Application of Microarray in Animal Disease Pathogenesis and Diagnosis

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Abstract

Microarray is a plethora of multiple genetic data. It is a collection of microscopic spots contains picomoles (10^{-12} moles) of a specific sequences (DNA, cDNA, cRNA, Oligonucleotides), known as *probes*. These probes hybridize with specific nucleic acid sequence called targets which are labelled with a fluorescent dye. Hybridization property between nucleotides were utilized in this and the signals produced are scanned and image data is converted to raw data using various softwares. Microarray has number of applications in the area of evolution biology, pathway analysis, toxicogenomics, pharmacogenomics, forensics, oncology and disease diagnostics and characterization. The genes responsible for several stages of cell to cell interaction of pathogens from adhesion to entry into the host cell and evading the host immune mechanism can be studied. Comparative genomic hybridization, not only yield information of individual genes in different tissues (normal or diseased, cancerous or non cancerous cells), but also on the regulation of different genes regulated pathways. Studying the molecular signature of pathogens paves an easy way for their detection. Moreover, these informations are utilized for better diagnostics, vaccine and drug development. Use of microarrays during outbreaks and/or disease surveillance would save time and help in early decisions to control the spread of disease. Thus, microarray proved to be a perfect drive in animal disease diagnostic. In veterinary field only few studies were done to interrogate the gene expression profile and detection of pathogens. While they have the potential, detection microarrays are yet to be used as routine surveillance tools in veterinary because of high sophistication, and cost per test is still high compared to other diagnostic methods. With all these advances many novel techniques born out of this technology with less cost and minimum training will play increasing role in disease diagnosis in near future.

Keywords: Microarray; Animal disease; Pathogenesis; Diagnosis

Introduction

Two decades ago scientists were using a number of molecular techniques for studying the genes, mapping them, mutating, cloning, sequencing and analyzing the protein that encodes. They studied one or few genes at a time. Hence microarray was proven to be a good tool for detection of thousands of gene simultaneously. Species specific primers in PCR reactions won't work well in detecting multiple pathogens in mixed infections. Microarray stands in between conventional method PCR and the latest technique like Next generation sequencing in terms of its cost, process time, sensitivity, specificity and the ability to detect unknown pathogen in the sample [1]. It's a rapidly evolving technique in clinical diagnostic and molecular research. Early diagnosis of an infectious disease is always desirable to prevent its spread among livestock species and thus reduce the economic losses both to the livestock owners and the country as a whole. Microarray was used to study infectious processes of pathogens, in diagnostics, and to study host-pathogen interactions. The genes responsible for several stages of cell to cell interaction of pathogens from adhesion to entry into the host cell and evading the host immune mechanism can be studied [2]. These informations are utilized for diagnostics, vaccine and drug development. Studying the molecular level pathogenesis in disease conditions helps in transfer of many basic science knowledge to clinical practices and it become fruitful for the prognosis of disease. Genome projects on many infectious organisms and the hosts provide a huge data of sequences, which made advantageous in developing more specific probe for diagnosing more diverse infectious agents. Microarray thus proved to be a perfect drive in animal disease diagnostic.

A microarray is a collection of microscopic spots arranged on a solid surface like glass, silicon, plastic or nylon. Each spots contains picomoles (10^{-12} moles) of a specific sequences (DNA, cDNA, cRNA, Oligonucleotides), known as *probes*. These probes hybridize with specific nucleic acid sequence called *targets* which are labelled with a fluorescent dye. Core principle behind working of microarray is hybridization property between the nucleotides. The property of complementary nucleic acid sequences is to specifically pair with each other by forming the hydrogen bond between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence mean tighter the non-covalent bonding of base pairs. After washing off, the tightly paired strand remains hybridized. Fluorescently labeled target sequences bind to probe and generate signals. The signal strength depends on the amount to target sequence bound to the probe. Relative intensity of a spot is compared to the intensity of another in different condition. The general workflow of an array is as follows (Figure 1).
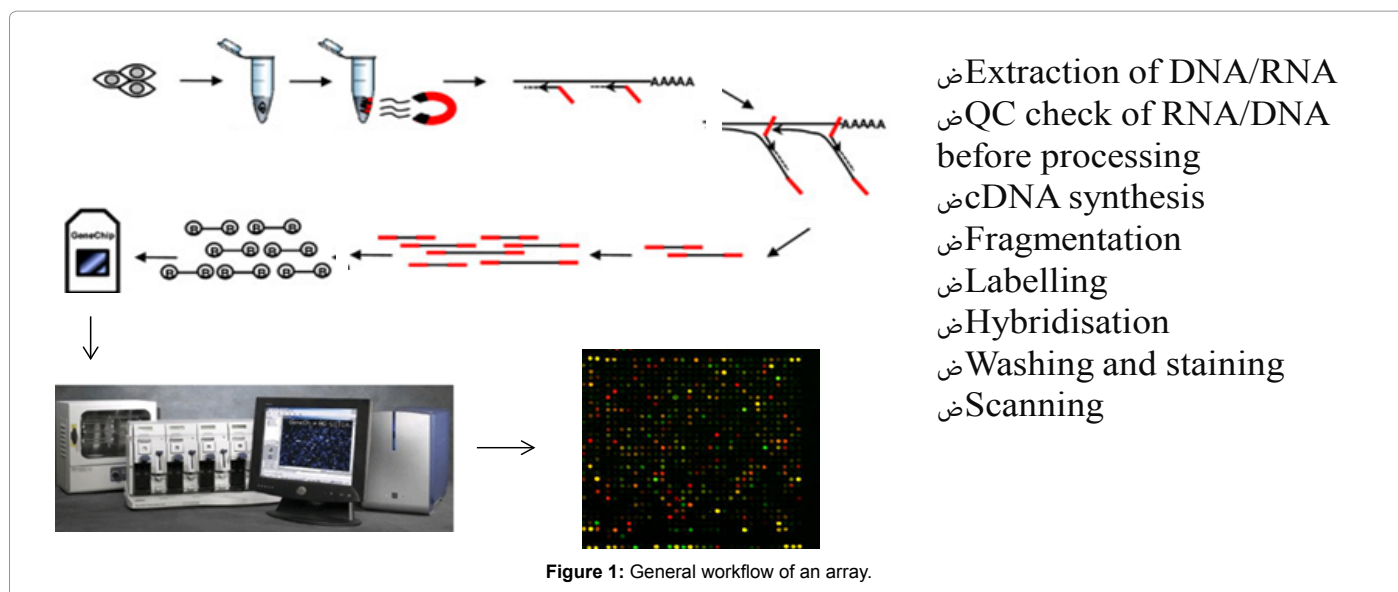
History of Microarray

Microarrays are powerful tool among the latest technologies for rapid, precise, reliable, and efficient with high through put for detection and diagnosis of pathogens causing diseases in animals and

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Figure 1: General workflow of an array.

ضExtraction of DNA/RNA
ضQC check of RNA/DNA before processing
ضcDNA synthesis
ضFragmentation
ضLabelling
ضHybridisation
ضWashing and staining
ضScanning

humans [3]. Microarray technique evolved from southern blotting technique in mid of 90's. The first microarray was commercialized in 1994 by Ron Davis and Patrick Brown of Stanford University. Array was commercialized in 1996 and in 1997 the whole genome expression of eukaryotic cell (*Saccharomyces cervisiae*) was first done using microarray. In 1999, Todd Golub and colleagues used microarrays for the first time to classify cancers. In 2002 when there is a havoc of SARS, the unidentified virus was identified as an unknown corona virus by using microarray [4]. The chip used had conserved probes for virus genera, hence the virus was identified as an unknown corona virus. The chip used for SARS-corona virus was subsequently developed as full-fledged microarray chip for virus diagnosis and was given the name Virochip. In 2004, Roche company released Amplichip CYP450, the first FDA-approved microarray for diagnostic purposes. In 2012, a chip was designed for the first time by IVRI scientists in the division of biochemistry for the detection of viral diseases affecting livestock's [5].

General Applications of Microarray

There are different types of microarrays are available in market nowadays. Among that the most widely used is DNA microarray. It has number of applications in the area of evolution biology, pathway analysis, toxicogenomics, pharmacogenomics, forensics, oncology and disease diagnostics and characterization. Major application of this technique was found in the field of oncology as classification of tumors, prediction of prognosis of tumors as an expression profile, single nucleotide polymorphism to study the mutation of gene. Also, to study the gene amplification, deletion, identify the copy number of genes using comparative genomic polymorphism. Re-sequencing arrays are used to identify the somatic mutation in cancers by sequencing a portion of genome. DNA microarray is used for the diagnosis of infectious diseases in humans of bacterial, viral, fungal and of parasitic origin. Recently emerging diseases like Nipah virus encephalitis, avian influenza, dengue fever, and West Nile encephalitis and re-emerging such as malaria, measles, foodborne pathogens; and antibiotic-resistant super bacteria such as methicillin-resistant Staphylococcus aureus, vancomycin resistant *S. aureus*, and multidrug-resistant *Mycobacterium tuberculosis* should be diagnosed as early as possible.

In veterinary field, only few studies were done to interrogate the gene expression profile of pathogens.

Applications of DNA Microarray in Veterinary Disease Diagnostic

Although microarray has been mainly used nowadays in research settings, for comparative genomic hybridization, gene expression analysis and mutation analysis of normal or diseased, cancerous or non-cancerous cells are studied [6]. Comparative Genomic Hybridization (CGH) of DNA explains the patterns of gains and losses which might prove to be useful for the classification of tumours, a risk assessment of premalignant lesions, and the prognosis prediction of cancers [7]. Gene expression analysis with cDNA, not only yield information of individual genes in different tissues, but also on the regulation of different genes regulated pathways. Veterinary diseases studied by gene expression analysis in dogs includes cardiomyopathy [8]; degenerative mitral valve disease [9]; atopic dermatitis [2]; pancreatic acinar atrophy [10]; and malignancies of the central nervous system [11]. In other animal diseases like bovine mastitis [12] and equine osteoarthritis has also been studied.

Application in Detection of Various Pathogens in Animal Disease Diagnosis

Viruses

The first broad range virus identification chip was used for detection of SARS-corona virus and in case of animals the chip used for identification of FMD (Foot and Mouth Disease) virus [13]. FMD DNA chip having 155 oligonucleotide probes, 35-45 bp long, designed from VP3-VP1-2A region of virus and it is serotype specific and labelled with Alexa-Fluor 546 dye. Animal Viruses Probe Dataset (AVPDS) is used for microarray based diagnosis and identification of viruses' probes. Currently the dataset contains 20,619 virus specific probes for 833 viruses and their subtypes and 3,988 conserved probes for 146 viral genera. Baner et al. [14] reported a chip was developed for three viral vesicular diseases (FMDV, Vesicular stomatitis and Swine vesicular disease) in animals. Multi pathogen or pan viral DNA microarrays for detection of orbiviruses have been developed [15]. Long oligonucleotide

microarray assay was also designed to identify viruses that cause vesicular or vesicular-like lesions in livestock animals. Using this array the genus level of FMDV, VSV, swine vesicular disease virus, VESV, BHV-1, orf virus, pseudocowpox virus, bluetongue virus serotype 1 and Bovine Viral Diarrhea Virus 1 (BVDV1), Enteroviruses [16] were detected separately. North American Veterinary Diagnosticians opined that DNA microarray as one of the suitable diagnostic tool for Blue tongue virus and Epizootic haemorrhagic disease affecting animals [17]. Similarly virus of genus Pest and Flavi, mainly classical swine fever virus, border disease virus, BVDV1 and 2 was also identified using a magnetic bead microarray [18]. Detection of FMD virus and PRRS (Porcine reproductive and respiratory disease) virus of swine by means of microarray chip was developed by Liu et al. [19].

In India, a microarray chip for diagnosis of virus in livestock was first designed by a team of scientists in IVRI. The chip contains unique and conserved probes for the identification of species and genera specific viruses [5]. In India, first case of Newcastle disease virus in non-avian host (sheep) and a mixed infection of Bovine viral diarrhoea and Bovine herpes virus in cattle were diagnosed using this chip [20].

Bacteria

Various diagnostic microarray chips developed in the field of microbiology for identifying pathogens. Among that the most important was Pathochip in which 23S rDNA and 16S-23S rDNA ISR sequence of bacteria was analysed. Probes for detecting drug resistance of *Mycobacterium* and an array used for detection of *Mycobacterium* spp. based on gene sequence upstream of 65 kDa heat shock protein gene also been reported [19]. Identification of factors leading to resistance of microbe such as Mycobacterial infections [21] were studied. Rapid tracking of the pathogen and its various strains helps in developing strategies for prevention and serotyping provide information of vaccine selection during an outbreak [13]. A spotted array has been developed in 2009 for bacterial pathogens, consisting of 489 (70mer) probes to detect 40 bacterial pathogens of medical, veterinary and zoonotic importance [22]. Emerging diseases and bio-defense agents are great challenge in the 21st century, so for identifying those biological threats a new forensic array was developed [23]. Microarray chip was developed to characterize 20 MRSA (*Methicillin resistance Staphylococcus aureus*) in livestock's in Switzerland [24]. Panmicrobial microarray (*Greene chip)* was found to be useful when an outbreak occurs in an area endemic for more than one pathogenic agent overlap with time and geography. This chip has a large number (about 29, 455 sixty mer) of oligonucleotide probes for different pathogens [25]. Easy Operating Pathogen Microarray (EOPM) was another high through put pathogen identifying array which consist of 2,110,258 bacterial 16S rRNA sequences, 621,351 fungal 18S rRNA sequences, and 1,735,744 parasitic 18S rRNA sequences [26]. Recently, an ArrayTube (AT) platform, with chromosomal and plasmid coded targets for the detection of *Coxiella burnetii* [27] was also developed. This is found suitable for detection, differentiation and genotyping of *Burkholderia mallei/pseudomallei, Brucella* spp.*, Bacillus anthracis,* and *Chlamydia* spp too. This kind of creating all in one assay is useful in screening of individual pathogens with similar symptoms and multiple pathogens in co- infection [28]. Typing of new emerging species/ strains, and understanding evolutionary relationships among bacterial species also be done with DNA microarray. Toxin array for genotyping of *C. perfringens* toxins based on oligo probes of six toxins [29]. DNA microarray is currently used to identify the presence of drug-resistance genes/plasmids in the infectious agents; the data thus obtained helps in developing better therapeutics [30].

Parasites

The gene expression during development of parasite at different stages of life cycle reveals the unique metabolic and physical properties of that developmental stage. During exponential growth phase of *P. falciparum* grown in red blood cells revealed that glycolytic and other metabolic enzymes are upregulated. At the late schizonts a reduced global transcriptional level except for a subset of genes whose transcripts encode proteins involved in signalling [31]. In case of *Toxoplasma gondii* transition from tachyzoites to bradyzoites shows the genes encoding metabolic enzymes and bradyzoite secretory antigens were upregulated [32]. Among different parasitic disease affecting the livestock, differentially expressed genes of *Eimeria spp*. were studied. To study the molecular basis of sporulation and invasion of the precocious line of *Eimeria maxima* was analysed using DNA microarray [33]. Han et al. [34] reported some new and important genes involved in the development and invasion of the early stages of *Eimeria tenella*. Gene expression changes in 3 different developmental stages like unsporulated oocysts, sporulated oocysts, and sporozoites of *E. tenella* was assayed using three subtractive cDNA libraries.

Pathogenesis of Certain Animal Diseases Studied Using Microarray

By understanding the molecular basis and the crosstalk between a pathogen and a host the knowledge about pathogenesis improves. This paves way for better diagnosis, prevention and treatment of diseases. Among different livestock animals the host pathogen interaction was studied frequently in bovine species. The work was mainly focussed on normal physiology, pregnancy, lactation, and parturition of the animal [35]. The response of bovine tissues to various pathogens using a bovine microarray was studied by Band et al. [36]. The molecular level pathogenesis of Bovine Spongiform Encephalopathy (BSE) in cattle was identified through the genes that are differentially expressed (DE) between experimentally infected and non-challenged cattle. It was found 182 DE genes were identified between normal and BSE-infected tissues (>2.0-fold change, P<0.01); majority (81) of the genes had ontology functions, which included synapse function, calcium ion regulation, immune and inflammatory response, apoptosis, and cytoskeleton organization; some (13) genes were found to be involved in 26 different Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. The expression of five DE genes associated with synapse function (tachykinin, synuclein, amphetamine-responsive transcript, and synaptosomal-associated protein 25 kDa) and three DE genes associated with calcium ion regulation (parvalbumin, visinin-like, and cadherin) [37].

In vivo study of bovine rota virus and corona virus suggested that 13% of gene expression for specific mucosal epithelial cells were similar [17]. Bovine leukemia virus becomes a study model for oncogenesis. The transcripts of genes included those of apoptosis, DNA transcription, cell cycle regulators, protooncogenes, and Tax responsive genes and it was found that Tax is the main factor which is responsible for malignancy in bovine leukaemia viral infection [38]. African swine fever viral virulence within the macrophage was examined in a swine cDNA microarray and found multigene family 360 and 530 (MGF 360/530) of ASFV affects viral growth and virulence in pigs. By comparing the pig macrophage infected with this gene caring virus to a mutant virus the actual mechanism of novel gene was defined. Cells have intact virus suppressed IFN response by ASFV MGF 360/530. Whereas mutant strains have a growth defective phenomenon due to its inability to suppress this gene [39]. Differentially expressed genes in susceptible

and resistant pigs in *Actinobacillus pleuropneumoniae* infection was studied [40].

A cDNA microarray was used to search signalling mechanisms of bovine mammary epithelial cells in response to *E. coli* LPS during mastitis and four genes were validated for this reaction [41]. When compared with paratuberculosis infected macrophages to *M. avium* subspp. *avium* the apoptosis regulating genes were found to be up regulated and immune related genes were down regulated [42]. The intracellular survival of salmonella was unknown in earlier days. With advances in using a DNA microarray of *S. enteric* serovar Typhimurium LT2 genome, the transcriptomes of the wild type and a mutant carrying point mutation in the polynucleotide phosphorylase (PNPase) gene were analysed. The point mutation in PNPase affected invasion genes in *Salmonella* Pathogenicity Island (SPI 1) and genes involved in intracellular growth of the organism in SPI 2 [43]. The transcriptome of intramacrophage *S. typhi* and *S. typhimurium* were studied to reveal the differential expression of various genes involved in the intracellular survival of the pathogen [44]. It was found that the genes encoding resistance to antimicrobial peptides were upregulated, whereas the flagellar apparatus, chemotaxis, and iron transport genes are downregulated in intracellular pathogens.

The genomic comparison between *Mycobacterium avium subsp. Paratuberculosis* strains of sheep and cattle (CM00/416 and 316v) by DNA microarray uncovered two new large sequence polymorphism and deletion of 20 ORF in sheep strain which is responsible for host specialization and pathogenicity between these strains [45]. The incidence of *E. coli* O157:H7 in food animals creates major public health concern. To study the pathogenesis and physiology of *Escherichia coli,* microarray is widely used [2]. The expression of genes of *Mycoplasma hyopneumoniae* in swine grown under different conditions was scanned on a microarray slide having 632 open reading frames of the pathogen. The study identified a differential expression of heat shock proteins [46]. DNA array analysis showed many genes were found to be upregulated, including regulatory genes, metabolic, and virulence-associated genes of *M. avium* subsp. *Paratuberculosis* during intracellular survival within mammary epithelial cells (MAC-T). In case of *Rhodococcus equi* in addition to plasmid virulence genes, the chromosomal virulence genes were associated with survival of organism within macrophages was assayed using a spot based microarray [47]. Another study by Cumming et al. [48] for 42 *Bordetella* strains revealed LPS O antigen was important in host susceptibility and fim2 gene loss as main factor in evolution of the organism.

Applications of Protein Microarray in Veterinary Diagnostics

Since DNA microarray can monitor up to mRNA level, there are certain proteins in post translation stage which are not regulated by genes. One of the fundamental problems in disease diagnosis is the limitation in detecting the serodiagnostics antigens. These factors lead to development of a novel technology based on protein array for microbial diagnosis. Protein microarrays are based on any miniturized ligand-binding assay that placed on a protein microarray chip. The product is formed with formation of an immobilized capture molecule on the chip and a target molecule present in the solution. Antigen antibody assay, nucleic acid protein interaction, sandwich immunoassay, protein- protein interactions, enzyme substrate protein microarrays are the different types of protein microarray. Among different types of protein arrays the most common is the antibody microarray.

Major applications of this array are detection of antigens and antibodies in blood samples, used to discover mechanisms of pathogenesis, profiling of sera to discover new disease biomarkers and monitoring of disease states and responses to therapy [49]. Mezzasoma et al. [50] reports protein microarray was used for detection of antibodies directed against *Toxoplasma gondii*, rubella virus, cytomegalovirus, and herpes simplex virus types 1 and 2 (ToRCH antigens) in serum samples of humans. Microarray based on *Brucella melitensis* proteins revealed 18 antigens were differentially recognized in infected and non-infected goats and 10 antigens differentially expressed in humans. Among that 2 antigens only found to be common for both in naturally infected humans and goats. This suggests different immune response was shown in goat and humans for *Brucella melitensis* infection [51]. Rozek et al. [52], diagnosed the changes in cellular protein expression of an equine infected with H7N7 and H3N8 using an antibody microarray. They found out in H3N8 a pro apoptotic and in H7N7 both pro and anti-apoptotic factors were induced. Veterinary sero diagnostics for canine lymphoma using peptide micro array provided valuable informations whether the lymphoma is present or not, whether it is of B or T cell lineage and whether the chemotherapy will be short or long [53].

Applications of Tissue Microarray in Disease Diagnostics

DNA and protein microarray gives a huge data about the genes and the various proteins in microbes. For validation of data obtained from cDNA microarrays and protein expression profiling of tissues, tissue microarray was used [54]. Wan, Fortuna and Furmanski described tissue array in 1987. Tissue microarray technology involves core needle biopsies of multiple tissues constructed in the same block. It composed of small 0.6-3.0 mm cores of tissue from donor tissue paraffin blocks arrayed at a high density in a single recipient paraffin block. Analysis of various biomarkers is the most important application of this technique. In a single slide a variety of samples and disease conditions were examined. Hundreds or thousands of tissue cores are analyzed by a single immunostaining or in situ hybridization reaction and other molecular detection techniques [55]. Another advantage is gene and protein expression validated in a large number of tissue samples simultaneously. For profiling of specific proteins in cancerous as well as non-cancerous tissues also it is used. TMAs can be generated from archival formalin-fixed tissues, paraffin-embedded tissues and from fresh or frozen tissue, combined with sophisticated image analysis software for reading TMA immunohistochemistry, and a staggering amount of useful information can be generated in terms of the biomarkers useful in predicting patient outcome. TMAs based on neoplastic tissues also termed as "Tumor TMAs" are broadly classified into three types - Multi-Tumor Arrays, Progression Arrays (based on stage of tumor) and Prognostic Arrays, where tumors with known clinical end points are arrayed. In Cryo-TMAs frozen tissues which are superior to formalin fixed tissues were used for RNA and protein analysis. Also, antibodies work much better with frozen tissues. The technique multi-tumour TMAs have many different types of tissues aligned on the slide and it was first used by Kononen et al. Progression TMAs is a type of tissue microarray which examines different stages of tumor (or disease) progression within a given organ e.g., examination of tumors in the breast. These slides can then be assayed for markers of interest, or biochemical analysis of the samples can be done. In Prognosis TMAs, samples such as tumour biopsies can be taken from patients and examined. These samples can be used for clinical follow-ups to monitor the patient's progression. Data is then analyzed and compared with other clinical data.

Tissue microarray is used as a standard for the validation of diagnostic and prognostic biomarkers. Genes such as *villin* and *moesin* were differentially regulated between ovarian and colon carcinoma was identified and validated using TMA [16]. High ezrin expression was associated with earlier metastasis and poor outcome was also demonstrated [56]. The expression of p53 and Bcl 2 in avian thymus and Bursa Fabricii after irradiation and IBDV infection was also studied using tissue microarray [57]. Non-neoplastic pathology research like nuero degenerative, dermatological, cardiac and placental diseases also been achieved. Clinical applications of TMA in Pathology departments are for testing of new antibodies and probes. The optimal staining conditions are determined by using small test [58]. Used to define the functional status of the tumor prior to therapy and also analysis of hundreds of specimens from patients in different stages of disease can be done. Quality control in IHC is one of the major problems in diagnostic pathology [59-61]. The tests performed using tissue array slides are Immunohistochemistry, Routine Staining e.g., H & E, In situ hybridization, Fluorescent in situ hybridization (FISH), *In situ* PCR, RNA or DNA expression analysis, TUNEL assay for apoptosis, Morphological and clinical characterization of many tissues [62,63].

Conclusion

Microarray technology is rapidly advancing with numerous applications especially in diagnosing infectious diseases and in oncology. High through- put assays plays a critical role in molecular researches and better development in disease diagnostics. Rapid advances in the field of biotechnology and bioinformatics provide improved diagnostics in animal diseases. Use of microarrays during outbreaks and/or disease surveillance would save time and help in early decisions to control the spread of disease [64,65]. The molecular signature of pathogens paves an easy way for detection of microbes. It offers wide-spectrum detection with the capability of discovery of unknown pathogens in the sample. Consequently, increased knowledge on molecular pathogenesis and host cell responses helps in phylogenetic classification of microbes and drug development. Combination of histology and gene expression analysis helps in developing a better therapeutics and vaccines for prevention of various emerging diseases. While they have the potential, detection microarrays are yet to be used as routine surveillance tools for veterinary use because of high sophistication, and cost per test is still high compared to other diagnostic methods. With all these advances, many novel techniques born out of this technology with less cost and minimum training will play increasing role in disease diagnosis in near future.

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