



Novel Molecular Diagnostics and Therapeutic Tools for Livestock Diseases

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14.1 Introduction

Increasing human population, urbanization and income sources are raising the demand of animal origin food (milk, meat, eggs) for fulfilment of protein deficit in developing countries. All over the world, the livestock production is gaining higher speed than other agricultural sectors, and by 2020, the livestock sector will be the most significant agricultural sector in terms of value addition.

However, the health of animals is under the constant threat of various dreaded infectious diseases, which may result in significant economic losses to livestock

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owners. Maintenance of good health status of animals by effective control and treatment of diseases lies in the access to various standard diagnostic tests which are rapid, reliable, precise and highly sensitive and therefore helps in confirming early detection of causative agent. The conventional diagnostic methods are too much demanding in terms of time and labour. Latest advances in the molecular biology and biotechnology have opened new avenues in disease diagnosis and therapeutics, but these technologies are in budding stage so need to be further explored to their full potential to safeguard the health of human in addition to their companion animals. Along with this there is a need to build up qualified, trained and competent manpower for bringing the existing disease diagnostic methods in laboratories to field of action. Because of the lack of skilled manpower in different institutions of public undertakings, it is highly desirable that the gained expertise and knowledge be disseminated to users of these techniques directly so benefits can be harnessed more efficiently and effectively.

Veterinary pathogens have been traditionally diagnosed by detecting the pathogens by culture isolation or serological detection of antigen or antibody such as neutralization tests, enzyme-linked immunosorbent assays, agar gel immunodiffusion and complement fixation tests. In the past diagnostic capabilities have been enhanced by complementing conventional assays with molecular diagnostics. Incredible progress in the molecular diagnosis and characterization of avian influenza virus infections in the past two decades is the true example of this. Moreover, recent advancements in the field of molecular diagnostics have made the speedy identification of group A influenza and H5 and H7 subtype viruses feasible, which is a major global cause for the spread of avian influenza from poultry to humans. The innovative, reliable, rapid and appropriate DNA-based diagnostic techniques have the potential to assist international efforts to check the incursion of exotic diseases into new geographic areas.

This write-up focuses on recent advancements in molecular diagnostic tools which can help clinicians in promoting human and animal health within 'One Health' concept.

14.2 Pathogen Genome-Based Diagnostics

The use of molecular diagnostics has been rampant in recent years for the detection of veterinary pathogens. These involves detection of pathogen either directly by sensing the presence of DNA or RNA in the host or indirectly by prior amplification of genome of infectious agent. These techniques have not only helped in earlier disease diagnosis but also animal disease control programmes. Further, modifications in PCR-based molecular detection techniques have generated a vast array of fast, reliable and specific assays which have widespread applications in veterinary diagnostics.

The sensitivity of any genome detection-based method can be enhanced to a very high degree by manipulating any of the three pillars of the assay, i.e. by amplification of target, signal and probe.

1. Target amplification: different assays as polymerase chain reaction (PCR), transcription-based amplification (TBA)/nucleic acid sequence-based amplification (NASBA)/self-sustained sequence replication (SSSR/3SR), strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP) come under target amplification.
2. Signal amplification.
3. Probe amplification: ligase chain reaction (LCR).

14.3 Target Amplification

14.3.1 Polymerase Chain Reaction

The revolutionary, very sensitive and specific PCR method was developed by Kary Mullis in the 1980s. It has allowed scientists to detect and amplify a unique region of DNA multiple folds under strict thermal conditions (Mullis 1990). The specificity of the assay is determined by complementarity of short stretches of synthetic DNA oligonucleotides or primers to the target sequence followed by amplification of the sequences between these primers by the thermostable DNA-dependent DNA polymerase enzyme. It is a faster technique than other methods of detection of pathogens (bacteria, fungi or viruses), which require initial isolation in culture. PCR has been used in veterinary diagnostics for specific genomic detection, e.g. infectious bovine rhinotracheitis virus, foot-and-mouth disease virus, bovine viral diarrhoea virus, buffalopox virus, ephemeral fever virus and many others. Mass level detection of avian influenza and Newcastle disease poultry pathogens in recent outbreaks in the USA has been made with PCR. The following is the list of the different types of PCR being used in the diagnosis of animal diseases:

1. Reverse transcription PCR (RT-PCR)
2. Semi-quantitative PCR
3. Nested/semi-nested PCR (nPCR)
4. Asymmetric PCR
5. Quantitative PCR (qPCR)/real-time PCR
6. Linear after the exponential PCR (LATE-PCR)
7. Multiplex PCR
8. Other variants of PCR

14.3.2 Reverse Transcription (RT)-PCR

Ever since the discovery of PCR, various changes in the PCR protocol have been developed (Erlich et al. 1991). Some of them effectively increased the diagnostic capabilities of PCR and enhanced its uses in the molecular diagnostics. Reverse transcription PCR (RT-PCR) is particularly designed for the amplification of RNA sequences by converting into complementary DNA (cDNA). After reverse

transcription, cDNA is amplified using PCR. The RT-PCR has played a vital role in diseases diagnosis especially RNA viral infections, for example, influenza viruses, rotavirus, bluetongue virus, foot-and-mouth disease virus, etc. (Erlich et al. 1991; Persing 1991).

Furthermore, *in vitro* gene expression study can be done by application of RT-PCR as the acquired cDNA retains the original complementary RNA sequences. The main confrontation in RT-PCR is the difficulties in handling low level of target RNA/mRNA. Moreover, low stability of RNA/mRNA and its susceptibility to be degraded by ribonucleases and pH change poses many problems to combat.

14.3.3 Semi-quantitative PCR

This technique helps in the approximate estimation of nucleic acids present in a given sample. For RNA quantitation, firstly, the RNA sample is converted into cDNA by RT-PCR. The internal controls/markers (e.g. Apo A1 and β -actin) are also amplified simultaneously. Amplified products are separated by agarose gel electrophoresis that are photographed on gel documentation system. The amount of ethidium bromide bound visible DNA is calculated by measuring its optical density using densitometer. The drawback of this method is the possibility of nonspecific annealing, producing false positive/negative results. The specificity can be enhanced by using highly specific primers.

14.3.4 Nested PCR

This PCR uses two sets of primer pairs for two rounds of amplification for increased sensitivity and specificity. The product of the first set of outer primer pair is used as template for the second PCR using the second set of inner primer pairs. The latter are specific to the amplified product sequence of the first PCR thereby verifying the specificity of the first round of PCR with the specific product availability of second PCR. Nested PCR (nPCR) has been used to detect a number of etiological agents of veterinary importance such as canine corona virus, West Nile virus and orf virus (Pratelli et al. 2004; Bora et al. 2015). The drawback of nested PCR is the possibility of contamination of the first amplified product. This problem can be overcome by using the primer pairs annealing at different temperatures. Secondly, ultrapure oil or wax can be used to make physical separation between two amplification mixtures as a contamination control measure (Erlich et al. 1991).

14.3.5 Asymmetric PCR

In a symmetric PCR, there is exponential growth of double-stranded DNA, whereas an asymmetric PCR generates a single-stranded DNA predominantly as it is governed by the concentration ratio of the primers (i.e. one of the strands of DNA by

the primer in excess) by linear amplification due to the use of unequal concentrations of two primers in a primer pair and a fraction of dsDNA. In the asymmetric PCR, the primer in lower concentration is quantitatively incorporated into dsDNA. Thus the diagnostic technique provides lower intensity signal in agarose gel leading to lesser sensitivity than symmetric PCR. Asymmetric PCR has been used in detection of gene mutations (Zhang et al. 2008).

14.3.6 Real-Time PCR

Real-time PCR is a modification of conventional PCR to address the need for robust quantification of nucleic acids. During this type of PCR, fluorescent signals arise due to the use of fluorescent dyes. These signals are directly proportional to the number of amplified products produced. The amount of product is measured after each cycle of PCR. The exponential phase data represent reaction yield or quantitative information based on the starting quantity of the target. Common real-time PCRs include (1) SYBR green method where the fluorescent dye SYBR green binds to random dsDNA and can also give nonspecific amplification and (2) dual dye-labelled probe method which involves the use of sequence-specific DNA probes that are labelled with a fluorescent reporter, permitting specific detection after hybridization of the probe with its complementary sequence.

There are several advantages of real-time PCR that include:

- (1) Its ability to monitor the reaction progress in real time.
- (2) It is helpful for accurate quantification of samples.
- (3) It has a wide dynamic range of detection, and being a single-tube method, post-PCR manipulations and contamination is not a problem.

RT-PCR is also extensively used for the genotyping and phylogenetic analysis (relatedness) of veterinary pathogens. Real-time RT-PCR is the most sensitive, informative technique yielding rapid results, with the only drawback of high cost of start-up and of reagents. It has been employed for detection of bluetongue virus (De Leeuw et al. 2015; Maan et al. 2015), foot-and-mouth disease virus and bovine piroplasmids (Criado-Fornelio 2007) etc.

The quantification of nucleic acids in RT-PCR is performed by two methods, i.e. the relative and absolute quantitation. In absolute quantification, exact numbers of target DNA molecules are calculated by using a calibration curve. The calibration curve is built with DNA standards. Limitation of this method is that sample and standard should have the same amplification efficiency. The relative quantification is depending on fold differences in the expression of target gene as compared to internal reference genes. The result is expressed in different expression levels of mRNA as cDNA (reverse transcription). It is an easy approach for quantification because there no need of making calibration curves.

14.3.7 Linear After the Exponential (LATE)-PCR

There are multiple reasons for considering conventional asymmetric PCR inefficient, as there are difficulties in optimization and it tends to promote nonspecific amplification and restricted concentration of one primer lowers the T_m (melting temperature) below the reaction annealing temperature. To overcome these limitations and to increase efficiency comparable to symmetric PCRs, linear after the exponential (LATE)-PCR was developed based on primer pairs purposely designed for use at unequal concentrations to yield specific single-stranded DNA products in a robust way (Pierce et al. 2005). In the LATE-PCR, detection step is distinct from the annealing and extension step, and it improves allele discrimination and increases signal strength significantly as compared to symmetric PCR (Sanchez et al. 2004). LATE-PCR does not reach the characteristic plateau like conventional PCR but ends in a nearly linear phase (Johann et al. 2015). LATE-PCR is highly appropriate for high-throughput field applications, e.g. clinical analysis, biodefense, forensics and DNA sequencing.

14.3.8 Multiplex Polymerase Chain Reaction (mPCR)

Multiplex PCR (mPCR) is a modification of PCR for concurrent amplification of many sequences by inclusion of different sets of genome sequence-specific primer pairs for different targets. mPCR is used for diagnosis of different disease pathogens in a single reaction. It can also be used to identify exonic and intronic sequences in specific genes. In multiplex PCR the design of various primer pairs is crucial so that they complementarily anneal to specific DNA sequences at more or less similar temperatures, i.e. annealing temperature should be the same for different primer pairs used in combination. A multiplex PCR assay to detect H5N1 and other human respiratory pathogens (Lam et al. 2007; Rheem et al. 2012) and mastitis in animals has been developed (Shome et al. 2011). Other variant and combination of this technique is multiplex one-step real-time PCR kits that are available with all plus points inherited (Gautam et al. 2016).

14.3.9 Other Variants of PCR

14.3.9.1 Hot-Start PCR

During the amplification of target DNA, sometimes amplification of nontarget sequences also takes place which are nonbeneficial, and their presence in huge amount decreases the amount of desired product that leads to complication in data analysis. For getting higher reaction specificity and yield, 'hot-start PCR' was developed, where the Taq polymerase enzyme stays inactivate/blocked by specific antibodies at temperatures below 72 °C, the optimal temperature of primer extension by Taq polymerase, thus avoiding non-specific amplification. The blocking

antibodies get denatured and removed at an initial step at 95 °C allowing the specific reaction to proceed (Kellogg et al. 1994).

14.3.9.2 Touchdown PCR

In this type of PCR, the annealing temperature (T_a) is deliberately brought down during the cycling process. At initial stage, T_a is set 5–10 °C higher than the T_m of the primers, while in the follow-up cycles, T_a is slowly decreased, and by the end of the amplification, T_a is below 2–5 °C from T_m (Don et al. 1991). The high temperature increases the specificity of primer and template binding leading to amplification of only target sequences.

14.3.9.3 Assembly PCR/Polymerase Cycling Assembly (PCA)

PCA involves two sets of PCR. In the first set of PCR, overlap primers are used. The product of the first PCR is used as a template for the second PCR, which amplifies final full-length product. PCA synthesizes DNA from a pool of long oligonucleotides having short overlapping segments and can be used as an alternative for ligation-based assembly (Bang and Church 2008).

14.3.9.4 Colony PCR

This PCR is specifically to screen the bacterial colonies. The bacterial colonies are transferred into a PCR master mix aseptically. The cellular DNA is released either by incubation at 95 °C (with standard polymerase) or by shortened denaturation step at 100 °C with a recombinant DNA polymerase (Pavlov et al. 2006). This technique is used for an established Fung's double-tube method for rapid detection and confirmation of *Clostridium perfringens* (Ruengwilysup et al. 2009).

14.3.9.5 Digital PCR/Emulsion PCR

The digital PCR performs simultaneous amplification for a large number of samples. These samples are present in an emulsion in a separate droplet, e.g. 454 sequencing platforms and Ion Torrent technology.

14.3.9.6 Suicide PCR

Suicide PCR is performed where very high specificity of the desired product is required, e.g. palaeogenetics. The target of this PCR is the genomic region, which has never been amplified in the laboratory with any sets of primers. This is to avoid false positive results from contaminating DNA from previous PCR reactions (Raoult et al. 2000).

14.4 Non-PCR Methods of Nucleic Acid Detection

Nucleic acid techniques (NATs) are sensitive, rapid and reliable diagnostics that are based on amplification of specific regions of pathogen genome. Though quantitative in nature, the non-amplification nucleic acid detection methods are not so commonly used because of their lower sensitivity compared to amplification methods.

These methods have less specific requirements for performing enzymatic process and also less dependency on good reagents. The property of less sensitivity of these methods is favoured to reduce carryover product contamination.

The different methods of nucleic acid amplification have been developed, e.g. rolling circle amplification technique and direct signal amplification system. In human medicine these techniques are presently being used for the detection of cytomegalovirus (CMV) and human immunodeficiency virus (HIV). These methods can be useful in the diagnosis of livestock diseases.

14.5 Signal Amplification

14.5.1 Branched DNA (bDNA) Assay

To minimize the chances of contamination during amplification, various molecular technologies have been developed. One alternate to enzymatic amplification of target nucleic acid is hybridized probe-based signal amplification. The most common signal amplification technologies are branched DNA (bDNA) and hybrid capture (HC) assays (Datar and Joshi 2001).

In bDNA technology, the presence of specific nucleic acid is detected by measuring the signal generated by specific hybridization of many branched labelled DNA probes on an immobilized target nucleic acid. One end of bDNA binds to the immobilized target DNA, and the other end of it has many branches of DNA which amplify the detection signals. The amplification of signals is linear and is achieved by sequential or simultaneous hybridization of synthetic oligonucleotides. Final detection uses alkaline phosphatase (AP) as it generates chemiluminescence. bDNA is a quantitative technology and is used in the determination of viral or pathogen load (Cao et al. 1995; Kern et al. 1996; Collins et al. 1997). This technology is a highly sensitive, specific, less labour-intensive, less contamination prone and reliable tool in the diagnosis of viral and bacterial infections and for monitoring disease status during the course of therapy. This technology has been proved versatile since it helped in the detection of infections by a wide range of microorganisms (Tsongalis 2006).

14.5.2 Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA, introduced in 1989, is an isothermal amplification method which is carried out at a constant temperature of 41 °C. It is also called self-sustained sequence replication and transcription-mediated amplification. Three enzymes, viz. avian myeloblastosis reverse transcriptase, RNase H and T7 DNA-dependent RNA polymerase, are involved in the process (Fakruddin et al. 2012). It is a sensitive, isothermal, transcription-based amplification system which is specifically designed for the detection of RNA targets (Van Gemen et al. 1995; Deiman et al. 2002).

The major advantage of NASBA is the production of single-stranded RNA amplicons. These amplified RNA products can be sequenced directly with a dideoxy method using RT and a labelled oligonucleotide primer. Additionally, the ssRNA amplicons can either be amplified or probed for direct detection. The technique can be easily adopted for the development of various pathogen detection kits (Fakruddin et al. 2012).

14.5.3 Transcription-Based Amplification System (TAS)

TAS was developed by Kwoh and his coworkers in 1989. In TAS, RNA is the target molecule as well as primary product (Kwoh et al. 1989). A copy of DNA is made from RNA, and then transcription produces million copies of RNA. The variations of TAS are transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA) and self-sustaining sequence replication (3SR). In the process, the primer complementary to the sequences that has the binding site for RNA polymerase is added to the targeted RNA molecule. Three enzymes, reverse transcriptase (RT), RNase H and T7 DNA-dependent RNA polymerase, are used in the amplification reaction. The primer anneals and then RT enzyme is used to produce cDNA copy of target RNA. RNase H then degrades the initial RNA from DNA-RNA hybrid. The second primer set binds to newly synthesized cDNA which is extended to produce double-stranded cDNA. Both the strands of this ds cDNA can act as a template for RNA polymerase for transcription.

TAS has few advantages over various amplification processes: (1) TAS is an isothermal process, negating the requirement of thermal cycling to drive reactions due to which it helps to minimize contamination risks (Guatelli et al. 1990). Apart from direct detection of RNA-containing viruses, e.g. hepatitis C virus, this technique can be used for detection of low amount of certain bacterial and fungal pathogens (Compton 1991). The only demerit of TAS is heating step which denatures the enzyme due to which enzyme is needed after every denaturation step.

14.5.4 Strand Displacement Amplification (SDA): Loop-Mediated Isothermal Amplification (LAMP)

14.5.4.1 Strand Displacement Amplification (SDA)

SDA, a non-PCR nucleic acid amplification technique, developed in 1991, involves DNA polymerase-initiated DNA synthesis. It works on a single-stranded nick followed by the displacement of the nicked strand during the process. The displaced strand is used as a substrate for further simultaneous nicking and displacement reactions. SDA uses specific primers, a DNA polymerase and a restriction endonuclease, to achieve exponential amplification of target. In brief, this method makes copies of the target sequence flanked by nickable restriction sites, allowing the exponential increase of these modified target sequences by recurrent nicking, strand dislocation and further priming of banished strands (Spargo et al. 2000).

SDA has two important advantages: (a) SDA is isothermal with the exception of the initial denaturation step and (b) it can be applied to both single- and double-stranded DNA (Kim and Easley 2011).

Loop-mediated isothermal amplification (LAMP) assay, initially described by Notomi et al. 2000, is an important method for disease diagnosis especially in the developing countries (Abdullahi et al. 2015). LAMP is a simple, quick, highly specific and cheap single-tube technique for the amplification of DNA. LAMP can be used as a simple test in the field or at the point of disease outbreak.

In LAMP test, four different types of primers, a forward inner primer (FIP) and a backward inner primer (BIP) set and a forward and backward outer primer set, are used. These primers recognize six distinct binding sites on target DNA. LAMP uses Bst DNA polymerase having strand displacement activity. Both primers and enzymes work together for LAMP cycling initiation and continuation of DNA synthesis with auto-strand displacement leading to the accumulation of 10^9 copies of target in less than an hour. DNA synthesis is primed by FIP and BIP, in combination with initial strand displacement mediated by the extension of forward and backward outer primers, resulting in the successive formation of a DNA molecule. This DNA molecule is looped out at one end, further by a dumb-bell DNA structure, which is rapidly transformed to a stem-loop structure which is the substrate for the elongation and recycling step of LAMP.

LAMP-based commercial detection kits for bacterial and viral pathogens are available. LAMP has also been developed for important animal pathogens, e.g. foot-and-mouth disease virus (Yamazaki et al. 2013), bluetongue virus (Maan et al. 2016), capripox viruses (Batra et al. 2015) and peste des petits ruminants virus (Zhan et al. 2009). The process demands the use of multiple primers where a major disadvantage of LAMP over PCR lies, that is, the need of frequently updated primer sequences in order to detect the prevalent virus strains with adequate sensitivity and specificity.

14.6 Probe Amplification

14.6.1 Ligase Chain Reaction

Ligase chain reaction (LCR) is a probe-based amplification technique and was first described by Wu and Wallace (1989) to detect point mutations. In this technique one probe is formed by ligation of two adjacent probes. The main characteristic of LCR is the second set of primer which is complementary to the first pair, designed with the nucleotide at the 3' end of the upstream primer indicating the sequence difference. When target DNA is present, the two adjacent probes are ligated by DNA ligase. If ligated product is absent, that means there is at least a single base-pair change in the target sequence. In the following steps, the ligated products can serve as templates and can be amplified by thermal cycling in an exponential manner (Wiedmann et al. 1994). In LCR contamination risk and variation in copy number of the plasmid containing the target is a problem (Umesha and Manukumar 2016).

14.6.2 RNA Aptamers

RNA aptamers are defined as RNA oligonucleotides (56–120 long) having variable and constant region that bind to a specific target with high affinity and specificity (Ellington and Szostak 1990). The method for isolation of aptamers is called systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990).

RNA aptamers are an important tool in RNA nanotechnology as compared to DNA and protein aptamers (Germer et al. 2013). They are easy to synthesize in huge quantity under controlled way having well-defined structure and stereochemistry. Similar to antibodies, RNA aptamers have low immunogenicity as compared to other macromolecule such as proteins. RNA aptamers are more thermodynamically stable and its unique tertiary structure leads to specific binding. It is small in size due to its single-stranded nature so it can easily enter into the cell.

RNA aptamers can be used in different fields of science, i.e. diagnostic, prognostic and therapeutic (Germer et al. 2013).

14.7 Rapid Whole-Genome Sequencing Technologies

Nowadays whole-genome sequencing techniques are being extensively utilized to study a wide variety of infectious agents. With the advent of high-throughput ‘next-generation’ sequencing technologies, detailed analyses of entire pathogen genomes have been made possible within a few days which previously took many years. Different rapid whole-genome sequencing platforms as Illumina, 454 and Ion Torrent have tremendously revolutionized the world of disease diagnostics by enhancing the ability to rapidly screen complex mixtures of genomes. It has become easier to make comparison between diseased and apparently normal/healthy states of patients. No prior knowledge is required about the infectious agent to be sequenced, so it plays an important role in the detection and identification of new and emerging pathogens. The latest sequencing methods hold promises in increasing the present knowledge regarding the evolution of pathogenic microorganisms especially with respect to the development of antimicrobial resistance. Expensiveness is always a matter of consideration when any diagnostic assay or kit is developed. At present whole-genome sequencing technologies particularly the next-generation sequencing (NGS) are very costly, but as per the increasing demand and use in different field of scientific research, diagnostics and many others, it can be expected to become cheaper in the future. Some of the NGS platforms are briefly described below.

14.7.1 454 Genome Sequencer FLX (Roche Applied Science)

The very first next-generation system brought in the market was Genome Sequencer (GS) instrument based on the principle of pyrosequencing by 454 Life Science in

2005 (Margulies et al. 2005). Traditional sequencing methods required cloning of the gene to be sequenced, but this demerit was removed by 454 GS pyrosequencing system. Briefly, the procedure involved initial random shearing of genome (may be enzymatic or physical by ultrasonication), followed by ligation of both the ends of each fragmented DNA genome with specific adaptors. These adaptors allowed the DNA molecule capturing on the surface of beads. Now these ligated fragments were amplified (emulsion PCR) and captured in emulsion droplet. Individual bead with amplified fragment arrayed in a well of a fibre-optic slide. The 454 GS followed the same sequencing principle as of most primitive Sanger sequencing method. In the 454 method, when a new base was incorporated in the growing chain, a pyrophosphate group was released and detected as emitted light by a CCD imager coupled to the fibre-optic array.

The main disadvantage of this system was high error rates as insertion and deletion mutations (Indels) were produced because of the false reading of template by misjudging the homopolymers. To increase the read lengths, a new GS FLX Titanium XL+ has been developed which is able to generate 700 Mb of sequence data with 99.997% accuracy. The system involves a relatively high cost and lower throughput than other developed next-generation sequencing methods and therefore has decreased its preferences.

14.7.2 Ion Torrent

Ion Torrent is known by different synonyms as ion semiconductor sequencing or pH-mediated sequencing or silicon sequencing. It is based on the principle of detection of hydrogen ions by ion-sensitive field-effect transistor (ISFET) ion sensor that is released during polymerization reaction of new DNA strand synthesis. This is a method of sequencing by synthesis, i.e. sequence data of template is generated, while complementary strand is synthesized. The labelled or modified nucleotides and optical instruments are not employed as done in other sequencing methods. This system also has various limitations like difficult enumeration of long homopolymer repeats. Signals obtained from high repeats of different nucleotides create obstacles as differentiation could not be made from that of similar but different number homopolymer repeats. It gives an average read length of 400 nucleotides per read, which is a shorter read length compared to pyrosequencing and Sanger sequencing methods. Thus this is best suited for small-scale applications as microbial genome/transcriptome sequencing, amplicon sequencing or quality testing of sequencing libraries.

14.7.3 Illumina

Illumina NGS technology was developed by Canard and Sarfati in 1994 as a second next-generation sequencing technology, which uses the reversible termination chemistry concept (Canard and Sarfati 1994). This concept allows the identification

of single-nucleotide base as it is polymerized into new DNA strand, i.e. sequencing by synthesis. It is multipurpose solving as has been used in whole-genome sequencing, transcriptome analysis, metagenomics, small RNA and methylation profiling and also helpful in the analysis of protein and nucleic acid interaction. It is also known as Solexa, released in 2006 as Solexa sequencing platform. Solexa platform uses sequencing by synthesis (SBS) technology where fluorescently labelled nucleotides are used as terminating base. The latter when removed leave an unblocked 3' terminus making the process of chain termination reversible.

The Illumina technology involves DNA fragmentation, ligation with specific adapters and followed by denaturation. The single-stranded denatured templates are then immobilized on one end on a flow cell surface which is already laid with complementary adapters. Immobilization of every fragmented template strand on flow cell surface occurs by hybridization of free end to the complementary adapter. It is followed by amplification, which results up to 1000 identical copies of every ssDNA template molecule named as DNA 'colonies'. During the cycling process of sequencing, as a single fluorescent dye-labelled dNTP is incorporated, the fluorescence of dye is imaged by the CCD camera for identification of the base, and then the dye is enzymatically cleaved, which allows incorporation of the subsequent nucleotide.

14.7.4 Microarray Technology

Microarray technology is very powerful and high throughput which can be used for expression studies, transcriptome analysis, detection and characterization of genetic variants, DNA-protein interaction study and detecting genome methylation. The important feature of this method is immobilization of different molecules (oligonucleotides, proteins, small drug-like compounds) onto a solid and activated surface as matrix (Skena et al. 1998). This matrix arrangement is called microarray. Microarray plate has specific point at which high concentration of immobilized molecules are present to interact with their targets. These are named as microchips, biochips, DNA chips or gene chips to detect DNA in diverse biological samples. The advantages of microarray technology are the following: the expression of the entire gene content of a genome of interest can be monitored and it also provides data analysis in field conditions because detection is simple and rapid and also provides real-time data analysis (Zhou and Tompson 2004).

14.7.5 Nanotechnology

The term 'nanotechnology' was given for the first time by Norio Tangiuch in 1974. This technology includes manipulation in atoms and molecules at very small scale that is nano in size (Savage et al. 2007; Medina et al. 2007). Till date so many types of nanoparticles are obtained from transition metals, silicon, carbon and metal oxides (Torres-Sangiao et al. 2016). This nanomaterial shows different type of

physico-chemical properties, thereby widening its application area. This technology is very helpful in designing molecular diagnostic assays, pen-side test or chip-based diagnostics for medical and veterinary fields. The very small size also increases its use in nanomedicine (Jos et al. 2009).

14.7.5.1 Gold Nanoparticles for Use in Diagnostics

Gold nanoparticles (AuNPs) are very flexible nanostructures and can be used in different field of biomedical science (Mirkin et al. 1996). AuNPs show so many useful properties like more flexible structure, defined size, shape, structure and better optical properties. These particles are being used for diseases diagnosis as biosensors (Dilbaghi et al. 2013; Mirkin et al. 1996; Shah et al. 2014).

14.7.6 Proteomics

The whole set of proteins expressed by a genome, cell, tissue or organism is termed as 'proteome'. The study and characterization of complete set of proteins is called as 'proteomics' (Anderson and Anderson 1998). Proteomics recently in the last two decades has emerged as a field of research and has developed rapidly (Ceciliani et al. 2014a, b).

Proteomics is a better approach than genomics for studying changes in metabolism under stress conditions. Various methods in proteomics, viz. 2-D gel electrophoresis (2DGE), MALDI-TOF/MS, etc., play a significant role in the analysis of novel proteins and in disease diagnosis. The expression of proteins depends on the status of the body and different environmental factors. This field of study has an important role in novel drug discovery and in the early-stage disease diagnosis (OIE 2016). Proteome maps are being derived from a range of veterinary pathogens (Mujer et al. 2002; Rout and Field 2001; Yatsuda et al. 2003). Proteomics-based diagnosis may be used to identify known or unknown disease markers in the future. Proteomics may have applications in the diagnosis of disease pattern development in combination with biochip technology, bead technology, mass spectrometry and other separation chromatographic methods. This type of combination of technologies can be useful for identification of pathogens that do not induce predictable serological reactions, i.e. bovine tuberculosis.

14.8 Conclusion and Future Applications

Molecular diagnostic assays are becoming more popular for confirmation of field-based diagnosis of livestock diseases on the basis of clinical signs and symptoms. Recent advancements of diverse biotechnological tools promise for improvement in the speed and accuracy of diagnostics for both human and veterinary medicine. This can also support the development of advance epidemiological tools and data for various animal and human pathogens, thereby allowing prioritization and implementation of appropriate biosecurity measures during outbreaks more effectively and saving valuable national funds.

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