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REVIEW ARTICLE

MOLECULAR PATHOLOGY: AN ADVANCED APPROACH FOR RAPID DIAGNOSIS OF DISEASES

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Abstract

In the recent times, we have witnessed some of the major outbreaks of various diseases in human beings and animals and the ultimate conclusion drawn is, the traditional diagnostic tools and techniques are not up to the mark at all particularly for a rapid diagnosis. Hence, there is a need of dynamic techniques with a wide spectrum of applications such as early disease diagnosis and characterization, genomic studies, early prediction of the outbreaks and novel drug development etc. The molecular diagnostic techniques are providing major break throughs in the above- mentioned fields of the medical and veterinary sciences. The advent of PCR has provided the pathologists a tool for the rapid disease diagnosis than the traditional techniques of isolation and culturing of the pathogens. Since, its introduction PCR has been a handy tool for the mass level screening of the pathogens (such as Influenza virus, Pox virus, FMD virus, and PPR virus); responsible for the major outbreaks across the world. Blotting techniques, the pivotal techniques of the hybridization techniques are based on a simple principle of immobilization of biological moieties such as DNA, RNA and proteins on a solid support system. But these techniques have a wide range of applications such as documentation of the mutations, screening of food samples for the potential threats of public health and detection of the bacteria which are difficult to culture. Microarray techniques basically traces its evolution from the blotting techniques. It is simply based on the collection of microscopic units/moieties of targeted sequences (DNA, RNA, Oligonucleotides) called as Probes. The introduction of these assays has enabled scientists to document information in the various fields such as toxicogenomics, forensics, oncology, disease diagnosis and most importantly early prediction of the outbreaks. The introduction of the Next Generation Sequencing techniques has enabled the pathologists for a rapid and accurate documentation of the whole genome sequences of almost all the organisms. These techniques have applications in almost all the fields of veterinary and medical sciences ranging from genomics to epidemiology such as identification of the new strains of pathogens, study of patterns of evolution and transmission of various pathogens during outbreaks, early prediction of outbreaks, study of mutations and the study of drug resistance. Proteomics is the most recent addition to the molecular diagnostic techniques; it is basically the study and characterization of the

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proteomes on a large scale. It finds its utility in the various aspects of the veterinary sciences such as drug development, finding of the molecular targets for the various drugs and vaccines, vaccine preparation, study of the pathogenesis and transmission of various viruses and bacteria and the investigation of the transmission of various parasites etc. The advent of molecular diagnostic techniques has dramatically enhanced the capacities of the researchers, scientists and pathologists to document the genomes of almost all organisms rapidly, predict the transmission patterns of the diseases, take early measures to counter outbreaks, identify specified molecular targets for the new drugs and the methods to overcome increasing issues of drug resistance. These high-throughput techniques are therefore, paving way for the detailed analytical studies of host-pathogen interaction at genomics, transcriptomics as well as proteomics levels; results of which are also utilized by the policy planners to formulate the prevention and control strategies and ultimately save the country from severe economic and other losses due to morbidity and mortality in population.

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Introduction:-

The need of rapid and specific diagnostic tool(s) for detecting a wide variety of viral, bacterial, fungal and parasitic diseases of man and animals has always been an area of considerable importance for clinicians. Many infectious diseases are diagnosed by isolating, growing and identifying the infectious agent from clinical specimens. These methods demand well equipped and staffed microbiological and Pathological laboratories. Days to weeks are sometimes spent in arriving at an unambiguous diagnosis which lead into failure to meet the need for an early management of the disease by the clinicians. Although isolation and identification of the microorganisms provides incontrovertible diagnosis of the disease but rate of success is limited (Chauhan,1995). Alternatively, methods detecting seroconversions to particular micro-organisms can be of great diagnostic importance. Laboratory based diagnosis of majority of infectious diseases involving seroconversion has largely been depended on the assays using antigen-antibody reactions. Historically, agglutination tests in various forms have been used for the diagnosis of diseases such as leptospirosis, salmonellosis, brucellosis etc. Some of these agglutination tests still in use indicating their usefulness to prevail despite some inherent problems associated with these assays. Tremendous advancement has been made in the field of immunodiagnosics with a view to develop a technology which should be able to provide rapid and specific diagnosis of infectious diseases. Amongst the various immunodiagnostic tools enzyme immunoassay becomes increasingly popular for the detection of humoral immune response (seroconversion) or microbial antigens for the purpose of diagnosis (Chauhan et al., 2001).

The most popular among the enzyme immunoassays is the Enzyme Linked Immunosorbent Assay commonly termed as ELISA which finds a wide application in studies of infectious diseases (Chauhan, 2010). The popularity of ELISA is primarily due to its high sensitivity, excellent specificity and relative simplicity as compared to other immunoassay methods. In general, ELISA results can be read or quantitated by readily available and relatively inexpensive spectrophotometers. Minimal training is required to perform ELISA, facility licensing or special handling of reagents and wastes is not required, and there is no radiation hazard. Reagents for ELISA are relatively cheap, stable, and easy to prepare and the technique lends itself well to automation. The time required for preparation of reagents is minimal. In the performance of the assay, the time required for successive incubations may be a limiting factor in applications, although incubation periods can be shortened significantly. Despite several merits of ELISA as outlined above, the assay has some important limitations. Particularly the requirement of an ELISA reader to obtain accurate measurements mainly restricts its application to laboratory settings. The situation in many areas, particularly in developing countries where application of electrical equipment is difficult due to non-availability of electricity, is quite different. For such areas it will be important to have an enzyme immunoassay which should be as excellent as ELISA and should not require any electrical equipment like spectrophotometer. The development of an enzyme immunoassay for screening of monoclonal antibodies which basically required no equipment including ELISA reader, was named as "Dot Immunobinding Assay" because the final outcome of the assay constitute a colored dot against a white background in positive reactions. Later several modifications of this assay were developed according to the demand and convenience, and scientists working in the field of diagnostics

have demonstrated that the Dot Immunobinding Assay is as good as ELISA for diagnosing infectious and non-infectious diseases. The other commonly used name for this assay is Dot-ELISA (Chand and Chauhan, 2001).

The basics of the Dot Immunobinding Assay are similar to that of any solid phase immunoassay such as RIA or ELISA except that nitrocellulose membrane is used as an adsorbent matrix instead of plastic surface. The antigen is dotted on to nitrocellulose membrane and the membrane then first incubated with test antibody, and second with enzyme conjugated antibody directed against the first antibody. Specific antigen-antibody reaction is developed as a colored dot against the white background at the site of antigen deposition (Joshi and Chauhan, 2012). In case of negative reaction, no color development takes place at the site of antigen deposition. Development of Dot Immunobinding Assay in the laboratory is easy and any laboratory involved in immune-diagnosis of infectious disease could standardize this assay for immune-diagnosis without much cost and effort because basically no equipment is required. Several formats have been evolved for carrying out this assay for the purpose of diagnosis of infectious diseases since its development (Chand et al., 1989; Chauhan and Singh, 1992). The idea of direct deposition of antigen on to nitrocellulose membrane came into mind by looking at solid phase systems of nucleic acid hybridization to nitrocellulose. This is commonly done in dot hybridization method in which, instead of being blotted DNA fragments from a gel, the DNA sample is applied directly to the nitrocellulose membrane as a spot or dot and detected subsequently using radio-labeled probes (Chauhan and Agarwal, 2006). Similarly, in Dot Immunobinding Assay the antigen is directly dotted on to nitrocellulose membrane and then exploited for the detection of specific antibody. The steps given below will provide an insight into the protocol of this assay as to how it is developed. Basic requirements to develop Dot Immunobinding Assay include: Solid phase matrix, Antigen, Serum sample in which antigen specific antibody is to be detected (Primary antibody), Anti-immunoglobulins conjugated to an enzyme (Tracer antibody), Substrate solution according to the tracer antibody enzyme conjugate, Chemicals, reagents, glass ware and plastic ware to prepare buffers and solutions required for the assay (Chauhan and Joshi, 2012). This assay could be developed to detect either antibody in the serum sample or antigen in the clinical sample. Depending on the interest the protocol of the assay could be modified accordingly. However, the basic requirements as given above remain the same. For detection of antibody in the serum sample the indirect method is most popular as with ELISA. But for the detection of antigen the direct method has commonly been used. Dot Immunobinding Assay is an efficient method for diagnosing infectious diseases of man and animals either by detecting the presence of antibody produced against infectious agent or by detecting the presence of etiological agent or its components in clinical samples. In principle, the assay can be used to diagnose any infectious disease of man or animals (Chauhan and Chandra, 2007; Pandey and Chauhan, 2008).

Immunofluorescence techniques are being used in diagnosis of several viral and bacterial diseases since long and are considered one of the confirmatory tests for the diagnosis of rabies in animals. This provides definitive diagnosis within a few hours and is the simplest way of identifying the new isolates of viruses. The immunofluorescence test involves the detection of antigen in specimen with the help of antibody commonly labelled with dye fluorescein isothiocyanate (FITC) through direct and indirect methods. Using immunofluorescence techniques, antigen can be demonstrated in frozen tissue sections, biopsy materials, exfoliated cells and scrapped cells, Besides, cell cultures can be used for identification of viral antigens. Formalin fixed tissue sections can also be used after digestion with proteolytic enzymes to get better results. This test is simple, rapid and quite sensitive but requires a fluorescent microscope for demonstration of fluorescence (Chauhan and Singh, 1992).

Immuno-peroxidase techniques are being successfully used for the detection of antigen in tissue sections in many diagnostic laboratories. This technique requires the antibody labelled with an enzyme known as horse radish peroxidase (HRP) instead of fluorescein dye used in immunofluorescence tests and the antigen-antibody complexes are detected using an enzyme substrate, which gives colored reaction visible to eyes. It has several advantages over immunofluorescence technique and it does not require the expensive equipment like a fluorescent microscope. In immune-peroxidase technique, clear non-fading permanent preparations are produced which are visualized under ordinary light microscope. The slides stained with immune-peroxidase can be stored for a long period in the same way as other histopathological slides. This technique is also useful in studying the role of dual infections by using a second antibody directed against another antigen. Further, the immune-peroxidase techniques are valuable for retrospective studies using previous paraffin blocks. The basic principle of immune-peroxidase techniques is similar to that of immunofluorescence for detection of antigen in tissues (Chauhan, 1998). As in case of ELISA, primary antibody labelled with horse radish peroxidase is used in direct method while in indirect method the secondary antibody is labelled with enzyme. The presence of antigen-antibody complexes is detected by using diaminobenzidine tetrahydrochloride (DAB) as substrate which gives insoluble brown colour precipitate

(Chauhan and Singh, 1999). The main disadvantage of immune-peroxidase technique is the endogenous peroxidase present in various cells like leucocytes, which may give false positive results. To overcome this problem, before applying primary antibody the endogenous peroxidase activity should be removed and control slides should always be run for comparison. The immune-peroxidase techniques are not only used for detection of antigen in tissues but also for demonstration of specific antibody producing cells. Besides, tissues, cell monolayers, impression smears, exfoliated cells and scrapings can also be used for demonstration of antigen by immune-peroxidase. In immune-peroxidase method, cryosections or the tissues fixed in formol saline or buffered formalin are used but for a better contrast of staining, tissues and cell monolayers should be preserved in formol sublimate; which constitutes 9 parts saturated mercuric chloride solution and 1 part of formaldehyde. Tissues are fixed in formol sublimate for 24 hours; thereafter, the fixative is replaced by 80% ethanol (Chauhan and Singh, 2001; Singh et al., 2007).

Animal diseases reflect a wide gamut or range of pathologies and pathogenesis. The basic classification broadly includes (a) hereditary diseases, (b) infectious diseases, (c) inflammatory diseases, and (d) neoplastic diseases. But within a particular group of diseases, say infectious diseases, the pathogenesis of all the diseases are unique such as some may affect gastro intestinal tract or others may affect respiratory system. Since it is now a well-established fact that most major diseases are the end results of deviant gene expression, and that the susceptibility to disease is significantly influenced by the pattern of gene expression in target cells for a particular type of pathology. The recent development in the field of genomics is redefining our approach to the diagnostics and therapeutics. And currently molecular diagnosis is playing an important role in the clinical management of a wide array of hematologic neoplasms, tumors, infectious diseases and inherited genetic disorders. Nucleic acid- based testing is becoming an important and reliable diagnostic tool for disease with genetic component. Molecular testing may have once been considered as a tailor- made component of the research laboratories but now molecular diagnostic and prognostic techniques are of great importance within the anatomic pathology laboratories especially in the ever- expanding sphere of infectious diseases (Ravindra et al., 2008; Ravindra et al., 2009a & 2009b). Molecular diagnosis is acting as the basic foundation for any successful application of gene therapy. It is showing great utility for assessing therapy response. At present, almost more than 5% of all laboratory testing is based on nucleic acid analysis. But ever- increasing advancement in the technology has paved way for more streamlined methods of testing. In this review we first describe some of the basic techniques used in molecular pathology laboratories followed by their applications to the diagnosis, prognostication and clinical management of various diseases. The aim of this paper is to give a basic knowledge about some of the molecular techniques in diagnosis of infectious diseases of animals.

Progress in the field of life science has been so rapid that molecular biology has become one of the basic pillars on which all biological, medical and veterinary sciences are based. The molecular biological techniques have given new dimensions to understanding the disease process and its etiology. On the basis of molecular biology, the classification of the etiological agents particularly of viruses have been reorganized. These techniques are nowadays very much in use in the sophisticated laboratories world over. Though, these techniques are performed with sophisticated instruments or equipments and require specialized trained personnel; some of them can be undertaken in routine diagnostic laboratories (Chauhan et al., 2007; Chauhan and Rana, 2010).

Polyacrylamide gel electrophoresis:

The polyacrylamide gels are prepared by mixing acrylamide and bis-acrylamide. The high molecular weight complex compounds are allowed to pass through gel matrix of the polymer of acrylamide by applying electric current. Various components of the compounds are resolved in the gel system on the basis of their size and electric charge. This method has high resolution capacity which can be further enhanced by addition of sodium dodecyl sulfate (SDS) which allowsthe resolution on the basis of molecular weight of the compound. In the polyacrylamide gel electrophoresis, one requires 0.2 M sodium phosphate buffer (pH 7.2), acrylamide-bis-acrylamide ratio (22.2:0.6), ammonium persulfate (15 mg/ml distilled water), TEMED (N, N, N', N'- Tetra methyl-ethylene diamine), bromophenol blue (50 mg/100 ml distilled water) and staining solution. The phosphate buffer is boiled on burner in order to remove the air dissolved in it and cooled at room temperature. In a beaker, take 15 ml phosphate buffer, 13.5 ml acrylamide bis-acrylamide solution and 1.5 ml ammonium persulfate solution (freshly prepared). Add 50 µl TEMED solution and mix the contents. Perform these procedures in dark or with minimal light source. Take clean and dry gel tubes (10 x 6 mm) and fill them with 2 ml of the above solution after closing one end with rubber stopper or the tubes are closely fitted on the rubber of the chamber of electrophoresis apparatus. After filling the tubes with acrylamide solution, a few drops of distilled water are placed on the top of the gel solution. Place these tubes under the fluorescent light at one feet distance. After about 30 min, an interface can be seen between water and acrylamide which indicates the solidification of gels. The water layer is then removed and the tubes are

placed in gel apparatus. In 40 µl solution of protein (sample, which may be bacteria/ virus/rickettsia/chlamydia/mycoplasma in purified form), add 10 ml of tracking dye (bromophenol blue 0.05%) to each gel. Also use positive control to compare the results. The gel buffer is prepared with dilution of stock solution with distilled water in the ratio of 1:1. Layer the gel buffer over each tube to fill the tubes; both the chambers of apparatus are filled with buffer. Apply current at the rate of 5 mA per gel tube with positive in lower chamber and negative in upper chamber. Under normal circumstances, tracking dye takes about 5 hours to resolve. After the electrophoresis, the gels are removed from the tubes by pushing water from fine needle and syringe between gels and glass. The length of gel and the distance by the dye is measured. The gels are kept in test tubes or culture tubes with screw cap and the tubes filled with coomassie blue solution (0.2% coomassie blue in 50% ethanol) for 2 hours. Thereafter, de-stain the dye by keeping the gels in de-staining solution of methanol-acetic acid solution (454 ml of 50% methanol + 46 ml glacial acetic acid). Gels can be stored in 7.5% acetic acid. The bands of protein can be observed with naked eyes. The standard can also be run along with sample for comparison.

SDS-polyacrylamide gel electrophoresis:

Sodium dodecyl sulfate is added in polyacrylamide gel solution in order to have better resolution on the basis of molecular weight of the molecule. The SDS-PAGE methodology given here is used for the diagnosis of rotavirus infection of calves. The rotavirus genome segments in polyacrylamide gels are detected by ultrasensitive silver staining instead of coomassie blue. This procedure involves the direct nucleic acid extraction from feces by diluting the feces of diarrhoeic calves 1:4 with 0.1 M sodium acetate buffer (pH 5.0) containing 1% sodium dodecyl sulfate, the normal sample size used being 0.25 gm feces, which provides enough extracts for analysis. An equal volume of 3:2 phenol chloroform mixture is added to the fecal suspension and the sample is mixed for 1 min. The emulsified mixture was then centrifuged for 10 min at 200 x g and the resulting clear upper aqueous layer is taken in a sterile vial. This sample is then prepared for electrophoresis by addition of 10 µl of 25% sucrose containing 0.2% bromophenol blue to 40 µl of the aqueous layer. The 50 µl samples are loaded on to 5% polyacrylamide gel tubes (acrylamide: bis-acrylamide ratio 37:5:1), which are polymerized with 0.01% TEMED and 0.05% ammonium persulfate. The gel and electrode buffer is 0.036 M Tris-0.03 M sodium hydrogen phosphate and 0.001 M EDTA (pH 7.8). The gel tubes are 11 cm long and have 0.4 cm diameter. The electrophoresis is performed at room temperature for 12 hours at 20 mA. The gels are stained by using a washing with 10% ethanol – 0.5% acetic acid for 30 min and then soaked in 0.01 M silver nitrate for 15 min. The gels are then rinsed briefly in distilled water and the reduction step is performed with a solution of 0.75M sodium hydroxide containing 0.1 M formaldehyde and 0.0023 M sodium borohydride. Although the bands appear at this stage, the reduction is continued for a maximum period of 10 min until the bands are clearly visible. The gels are then placed in 0.07 M sodium carbonate and the intensity of staining of both the bands and the background increases slightly in 20-30 min. After that the gels are placed in fresh carbonate solution which can be stored in screw cap culture tubes for a longer period. On SDS-PAGE, 11 bands of ds RNA genome appear in gel which can be grouped into four classes -based on their electrophoretic mobility. Class I consists of segments 1-4, class II of segments 5 and 6; the tightly migrating triplet of segments, 7, 8 and 9 fall into class III and class IV includes segments 10 and 11. This unique grouping of ds RNA segments in polyacrylamide gels is considered as characteristic of rotavirus genome. In place of tube gels, one can also use the vertical slab gels for the resolution of protein or nucleic acid. The dimensions of the gels can be standardized according to the materials used for resolution (Chauhan and Singh, 1993).

Nucleic acid hybridization:

After electrophoresis, the gels are blotted on the nitrocellulose paper. For this the nitrocellulose paper is applied on both sides of the gels to get duplicate blots of the gel. After overnight blotting, the nitrocellulose membranes are slightly rinsed in distilled water and heated at 80°C for 2 hours. The membranes are then kept at room temperature till hybridization. For hybridization, specific probes are used in order to make a confirmatory diagnosis. The probes are purified, characterized, nucleic acid sequence, which can be specific for a given species of organisms (Joshi et al., 2012). The probes are of two types.

Radio Labelled Probes:

The probes are labelled with radioactive material like ^{32}P , ^{125}I or ^3H , which facilitate the identification and detection of the probe after hybridization. The blotted membranes are hybridized with the radio labelled probes for 16-18 hours at 50°C. It varies from organism to organism like bacteria/virus/ mycoplasma which have separate procedures. It requires a specific procedure unique for each disease causative agent. The hybridized nitrocellulose membranes are exposed to X-ray film for autoradiography and characterization of the blots.

Biotin Labelled Probes:

Instead of radioactive material, the probe is labelled with biotin which is detected by avidin-peroxidase conjugate by using an enzyme substrate like diaminobenzidine tetrahydrochloride. The details of avidin biotin complex assays are given elsewhere in this book. The colour developed by the substrate at the reaction site is visible by naked eye; and the blotted nitrocellulose sheets can be stored for long period. The electrophoresis of nucleic acid and protein blotting and its detection by using probes is classified into three types.

1. Southern blotting: When the DNA is electrophoresed and blotted on nitrocellulose paper and is detected by the nucleic acid probe. It is known as southern blotting.
2. Northern blotting: When the RNA is electrophoresed and blotted on nitrocellulose paper and is detected by nucleic acid probe, it is known as northern blotting.
3. Western blotting: When protein is electrophoresed and is detected by using monoclonal or polyclonal antibodies and enzyme conjugates and substrate and by producing a coloured visible reaction; it is known as western blotting or immunoblotting.

DNA Finger Printing:

The nucleic acid of the causative organisms is isolated and cut into fragments by using restriction endonucleases. These fragments are resolved in agarose or polyacrylamide gels by electrophoresis and are stained by ethidium bromide or silver stain, which gives an endonuclease fragment pattern specific to particular organism, it is known as DNA finger printing (Nandi and Chauhan, 2006).

Polymerase chain reaction:

This is for the amplification of nucleic acid so that the small amount of nucleic acid present in the clinical sample may also be detected. In this the DNA is denatured at 90-98⁰C into two segments through de-annealing process. By using two primers complementary to each strand and bordering sequence to be amplified, the DNA template are mixed, cooled to 40-60⁰C for annealing of primers to the complementary sequence. Addition of DNA polymerase and nucleotide triphosphate allows the synthesis of complementary strands to each primer. The process is continued and new copies are synthesized, thereby increasing the number of DNA fragments. The amplified DNA fragments are electrophoresed into gel system and can be detected by probes. This technique can detect the very small amount of nucleic acid present in the clinical sample. Since, this technique is quite cumbersome and requires sophisticated equipment, its use is limited only to the well-equipped laboratories having trained manpower. PCR is used to amplify the specific DNA fragment that lies between two regions of known sequences. It consists of repetitive cycles of DNA denaturation, primer annealing and extension by DNA polymerase. The amplification is achieved by extension of short single stranded oligonucleotide primers on single stranded DNA template by a DNA polymerase. Each new strand of DNA synthesized becomes a template for any further cycle of amplification. However, the first two cycles of amplification do not produce any target sequences of defined length. Such target sequences are synthesized in the third cycle only and it increases exponentially from the fourth cycle onwards. So, the final number of copies of target sequence is expressed by the formula $(2^n - 2n) x$, where n is number of cycles, 2n is products of undefined length obtained in cycle 1 and 2 and x is the number of copies of the original template. At the end of 20 cycles it is expected that there is two- fold increase in the product under standard conditions (Nandi et al., 2008a, b& c).

Since its introduction in 1980s (Mullis,1990), PCRhas been constantly modified to be an efficient and sensitive method of studying the molecular pathology of primary, metastatic neoplasms(Bohlmeyer et al., 1998; Westraet al., 1996; Pulte et al., 2005; Bremnes et al., 2005; Pan et al., 2005) and infectious diseases(Raadet al,2002;Nogeeet al.,2001; Lordanet al., 2002). Firstly, a pair of priming complementary sequences are used to flank a location of interest and then DNA copying enzymes are introduced to obtain millions of copies of a targeted genetic sequence. Since the DNA primers only recognize the DNA for which they have been specifically designed, only that specific segment of DNA is preferentially amplified.After repeated cycles, more than a billion copies are generated. These copies are identical in electric charge and molecular weight and hence when subjected to electrophoresis, they migrate simultaneously forming a single identifiable band. The target sequence can be identified in a sample by the use of PCR products (also known as Amplicons) run on polyacrylamide electrophoresis in an instrument called as capillary electrophoresis instrument or Southern blotting with probe hybridization, comparing the lengths of the targeted DNA sequence with DNA "ladder" markers. DNA sequencing can be performed on the amplicons, to study mutations(Bevan et al.,1992; McPherson et al.,2000; Rattaet al.,2006).

Reverse transcriptase-PCR:

Reverse transcriptase-PCR is used to amplify RNA target sequence by converting it into a double stranded nucleic acid sequence (cDNA) using Reverse Transcriptase enzyme obtained from Retroviruses. RT-PCR can be used to examine genes (so far used in human medicine) that are expressed, over expressed, under expressed or not expressed in a specific cell type by the isolation of specific mRNA (D'Cunha and Maddaus, 2006; Lametal., 1998; Singhal et al., 2003; Dagnonet al., 2005). The recent development of real time PCR called as Quantitative PCR (Q-PCR) is replacing traditional PCR. This technique eliminates the need for post-PCR analysis (Lie and Petropoulos, 1998; Heid et al., 1996) as it couples the amplification and quantification of the targeted DNA sequence. It measures the amount of fluorescence emitted from a dye intercalated into the double helix DNA after completion of each cycle. The amount of fluorescence is directly proportional to the number of copies of the amplification target. The process is continued till a critical copy number is achieved which is indicated by the exponential rise in the amount of fluorescence. The cycle in which the critical copy number is reached is directly dependent on number of target DNA copies that were present in the given sample before further processing i.e. amplification. Q-PCR is advantageous as no post-PCR analysis is required. It is less prone to contamination since whole process is carried out within a single sealed tube.

Nested-PCR:

In this technique two sets of primer pairs are used for two rounds of amplification resulting in the increased specificity and sensitivity of the test i.e. the working principle of this technique involves the use of two primer sets and two successive PCR reactions. The first round of PCR uses outer primers to amplify a large fragment of rRNA gene. The product of first round is then used as a template for second round of PCR which targets a smaller region of the amplicon using inner primers (Carret al., 2010).

Applications of PCR in Animal Disease Diagnosis:

RT-PCR has been used for the diagnosis of RNA viruses like Influenza virus, Rotavirus, FMD virus (Erlich et al., 1991; Persing et al., 1991) and rabies virus (Araujo et al., 2008) with good results, even in decomposed samples. RT-PCR coupled with restriction enzyme digestion was used for the characterization of an Indian Bluetongue virus isolate directly in blood samples taken from sheep (Tiwari et al., 2000). Nested PCR assay is used in the detection of Bovine Herpes Virus-1 (BHV-1) in a naturally infected bovine fetus as the nested PCR assay was faster and easier to perform than the traditional virus isolation (Rocha et al., 1999). The use of Nested-PCR is also remarkable in detection of various viruses of veterinary importance like West Nile virus and Orf virus (Bora et al., 2015). The presence of M and S gene of canine corona viruses in the diarrheic faeces of naturally infected puppies were revealed with the help of RT-PCR (Pratelli et al., 2004). Real time PCR, a modification of conventional PCR, is being well employed for detection of FMD virus and Bovine piroplasmids (Criado-Fornelio et al., 2007). Ruengwilysup and colleagues applied colony PCR with an established Fung's Double Tube method to specimens obtained from food products, sewages and clinical samples for rapid detection (2 to 3 days lesser than conventional methods) and confirmation of *Clostridium perfringens* (Ruengwilysup et al., 2009). The use of multiplex PCR is quite relatable in diagnosis of various pathogens in a single reaction (Maan et al., 2018). A multiplex PCR-assay for detection of mastitis (Shome et al., 2011) in animals has been developed already. A study was conducted on large scale in western Europe to determine the risks associated with Bluetongue vaccine (De Leeuw et al., 2015) associated RNA in blood and spleen of cattle, the results indicated the fact that vaccine viral RNA can reach to blood circulation in sufficient amounts to get detected by real time- RT-PCR-assay (RT-qPCR). A "TaqMan" fluorescence-probe based quantitative real time RT-PCR assay has been described for detection of Seg-9 from 26 Bluetongue virus serotypes (Maan et al., 2015).

Hybridization or blotting techniques:

Hybridization or blotting consists of the immobilization of the biological molecules (like DNA, RNA or protein) on to a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is considered as the central technique for hybridization studies. The annealing of homologous nucleotide sequences indicates that each strand of DNA is from a different source. This specific base pair interaction is used to locate the targeted nucleotide sequences in an unknown sample. This forms the basis of DNA probe technique as only the homologous DNA from separate source will hybridize the DNA immobilized on the membrane. The probe is labelled with a marker to facilitate identification and quantification of the desired sequence.

Different types of Hybridization Techniques:

All blotting procedures start with a standard process known as Gel Electrophoresis. Different biological molecules like DNA, RNA or proteins are loaded on gel and then run through an electric field. Generally, two types of gels are used namely, Agarose gel and Acrylamide gel. Agarose gels are used to separate DNA and RNA; and polyacrylamide gels are used to separate proteins. These molecules are separated according to their size or charge under the influence of electric field. Among the basic blotting techniques Southern blotting is used for DNA sequence analysis; Western blotting for detection and analysis of proteins. Similarly, Northern blotting is the technique for analysis and detection of RNA sequences in a sample. After transferring the molecules from the gel surface to the blotting membrane, the sequences are visualized using stains like Ethidium bromide, Crystal violet, Osmium tetroxide etc.(Aboudet al.,2013;Itodo et al.,2014).A number of factors like probe concentration, complexity of probe sequence, probe size, ionic strength of hybridization medium etc. can influence the rate of hybridization. In traditional probe hybridization, nucleic acid probes labelled with enzymes, antigenic substances, radioisotopes or chemiluminescent moieties bind with the complementary sequences of nucleic acid with high specificity to form double stranded molecules, generally probes can be 20 to 20,000 bases long. Oligonucleotide probes (< 50 bp in length) can target the molecules more rapidly. In-situ hybridization describes the annealing of the specific sequences of single stranded cell or tissue bound DNA and RNA with single stranded probes of complementary sequences(Leitch et al.,1994; DeLellis,1994).Due to the presence of unique sequences in the cells and tissues of every pathogenic organism, In- situ hybridization can be used in identification and localization of single copy genes and mRNA transcripts in samples having <10 copies per cell (Beesley,1993).

Fluorescent in-situ hybridization (FISH) involves the use of fluorescence labelled oligonucleotide probes which bind to their complementary DNA sequences specifically on the genome and that region is labelled with fluorescent dye/color (e.g. Texas red, FITC green, Acridine orange). The labelled region is visualized by using fluorescence microscope.

Applications of Hybridization techniques in Veterinary sciences:

Hybridization techniques led the foundation for most of the modern- day mutation detection studies. Being effortlessly automated made these techniques to emerge as reliable and efficient tool for high throughput mutation screenings. Direct probe hybridization assays with labelled oligonucleotide probes are currently being used at wide scale for identification of slow growing organisms in clinical or food samples. Commercial probes are available for the identification of Mycobacteria and its related species (Woods,2001)and dimorphic fungi like Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis. Commonly in clinical microbiology laboratories, commercial probes produced by Gen-Probe Inc.,San Diego(Bourbeau et al.,1997) are available for testing clinical samples. Similarly, Gene Trak dipstick assays are being used for detection of food borne pathogens(Stewart et al.,2002).The commercialized probes by Gen-Probe use probes labelled with acridinium ester specifically directed at species specific rRNA sequences. These probes are available for organisms like Mycobacterium tuberculosis, Cryptococcus neoformans, E. coli, Staphylococcus aureus, Streptococcus pneumoniae, Hemophilus influenzae, Listeria monocytogenes. A co-cultivated method was used to infect primary fetal bovine lung cell culture (FBL) with cytotoxic Bovine Immunodeficiency-like-virus (BIV). The pelleted viruses (produced from infected culture) and detergent. Solubilized infected cell lysates from infected cell cultures produced the best Ag for Western blot assay. Sheep and goat were then inoculated with BIV. These experimentally infected animals produced sera which reacted in Western blot assay with different BIV viral induced polypeptides (Whetstone et al.,1991).Western blot assay can be used for diagnosis of Transmissible Spongiform Encephalopathies. A study on western blot detection of disease associated prion protein for diagnosis of Scrapie was constructed by using paraffin-embedded brainstem sample from an experimentally infected sheep(Kunkle et al.,2008). Studies reveal the application of western blotting and SDS-PAGE in determination of protein bands of excretory/secretory (E/S) Ag of Dicrocoelium dendriticum(Lancet fluke) in sheep(Simsek et al.,2006).A western blot assay developed by using partly purified LPS containing Ag of Burkholderia mallei strains was proved to be of higher diagnostic specificity as compared to complement fixation test of Glanders (Elschner et al.,2011). Hence, it was justified that western blot assay can supplement CFT to lessen the false positive results for Glanders in Equidae. Swine chlamydial nucleic acid was demonstrated with high specificity and sensitivity by using non- radioactive labelled DNA probe(Chae et al.,1999). In-situ hybridization technique was also applied to detect other diseases affecting swine like Pseudo-rabies(Belaket al.,1989),Proliferative Enteritis(Gebhart et al.,1994), FMD(Brown et al.,1995), Transmissible gastroenteritis(Sirinarumitret et al.,1996),Porcine Reproductive and Respiratory Syndrome Virus (Haynes et al.,1997;Larochelle et al.,1997), Swine Vesicular Disease (Mulder et al.,1997), Porcine circovirus(Allan et al.,1998) and Pneumocystis(Ramos-Vara et al.,1998).

FISH is evolved after establishment of some modifications in traditional probe hybridization assay. FISH emerged as a rapid non-culture- based tool for detection of pathogenic bacteria in different clinical samples. FISH is particularly used as a research tool to identify the bacteria which are hard to identify within complex cultures(Cai et al.,2014).Difficult-to-culture bacteria, mainly concerned with intestinal infections, can be rapidly diagnosed with the application of FISH technology. Model uses of FISH in veterinary diagnostics mainly comprises the detection of *Helicobacter* species of canine and feline origin(Jergens et al.,2009),Feline entero-adherent *Enterococcus hirae*(Nicklas et al.,2010), *Flavobacterium psychrophilus*(Strepparavaet al.,2012),*Spirochaetes* in bovine digital dermatitis(Capionet al.,2012), bacterial flora on Equine gastric mucosa(Perkins et al.,2012)and other bacteria in murine model of Crohn's disease(Craven et al.,2012).

Microarrays:

The use of microarrays for gene expression studies allow the formation of a single assay which provides the assessment of expression rates of several thousands of genes present in a sample. Initially, the microarrays were designed for gene expression(Call,2001b)and DNA analysis studies(Heller,2002); but with the passage of time, these are now being used as a powerful modern tool in novel areas of immunological research, proteomics, diagnostics, and DNA sequence analysis (Call,2001a;Heller,2002).The two main types of microarray techniques, which are being used in common, are cDNA microarrays and oligonucleotide chips. The microarrays consist of a solid flat media, like glass slide or silicon chips, on which complementary DNA sequences to variety of mRNA from several thousand of genes are bound systematically. The targeted sequence to be detected in a given biological sample is enzymatically labelled with fluorescence dye and is hybridized on the surface by complementary base pair binding. The fluorescent tag at each probe location quantitatively determines the amount of targeted sequence present in the tested sample. There are three major steps which are to be followed in microarray technique viz. preparation of microarray probes, their hybridization and followed by the scanning, imaging and analysis of data (Singh and Kumar,2013).

As DNA microarrays (originally constituted for explication of whole genome gene expression) are incapable of reading beyond mRNA level, it cannot monitor some proteins in post- translation stages which are not regulated by genes. This limitation of DNA microarrays lead to the introduction of a novel technology based upon protein array (Beena et al.,2016)). In protein array (protein chip), purified protein molecules are immobilized on a slide and these are used for simultaneous detection of a number of analytes from test samples in a single experiment. The reaction between probe and immobilized protein is interpreted by detecting fluorescent or radioisotope labels using laser scanner. The two main categories of protein microarray include Functional and Analytic Protein Microarrays (Chen and Zhu,2006). Functional protein arrays are applied in studying protein-protein interactions, nucleic acid-protein interactions, biochemical activities and immune responses. Analytic protein microarray includes antibody microarray which is most commonly used in protein microarray.

Wan, Fortuna and Furmanski, in 1987 (Wan et al.,1987), developed a high throughput molecular technique, i.e. Tissue Microarray, for assessment of multiple novel markers, identified at gene level, simultaneously on hundreds or thousands of tissue samples (Kumar et al.,2004). Tissue Microarrays mainly consist of composite paraffin blocks which are constructed by embedding tissue cores acquired from donor block on to a empty paraffin block (recipient block). The tissues arrayed on the paraffin block can be analysed by variety of molecular detection techniques like in-situ hybridization, immuno-staining (chromogenic or fluorescent visualization), histochemical staining etc. Tissue microarrays, hence, allow the analysis of hundreds or thousands of tissues in a single batch on a single slide (Aktas, 2004).

Applications of microarray technique in disease diagnosis:

This technique, in the field of veterinary disease diagnosis, is still in developing stage. As it has the potential to create an all-in-one assay for a single pathogen or multiple pathogens causing co-infections, hence it is now emerging as a modern- day advancement in the field of molecular diagnosis. The advantage of detecting and differentiating the pathogens of interest at subspecies level(Schmitt and Henderson,2005)makes it a high throughput method of analysis.

Parasitic diseases:

The unique gene expression shown by different life cycle stages of parasites are due to distinct physical and metabolic properties of that particular developmental stage. A suppression subtractive hybridization and cDNA microarrays can be combinedly used to analyze the differentially expressed genes of early stages of *Eimeria*

maxima(Donget al.,2011). Out of 60 valid expressed sequence tags used by Dong and co-workers,microarray represented 32 unique sequences. Also, combined suppression subtractive hybridization and microarray techniques can be used during invasion and development of early life cycle stages of *Eimeriatenella*. The study involved the assessment of gene expression changes of 3 life cycle stages viz. unsporulated oocysts, sporulated oocysts and sporozoites of *E. tenella* using microarray analysis(Han et al.,2010).

Viral diseases:

Now a days, many commercialized chips based on microarray technology are available. First broad range chip was used for FMD virus identification(Baxiet al.,2006). Pan viral DNA microarray are available for identification of Orbivirus (Gardneret al.,2013).Animal viruses like FMDV, Vesicular stomatitis virus, swine vesicular disease virus, BHV-1, BVDV1, VESV (causing vesicular lesions in livestock species) and Enterovirus(Nishizukaet al.,2003; Nandi and Chauhan,2006), were detected by using long oligonucleotide microarray technique.Recently, a team of scientists developed a microarray chip for livestock virus diagnosis at IVRI, India(Yadav et al.,2015). This chip was used for detection of first case of New Castle Disease Virus in sheep (i.e. in a non-avian host) and mixed infection of BVD and BHV in cattle(Rattaet al.,2014). An antibody microarray was used to diagnose the changes in cellular protein expression of an equine having H7N7 and H3N8 infection. This study revealed that a pro-apoptotic factor and both pro and anti-apoptotic factors were induced in H3N8 and H7N7 respectively (Rozeket al.,2013). Peptide microarray conferred considerable information regarding serodiagnosis of canine lymphoma along with its lineage (B-cell or T-cell) (Johnston et al.,2014). Tissue microarray was used to study the expression of p53 (tumor suppressor gene) and bcl-2 (proto-oncogene) in avian thymus and bursa post-irradiation and IBDV infection (Fridmanet al.,2006).

Bacterial diseases:

The use of microarray assay for detection of specific disease causing bacteria of livestock importance is showing great promise and development(Cai et al.,2003).The use of microarray technique for the animal pathogens which are difficult to isolate under laboratory conditions was also explained.A genotyping array has been constructed for detection of *E.coli* O157:H7 strain which is mainly found in the food samples(Call,2001b). A low- density oligonucleotide assay has been formulated which targets the genes responsible for Shiga toxin in *E.coli* O157 strains (Quinones et al.,2011)resulting in early and rapid detection. A recent development in the field of microarray-based techniques is Arraytube™ (AT). It was devised for the detection of *Coxiellaburnetti*(Schmoocket al.,2014), but it was found to be suitable for the identification and genotyping of several pathogens of veterinary importance such as *Burkholderia mallei*, *Bacillus anthracis*, *Chlamydia* species and *Brucella* species.Microarray technique has been successfully used for detection of pathogens in routine clinical samples to determine the presence of pathogen before the clinical onset of disease.Easy Operating Pathogen Microarray (EOPM) is a high throughput pathogen microarray platform (Huang et al.,2013). It represents numerous complete and partial viral genomes, 2,110,258 bacterial 16s-rRNA sequences, 621,351 fungal 18s- rRNA sequences and 1,735,744 parasitic 18s-rRNA sequences. The EOPM chips are used for large scale pathogen surveillance and are able to distinguish 124 bacterial genes, 38 fungal genes, 47 genera of parasites and 2,544 viral species(Huang et al.,2013).

Genomics (dna sequencing and its various advancements):

Earlier, detecting the sequence of very small nucleic acid consisting of 5 to 10 nucleotides was very hectic procedure and it used to take a lot of time. But, the advent of a new technique described as DNA Sequencing, in late 1970's, has revolutionized the scopes of detecting genomic sequences.It is a well-known fact that PCR has single handedly led the way for the expansion of the molecular diagnostics but due to some limitations the need of new technology for identifying the genomic sequences was felt. One of the major-limitation of PCR is that there have been some issues for the Taq polymerase to amplify the regions rich in CG repeats. Due to such kind of technical limitations, the positive results needed to be confirmed by other alternative techniques leading to a costly and time-consuming process. Since its introduction, DNA Sequencing has been a handy and reliable tool in the field of molecular diagnostics and research as several genomes of the microbial world has been identified and sequenced using this very technique.Basically, two different methods for sequencing of DNA were developed (in 1977) namely, Chain Termination Method and Chemical Degradation Method.

Chemical Degradation Method, popularly known as the Maxam-Gilbert Method (developed by A. Maxam and W. Gilbert in 1976-77); was based on the principle of chemical modification of DNA and cleavage of specific bases subsequently(Maxam and Gilbert,1977). Initially, it was more popular; hence more in use as compared to Chain Termination Method(Sanger Method). This is because inthe Maxam-Gilbert method, purified DNA was used

directly; but in the Sanger Technique, cloning of each read start was the basic requirement before the initiation of the process. With the time, Sanger Method was studied and developed more. Due to some technical advancements in the Sanger Method, the Maxam-Gilbert Method became outdated as now it was more complex with the technical requirements and extensive use of hazardous chemicals. Basically, Sanger Method uses di-deoxynucleotide triphosphates (ddNTPs) as the DNA chain terminating moieties (Sanger et al., 1977). These two basic techniques of DNA sequencing are popularly known as the First- Generation Sequencing Techniques.

Next Generation Sequencing Techniques (NGS):

These techniques can be described as rapid and efficient tools which are now providing the major breakthroughs in the field of sequencing as the genomic research projects, which used to take several years in completion with the basic Sanger technique, can be completed within weeks, that too with an exceptional efficiency and accuracy, using these advanced techniques. Next Generation Sequencing provides quick results as it can determine the sequence data from amplified single DNA fragment, unlike the basic Sanger Method (first generation technique) where the cloning of the DNA was a pre-requirement.

454 Genome Sequencer FLX instrument (Roche Applied Science):

This device is based on the principle of pyro-sequencing or the pyrophosphate detection described in 1985 (Nyren and Lundin, 1985). The well-functioning system based on this very principle was developed 3 years later (in 1988) (Hyman, 1988). It uses a charge couple device camera to detect the light produced due to the conversion of pyrophosphate into ATP which is followed by the luciferase stimulation (Ronaghi et al., 1998). The Genome Sequencer was developed by 454 Life Sciences and it was introduced as the first Next Generation Sequencing System in market in 2005. The basic limitation of first-generation technique, i.e. the need of cloning of the targeted gene, was eliminated by this device. Though it was marketed as an advancement of the traditional Sanger Sequencing Technique, but it has a major drawback of higher error rates. Due to the false reading of a template by misjudging the homopolymer, there was production of deletion and insertion mutations resulting in high error rates. A recent advancement of this system has been introduced as GS FLX Titanium XL+ (<http://www.454.com>). It is capable in generating 700MB of sequenced data with an incredible efficiency (almost 99.997%). But despite of being an advancement to the basic Sanger Method, this system is losing its popularity due to low throughput and higher operational cost.

The Illumina (Solex) Genome Analyzer:

This technology was developed in 1994 by Canard and Sarfati. It is based on the principle of reversible termination chemistry concept (Canard and Sarfati, 1994). It uses the concept of sequencing by synthesis. It was commercialized in 2006 as Solexa Sequencing Platform and it allows the identification of single nucleotide base as it is polymerized into new DNA strand. It is a quite versatile system as it can be used for whole-genome sequencing, transcriptome analysis, metagenomics, small RNA and methylation profiling and also as a reliable tool for the analysis of protein and nucleic acid interaction. An advanced version of this system was introduced by Illumina in 2008, commercialized as Genome Analyzer II, it triples output per paired end run (from 1GB to 3GB) as compared to the previous version (<http://www.illumina.com>).

Ion Semiconductor Sequencing:

It is also known as Ion torrent or pH-mediated sequencing or Silicon Sequencing. It is based on the principle of detection of hydrogen ions which are released during the polymerization reaction of new DNA strand synthesis. Hydrogen ion is detected by ion-sensitive-field-effect transistor (ISFET) ion sequencer. It is based on the concept of sequencing by synthesis i.e. sequence data of template is generated while there is synthesis of complimentary strand. There is no use of labelled nucleotides and optical instruments in this method, unlike other sequencing techniques. This technique is suitable for small scale applications such as microbial genome or amplicon sequencing. Major limitation of this technique is difficulty in enumeration of long homopolymer repeats.

Applications of DNA Sequencing in Veterinary Sciences:

NGS technology is playing a key role in the study of etiology, genomics and epidemiology of various animal infectious diseases. NGS systems have played the central role in the completion of large animal genomes and also in the studies of genomic variations. Hence, the whole genomic sequences of bovines, pig, sheep equines and avian species are now available (Bai et al., 2012). NGS have been helpful in the documentation of intra-host genetic diversity in the *Cryptosporidium parvum* by targeting specific polymeric genes in its genomic sequence (Grinberg et al., 2013). NGS techniques have facilitated the studies on the transcriptomes of different parasitic species and their

developmental stages (Cantacessi et al., 2012), e.g. characterization of the transcriptome of *Eimeria* species from chicken (Matsubayashi et al., 2013) and *Taenia* species from sheep (Wu et al., 2012). The RNA sequence data has also been helpful in predicting potential drug targets (Romine et al., 2013) and the genes responsible for anti-helminthic resistance (Cwiklinski et al., 2013). The evolution of FMDV intra-sample sequence diversity (Morelli et al., 2013) was studied by using DNA sequencing techniques, under the experimental conditions of serial transmission in the ovine hosts, which resulted in the documentation of fine-scale evolution of FMDV.

At present, majority of veterinary researchers are using NGS to study animal infectious diseases at the animal to animal transmission level. These platforms are playing a key role in characterization of pathogens within a single host and this obtained data is being used as a source of important information in terms of managing outbreaks. E.g. NGS techniques were used to study the genomic complexity and horizontal gene transfer in food borne *Campylobacter* species (Lefebvre et al., 2010). The local spread patterns of *Mycobacterium bovis* in cattle and wildlife reservoir hosts in 5 farms was also studied using whole genome sequencing (Biek et al., 2012).

Illumina Sequencing Platform played a vital role in the identification of the new strain of West Nile Fever Virus during its outbreak (2006-2007) in US (Grinev et al., 2013). Ion Torrent System of Sequencing (for RNA sequencing) was utilized to identify a novel Coronavirus in a survey of Chinese Domestic Fowl (Chen et al., 2013) and it helped in accessing the diversity and distribution patterns of avian corona virus. The advent of NGS has paved way for the beginning of new era in the field of viral genomics as the genomic sequencing for Avian Influenza Virus (Ramakrishnan et al., 2009; Dugan et al., 2011; Croville et al., 2012; Van Bormet et al., 2012), Classical Swine Fever Virus (Leifer et al., 2013) and Bluetongue Virus (Rao et al., 2013) has been completed with incredible accuracy and in cost effective manner. In a study on Lyssa virus from brain and cell culture; the 454 Genome Sequencer has been used to document universal non-specific RNA viral genome (Marston et al., 2013). NGS transcriptomics was used to study the bovine resistance and tolerance traits to parasitic infections (Glass et al., 2012).

Proteomics:

Proteomics can be described as the study and characterization of entire protein complement or the whole set of proteins called as the proteome of cell, tissue or the whole organism on a large scale (Anderson and Anderson, 1998). The identification and documentation of the entire genomic sequences of various organisms has paved way for the development of Proteomics, but the characterization of whole set of proteins even in simpler biological systems or life forms still seems to be a distant goal (Burgess and Burchmore, 2012). The presence of proteins across a wide dynamic range makes them complex to characterize than that of encoding the whole genomes (Corthals et al., 2000); the proteome is also influenced by the environmental parameters and developmental progress of individual organism hence, making it difficult to document the whole set. But, the field of protein characterization holds a great importance as proteins are the primary effector biological entities and they can also act as the major drug target molecules. Hence, this field is developing rapidly and emerging as an important research opportunity since the last two decades (Cecilian et al., 2014a; Cecilian et al., 2014b). Electrophoretic (used for intact proteins) and chromatographic (applied for peptides) are two major protein fractionation systems which are used in proteomics. At present, two Mass Spectrometry systems are commonly used; having a basic difference of operational mechanism of ion generation. The ion sources are described as matrix-assisted laser desorption/ionization (MALDI) and Electrospray Ionization (ESI). There is one more difference between these two which is the feeding of sample, MALDI works only on the samples in solid state while ESI on a volatile solvent sample. Despite of different working principles, the ultimate goal of the both systems is to deliver homologous populations of peptides, that too unique to each species. 2-Dimensional Electrophoresis (archetypal orthogonal separation technique) is most commonly used in proteomics for separation and characterization of proteins, as it enhances the resolution. But, 2-DE is known to be time consuming and technically complex system and has very limited advantage over automated chromatographic systems. Chromatography based techniques of proteomics are more sensitive than electrophoresis because there is no need to recover proteins (or peptides) from a gel matrix. To counter inter-se limitations, chromatography is coupled with ESI-MS, resulting in an automated system which reduces the sample loss and less time consuming. This coupling forms the most efficient and technically sound fractionation system (Xie et al., 2011).

Applications of Proteomics in Veterinary Sciences:

To study the changes in metabolic parameters of organisms under the stress conditions, proteomics is far more better than the genomics as the expression of proteins depends on the environmental stimuli and the body condition of the organism. 2-DE is used to study the effects of environmental and physiological stressors (weaning and transportation) in the development of respiratory diseases (Mitchell et al., 2008) in the livestock species. It is an

important tool for research in new drug development and an early disease diagnosis(OIE,2016).Since, Mastitis the most common production disease responsible for the huge economic loses worldwide; so, proteomics is being applied to study the intra-mammary infections with *Staphylococcus aureus* and *E. coli*. In an experimental study, acute mastitis was induced by *E. coli* and peptide sequencing with MALDI was performed which led to the identification of differentially expressed proteins(Boehmer et al.,2008).Proteomics is used to study the biofilm formation modulated by alpha-casein and beta-casein in case of *Streptococcus uberis*infection (Varhimo et al.,2011).An experimental study to investigate the pathogenesis of *Haemonchus contortus*infection in sheep was carried out which was targeted on the documentation of quantitative expression of proteins(Nagaraj et al.,2012).Protein characterization of the hydatid cysts fluid obtained from sheep, cattle and humans, the involvement of immune and inflammatory defense mechanisms in *Echinococcus granulosus*was confirmed. This study also confirmed that absorption of host proteins across its outer germinal layer helps *Echinococcus granulosus* to evade the host defense machinery(Aziz et al.,2011).

Study based on the pathogenesis of Classical Swine Fever (mainly based on the primary porcine endothelial cells) suggested that the CSF virus induces the down-regulation of the proteins which are a part of the energy metabolism system and upregulates the proteins which inhibits the proliferation of endothelial cells (Li et al.,2010).

Involvement of cytoskeletal proteins was evidenced after macrophage infection in a study of Post Weaning Multisystemic Wasting Syndrome (Cheng et al.,2012)caused by porcine circovirus-2.DiGE and MALDITOF-MS of pig mesenteric lymph nodes(Martins et al.,2012)were used to study the transmission of *Salmonella typhimurium* through pork, and as result a complex interaction between pathogen and pig immune system was identified.DiGE along with western blotting (Costa et al.,2011)has been used to study novel Ag from *Leishmania chagasi* to aid in early diagnosis and development of vaccine. Characterization of proteins from a large variety of pathogens of veterinary importance is currently under process, such as *Brucella melitensis*(Mujeret et al.,2002), *Trypanosoma brucei*(Rout and Field,2001), *Haemonchus contortus*(Yatsuda et al.,2003). Some of the dog parasites were also investigated using proteomics such as *Ancylostoma caninum*(Mulvenna et al.,2009)and *Echinococcus multicularis*(Kouguchiet al.,2010).

Conclusion:-

With the ever-evolving pathogens, there is need of more technically sound, advanced and accurate approaches of disease diagnosis, new drug development and treatment. In recent years, molecular diagnostic techniques are becoming more popular for their rapid and more accurate diagnostic results for various old and emerging diseases of animals.Currently, there is a whole spectrum of molecular diagnostic techniques such as RT-PCR, FISH, DNA sequencing, hybridization and proteomics which proving to be an indispensable tool for the evaluation of genomic composition (through DNA and RNA sequencing) and the investigation of changes in protein complement set (through proteomics). These are also helping to counter the outbreaks by facilitating early prediction resulting in the better and rapid implementation of biosecurity measures.Since, the advent of molecular diagnostic techniques PCR and its associated techniques have been the sole torchbearer on the field but with the increasing research work and advancements, alternative techniques have arisen on the horizon; with greater efficiency and accuracy. Microarray assay-based techniques are helping in the prediction of patterns of disease transmission during outbreaks or surveillances. Coupling of histology with DNA sequencing is leading the way for the development of better approaches of therapeutics and vaccine development.Hybridization techniques such as blotting has been a cornerstone in the field of molecular diagnostics as these techniques have a wide range of applications as discussed earlier. Although, the traditional hybridization techniques still are of great utility, but due to rapid advancements these are being replaced by other techniques such as immunochemistry, flow-cytometry or immunofluorescence due to their more sensitivity, precision and a better working principle.Proteomics, is emerging as a more sensitive, diverse and practically non-invasive tool for the pathologists all over the world and hence it has witnessed a rapid development since the last decade, but proteomics in veterinary sciences (in terms of techniques, advancements, research and applications) is still lagging than human medicine and pathology. With the increasing focus of researchers on veterinary sciences, more and more information related to the molecular mechanisms of the various prevailing and emerging animal diseases is being documented on the daily basis. Hence, by increasing the use of these techniques the economic losses due to animal diseases can be minimized.But, there are some issues which are needed to be addressed such as (a) determining clear targets and the most suitable technique prior to any investigation, (b) tests to be conducted, (c) approach to be taken and last but not the least (d) cost or economics of the whole procedure; as there has always been an issue over the allotment of budget for research in the veterinary

sciences especially in the Asian countries such as India even though we are the largest country in terms of livestock population.

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