays. PCR results obtained in Sweden by Kennan *et al.* [125] showed that the gene for the major subtmit of F107 fimbria was present on approximately half of the strains not expressing K88, K99, 987P and F41 fimbria isolated from piglets older than 1 week with diarrhoea. This suggested that F107 fimbria is of major importance among ETEC strains causing post-weaning diarrhoea. Comparisons of PCR and microbiological cultures for the detection of salmonellas in drag-swabs from poultry houses have revealed that PCR is significantly more sensitive than culture for environmental monitoring. Conventional methods to detect leptospires in blood are either unreliable or too slow to give early results [126]. PCR is a promising tool for early detection of leptospires in blood, urine or CSF in the period between the first appearance of clinical symptoms and the time when antibodies become detectable [127].

**Isothermal Amplification for Nucleic Acid Identification:** Related advanced technologies, such as multiplex PCR, nested PCR, real-time PCR and reverse transcription PCR (RT-PCR), have been used for bimolecular analysis. However, there are numerous features confining the applicability of PCR. The approach requires thermal cycling instrumentation, considerable expertise and a substantial amount of space in routine diagnostic laboratories, thus limiting its use to highly sophisticated facilities. These limitations in current PCR-based techniques have spurred the development of a new molecular-biological technique known as isothermal nucleic acid amplification [3, 4, 128]. The major difference between PCR and isothermal amplification are the temperature reaction condition requirements. Stringent reaction conditions, including thermal cycling steps at specific temperatures, are employed in PCR, whereas only a single optimal reaction temperature is required for the entire isothermal amplification reaction, thus providing simpler and more effective reaction conditions without expensive equipment. Additionally, isothermal DNA amplification produces longer DNA fragments than the conventional PCR technique. Overall, isothermal nucleic acid amplifications have greater amplification efficiency and produce higher DNA yields than PCR owing to their undisrupted and sustained enzyme activity [128].

Isothermal approaches can facilitate rapid target amplification through single-temperature incubation, reducing system complexity compared to PCR-based methods. Established isothermal amplification methods differ in terms of complexity (multiple enzymes or primers), attainable sensitivity and specificity [129]. The main isothermal methods has been mentioned that have been used in diagnostic systems, including nucleic acid sequence-based amplification, strand displacement amplification and rolling circle-based, loop-mediated, helicase-dependent amplification and potential of new isothermal methods such as beacon-assisted detection amplification and hybridization chain reaction [129].

## CONCLUSION

PCR has already played an important role in studies of the epidemiology, taxonomy and patho-genesis of micro-organism infections in animals but is not yet used routinely for the diagnosis of any animal infectious disease. Especially, in developing countries, it is unusual to use its application for the diagnosis tool of animal and human disease. In fact, PCR has become a routine tool only in research laboratories. However, infectious diseases will remain among the major areas for application of PCR detection and genotyping, offering the potential to analyze most micro-organism of veterinary importance by a single technique. Most of the assays to detect microorganisms have high sensitivity with purified DNA samples, but advances in sample preparation and detection of amplified products under field or clinical laboratory conditions are needed in order to achieve high sensitivity with animal specimens. Diagnosis of viral diseases should be a major target for PCR application because laboratory tests for identification of viruses are either slow, expensive or insensitive. Eradication programmes must include the diagnosis of sick animals, asymptomatic carriers and vectors and often involve the rapid screening of a large number of samples for which PCR would be very useful. In general, PCR will most likely become the standard diagnostic test in situations where either the micro-organism level is low, differentiation between morphologically identical organisms is required, or whether the immune response to the infection is uninformative. Based on the above conclusion the following recommendations are forwarded:

- PCR should be used as a routine diagnostic tool at clinic level beyond in the research laboratories.
- For confirmatory diagnosis and treatment of diseases PCR based diagnosis should be used.
- Isothermal nucleic acid identification practices should be accustomed for the diagnosis method of infectious diseases
- Further advancement of technology is needed to improve automation, optimise detection sensitivity and specificity and expand the capacity to detect multiple targets simultaneously (Multiplexing). This review provides an up-to-date look at the general principles, diagnostic value and limitations of the most current PCR-based platforms as they evolve from bench to bedside.

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