



Review Article

New Approaches for Diagnosis of Viral Diseases in Animals

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ABSTRACT

Identification and control of viruses that causes diseases in human, domestic and wild animals represent continuous challenges to medical and veterinary sciences. From centuries many diagnostic techniques have been tried to identify individual infectious agents by their clinical signs and symptoms produced in the susceptible host. But it is not always possible to identify or differentiate the disease on the basis of its signs and symptoms and thus requires other advanced diagnostic procedures to clearly identify and confirm the pathogens. Rapid detection of infectious agents in animals as well as in environment is essential for effective control of infectious diseases. There are various approaches for detection of viral diseases which includes conventional as well as modern approaches. Several conventional techniques such as isolation in cell culture, serology and histological identification are traditionally used to identify viral pathogens. However, the latest molecular diagnostic technologies are more advantageous as they are offer more sensitive, less time consuming, high throughput result. Other novel technologies such as polymerase chain reaction (PCR), Reverse Transcriptase PCR (RT-PCR), Real Time-PCR, DNA probe, nucleic acid sequencing etc. provide a thorough understanding of accurate diagnosis and discrimination of present and emerging diseases. The aim of these new tools is to detect the presence of pathogen before the onset of disease. Here we have discussed the advancement of diagnostic tools and their applications for important animal viruses of domestic and wild animals.

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INTRODUCTION

Viruses are serious threats to human and animal health. Only few successful antiviral treatments are available for viral infections. Therefore, early and rapid detection and characterization of viral pathogens are essential. The viral disease diagnosis is important for not only deciding the treatment strategies but also to identify the prevalence of viruses in different forms such as serotypes or isolates. Many viral diseases like bluetongue virus, peste des petets virus, classical swine fever virus, Newcastle disease virus of poultry, foot and mouth disease virus have continuously raising serious economic problems in the field. Bluetongue virus (BTV) has been in the forefront of molecular virology and is one of the best understood viruses at the molecular and structural level (Roy, 2008). For bluetongue virus (BTV), specific peptides representing its antigenic epitopes and ssRNA-binding domains have been utilized to develop unique diagnostics that can differentiate whether an animal is vaccinated with BTV vaccine or infected by BTVs. The gene-silencing tool has been used for RNA interference (RNAi) for determination of gene functions and as potential therapeutics for Bluetongue disease. The signature amino acid sequences have been identified to develop a peptide-based assay to differentiate a BTV-vaccinated animal from

an animal infected with BTV. Other viruses such as *Rotavirus* (Niture et al., 2008; Minakshi et al., 2004), *Parvovirus* (Savi et al., 2009), *Caliciviruses* (Yu et al., 2013), *Paramyxoviruses* (Killip et al., 2012) and *Influenza virus* (Tonnessen et al., 2013) have also been extensively studied at the molecular level. Various molecular techniques such as polymerase chain reaction (PCR), probe hybridization (Minakshi et al., 2005), microarray, nucleic acid sequencing etc. have been extensively used for this purpose. In this paper molecular techniques for detection of viruses are reviewed and the potential for their application are discussed. The application of new techniques as a routine tool in a diagnostic laboratory is an area where relevant literature is not available and this may contribute to the acceptance of these methods.

Conventional Approaches

The viral disease diagnosis includes the isolation of live virus in different cell cultures, embryonated eggs or through animal inoculation. The detection of viral proteins (antigens) and viral genome fragments (viral nucleic acid) are also carried out. In general, various viral disease diagnostic tests can be grouped into 3 broad categories:

Direct Examination of Specimen

Direct examination methods are often used for rapid diagnosis of viral diseases. This is highly useful in clinical management of diseases especially when treatment depends on the rapid availability of laboratory results.

Antigen Detection

Virus isolation is the most effective and standard method for viral disease diagnosis. Growth of viruses was initially

restricted to animal inoculation. For BTV diagnosis initially sheep inoculation was used as the diagnostic method. Subsequently developing chicken embryo was used for isolation of the virus. Now a day, cell culture is used for production of large quantities of live viruses (e.g. BTV in BHK-21 or Vero cell line) to produce diagnostic antigens (Figure 1).

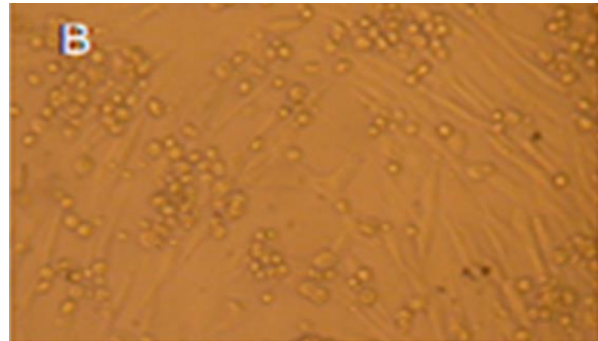
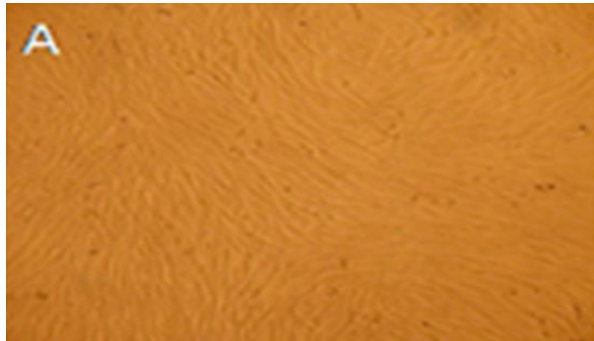


Figure 1: Isolation of bluetongue virus in BHK-21 cell line; A: Normal uninfected BHK-21 cell monolayer (48 hours); B: BTV infected BHK-21 cells (48 hours) showing cytopathic effect characterized by rounding and degeneration of the infected cells

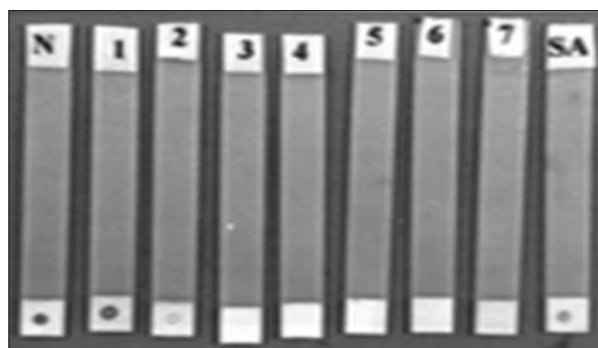
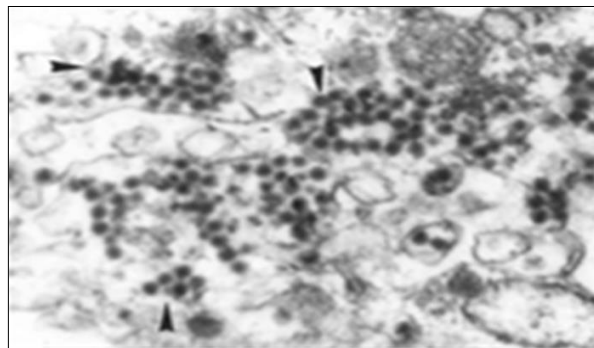


Figure 2: BHK-21 cells infected with BTV, exhibiting several virus particles in the cytoplasm of the cells by transmission electron microscopy; Figure 3: Dot immunobinding assay showing dots of antigen on the dipstick membrane with BTV positive sera

Microscopic Examination

The live replicating viruses often produce cytopathic changes in infected cells which can be easily observed by light microscopy. However, low sensitivity is the biggest limitation of this method as a diagnostic tool. In electron microscopy, specimens are stained negatively with phosphor-tungstate, or sometimes uranyl acetate and then scanned by electron microscopy (Figure 2). The specific modification in the basic EM was made by using immune-gold electron microscopy by adding specific antibodies labeled with gold (Prasad et al., 1993).

Indirect Examination/Virus Isolation

Virus isolation is one of the best methods for viruses that can be grown *in vitro* in cell culture, such as FMD virus (FMDV), Newcastle disease virus, Avian influenza virus (AIV) etc. Although it is time consuming, but this method is very sensitive, and offers the maximum opportunity for subsequent viral characterization. Cell cultures, chicken embryo and animal inoculation may be used for virus isolation. Various cell lines have been established for viral isolation such as N₂A for rabies virus, porcine kidney cell line for classical swine fever virus, Vero for NDV and PPRV,

BHK-21 for BTV, MA104 for rotaviruses, MDCK for canine parvovirus. Viruses can be adapted in different cell lines which include primary (Monkey Kidney), semi-continuous cells (Human embryonic kidney) and continuous cultures (HeLa, Vero cell line). Confirmation of the presence of virus in cell culture may be carried out using neutralization, immunofluorescence or molecular tests (Mishra et al., 2002; Prasad and Minakshi, 1999) (Table 1).

Serological Examinations

Several types of serological tests are available for diagnosis of viral antigen or antibodies. Some of the assays such as enzyme immuno assay (EIA) and radio immuno assay (RIA) are used specifically for IgM or IgG, whereas other assays such as complement fixation test (CFT) and Haemagglutination Inhibition (HAI) can only detect total antibody comprising mainly IgG. Newer techniques such as EIAs offer better sensitivity, specificity and reproducibility than classical techniques such as CFT and HAI. Certain viruses such as influenza, parainfluenza, flaviviruses etc. have been detected using Haemagglutination Inhibition (HAI) test. The Agar gel immunodiffusion (AGID) test and dot immunobinding assays were employed for canine

Table 1: Common diagnostic method and cell line for isolation of animal viruses (OIE, 2013; FAO, 2000; CDC, 2012)

Sr. No.	Viral pathogen	Cell line/culture used	Common diagnostic methods
1.	Swine pox virus	Primary pig kidney monolayer	IFAT (Indirect fluorescent antibody test)
2.	Swine influenza virus	MDCK	HA, FAT, IHC, ELISA, PCR, qPCR
3.	Foot and mouth disease virus	BHK21, IB-RS-2	ELISA, VNT, CFT, RT-PCR
4.	Caprine and ovine pox virus	LT monolayers	VNT, AGID, ELISA, PCR
5.	Swine vesicular disease virus	IB-RS-2	VNT, ELISA, PCR
6.	African swine fever virus	Porcine kidney monolayer	PCR, ELISA, IFAT
7.	Classical swine fever virus	FS-L3, PK15-A	ELISA, Fluorescent Antibody Virus Neutralization (FAVN), Neutralizing peroxidase-linked assay (NPLA)
8.	Peste des petits ruminants virus	Vero cells, BHK-21	VNT, ELISA, RT-PCR, AGID
9.	Bovine adenovirus	Calf kidney cells	IFAT, VNT, IHC (Immunohistochemistry test)
10.	Bluetongue virus	BHK-21, Vero, L929	AGID, ELISA, IF, VNT, RT-PCR
11.	Epizootic hemorrhagic disease virus	BHK-21	IFAT, PCR
12.	Bovine ephemeral fever virus	BHK-21	IFAT, ELISA, RT-PCR
13.	Bovine respiratory syncytial virus	Bovine turbinate cells	ELISA, IFAT, EIA, RT-PCR
14.	Bovine viral diarrhea	LT monolayer, MDBK	IFAT, ELISA
15.	Bovine herpes virus 1, 4	MDBK	IFAT, PCR
16.	Lumpy skin disease virus	LT monolayer	VNT, PCR, IF, AGID, ELISA
17.	Rabies virus	NG108-15, N2a, CCL-131, HEK-293	FAT, ELISA, VNT, IHC, RT-PCR
18.	Rotavirus (bovine and porcine)	MA-104	IFAT, RT-PCR, RNA-PAGE
19.	Vesicular Stomatitis Virus	Vero, BHK-21, IB-RS-2	LP-ELISA, C-LISA, VNT, CFT, AGID
20.	Equine arteritis virus	RK-13	VNT, ELISA, IFAT, PCR
21.	Equine encephalitis viruses	Vero cells	IFAT, PCR (group specific)
22.	Equine herpes virus 1,4	Primary equine fetal kidney monolayer	IFAT, PCR
23.	Equine influenza virus	MDCK	ELISA, HI, RT-PCR
24.	Ovine adenovirus	LT monolayer	IFAT, PCR
25.	Porcine adenovirus	Primary pig kidney cells	IFAT, PCR
26.	Pseudo rabies virus	PK-15, SK6, MDBK	VNT, ELISA, Latex Agglutination
27.	Porcine epidemic diarrhea virus	Vero cells	ELISA, PCR, IFT, IHC
28.	Porcine circovirus 1, 2	PK15-A	IFAT, PCR
29.	Swine influenza virus	MDCK	RT-PCR, Real-time PCR
30.	Swine pox virus	Primary pig kidney cells	IFAT, AGID
31.	Transmissible gastroenteritis virus (TGE) and Porcine respiratory corona virus (PRCV)	Primary pig kidney cells	ELISA, PCR, FAT
32.	Canine adenovirus 1 and 2	MDCK	IHC, IFAT, PCR
33.	Canine distemper virus	MDCK	PCR, Real-Time PCR
34.	Canine parvovirus	MDCK, CRFK	IFAT, ELISA, HA
35.	Feline calicivirus	CRFK	IFAT, Real-Time PCR
36.	Feline infectious peritonitis virus	CRFK	IFAT, PCR
37.	Feline panleukopenia virus	CRFK	IFAT, PCR
38.	Feline herpes virus 1	CRFK	ELISA, IFAT
39.	Japanese encephalitis virus	Vero, MDBK, BHK-21, C6/36	VNT, HI, CFT, RT-PCR
40.	West Nile virus	Vero cell, Rabbit kidney cells	ELISA, HI, RT-PCR

distemper virus (CDV) and feline parvovirus (FPV) specific antibody detection in Asiatic lions.

Enzyme Linked Immunosorbent Assay (ELISA)
 ELISA is now the most widely used form of immunoassay for the rapid detection of viral antigens and antibodies.

There are many different formats of ELISA are available such as DOT blot (Figure 3) and Plate method. DOT blot method has been used for Rotavirus, Bovine herpes virus and BTV detections. Commercial ELISA kits were also used for diagnosis of several viral diseases such as feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) (Ramanathan et al., 2007). The antibodies against bluetongue virus in deer, camel and sheep have been detected using AGID (Naresh et al., 1996). The bluetongue specific antibody in domestic and wild ruminants in and around Sariska tiger reserve in Rajasthan has been detected in deer as well as sheep (Prasad et al., 1998). Peptide based ELISA are also available for diagnosis of various viruses like infectious bursal disease virus, PPRV, FMDV, SARS and Infectious bronchitis (Dechamma et al., 2006, Jackwood et al and Hilt, 1995, Joshi et al., 2013a).

Immunofluorescence

Immunofluorescence (IF) assay is commonly used for the rapid detection of virus infections by the detection of virus specific antigen in clinical samples, as well as the virus-specific antibody. The technique is primarily based on reaction between fluorescein- labeled antibody and virus specific antigens in specimens (cells). The cells containing virus specific antigen fluoresce under UV illumination (Figure 4). The virus specific antibody can be detected using indirect IF whereas virus specific antigen is detected using direct or indirect IF. The *in-vitro* susceptibility of goat mononuclear cells to bluetongue virus has been studied in Haryana using IF test (Garg et al., 1996).

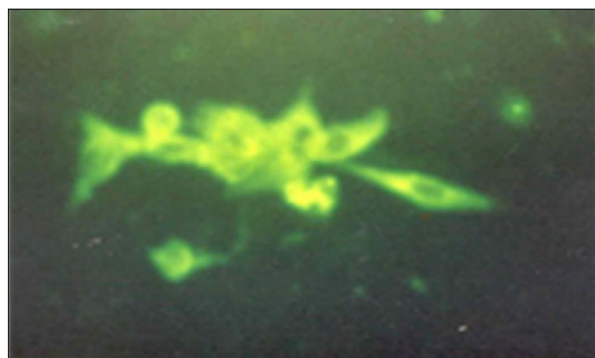


Figure 4: BHK-21 cell line infected with BTV and stained with FITC. The infected cells are showing extensive green fluorescence.

Molecular Approaches

Various molecular biology techniques have been used for direct detection of viral genome (nucleic acid) in the specimen which plays an important role in clinical virology. In the molecular techniques, DNA is extracted from the sample of interest which can be probed by DNA hybridization and analyzed by restriction fragment length polymorphism (RFLP). More commonly, DNA is amplified by the polymerase chain reaction (PCR) using pathogen specific primers for diagnosis. The amplified PCR products can be sequenced for confirmatory diagnosis. A number of assays like RNA-polyacrylamide gel electrophoresis (RNA-PAGE), reverse transcription polymerase chain reaction (RT-PCR) etc have been employed for Newcastle disease virus, rotavirus, parvovirus and bluetongue virus diagnosis

(Dalal et al., 2008; Kovi et al., 2007; Dahiya et al., 2005, Mase and Kenchira, 2012).

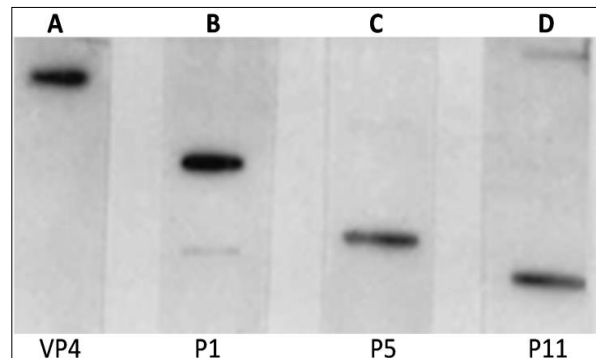


Figure 5: VP4 gene based genotyping of bovine group 'A' rotavirus by radio-labeled probes. Membrane A: detection of homologous dsRNA by full length vp4 probe; membrane B: P1 type and membrane C: P5 type and membrane D: P11 type.

Nucleic Acid Probes

Nucleic acid probes are segments of DNA or RNA that have been labeled with enzymes, antigenic substrates, chemiluminescent moieties or radioisotopes. They are highly specific to pathogen specific nucleic acid. Probes can be directed to either DNA or RNA targets and can be from 20 to thousands of bases long. The presence and the quantity of hybrids are determined by the detection of the labeling of probe. A variety of labeling and detection systems exist for nucleic acid probes such as radiolabelled (Figure 5) and non-radiolabelled methods. These include labeling with a variety of haptens such as biotin or digoxigenin (DIG) and detection by antibody binding coupled with fluorescent, chemiluminescent or colorimetric detection methods (Minakshi et al., 2005). Recently new category of nucleic acid probe called peptide nucleic acids are also used for molecular diagnosis of viral diseases (Joshi et al., 2013b).

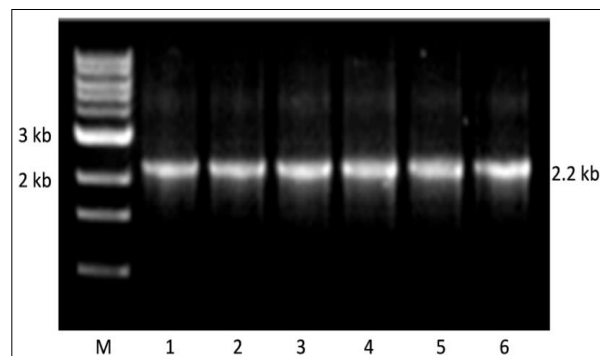


Figure 6: PCR products of VP2 gene of canine parvovirus yielding 2.2 kb product. Lane M: Marker 1kb plus, Lane 1-6: sample1-sample6 of canine parvovirus.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a technique for *in-vitro* amplifying a specific region of DNA by a thermo stable DNA polymerase (Figure 6). PCR reaction uses short oligonucleotide primer sequences, dNTPs, DNA template and thermo stable DNA polymerase enzyme. PCR reaction involves three steps viz. denaturation, annealing and extension repeatedly for 30-40 cycles in thermal cycler. It

can be used for detection of virus specific nucleic acid. There are several modifications of PCR such as RT-PCR, Real-Time PCR, Multiplex PCR and Multiplex real-time PCR etc. used in molecular diagnosis of viruses. The PCR

amplified products can be used for nucleic acid sequencing to confirmatory diagnosis and detection of phylogenetic variations (Figure 7) and new strains of pathogens (Susmitha et al., 2012).

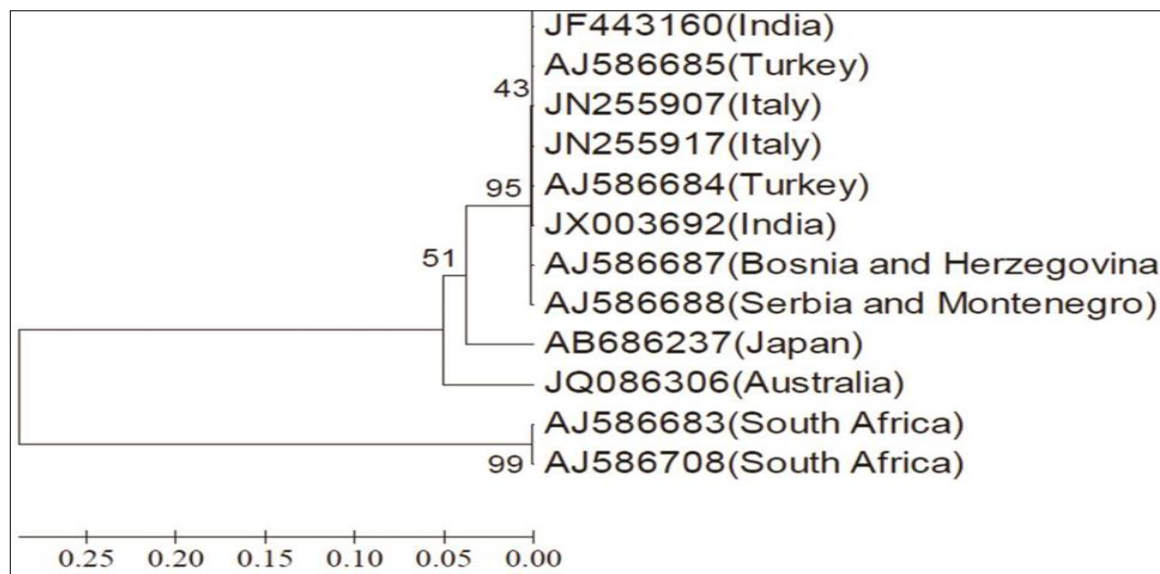


Figure 7: Vp5 gene based phylogenetic analysis of Indian isolate of BTV9 along with other global isolates.

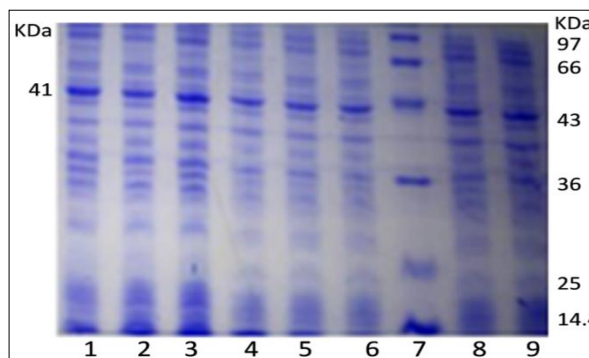
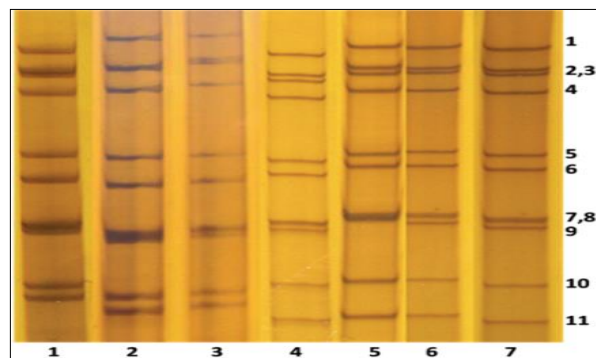


Figure 8: Electrophoretic migration pattern of human rotavirus isolates. Lane 1-3: Short electropherotype, Lane 4-7: Long electropherotype. The numbers on the right side indicate 1-11 RNA segments; Figure 9: SDS-PAGE for recombinant protein expression

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase enzyme (eg. Moloney murine leukemia virus enzyme). The cDNA is then used as a template for exponential amplification using pathogen specific oligonucleotide primer in PCR. The RT-PCR is currently the most sensitive method of RNA detection available. In molecular virology RT-PCR is used for diagnosis of RNA viruses such as Rotavirus and BTV. The sensitivity and specificity achieved in a well-designed RT-PCR make it an ideal tool for use in the surveillance and monitoring of many viral infections. It has been used to detect and identify the specific G and P genotypes present in rotavirus (Harimohan, et al., 2012). Similarly, segment 2 and segment 6 based RT-PCR has been standardized for BTV diagnosis (Kumar et al., 2013; Ranjan et al., 2013).

Poly Acrylamide Gel Electrophoresis (PAGE) Analysis

The RNA-PAGE has been used as a confirmatory tool for the identification of segmented genome dsRNA viruses such as Rota virus and bluetongue virus. Viral dsRNA are extracted either from cell culture grown samples or directly from various biological samples such as blood, semen or vector. The dsRNA can be analyzed by 8% RNA-PAGE followed by silver staining using the discontinuous buffer system without SDS (Laemmli, 1970; Svensson et al., 1986). Recently, a highly sensitive silver staining method for BTV has been developed (Minakshi et al., 2013). The characteristic genomes of Rota virus or BTV are visualized after silver staining (Minakshi et al., 2011a) (Figure 8), RNA-PAGE is a sensitive test and can detect up to 10^3 TCID₅₀/ml of BTV genomic segments (Prasad and Minakshi, 1999). RNA-PAGE can be used for electropherotyping in rotavirus (Harimohan et al., 2012) where several distinct RNA patterns can be observed (Figure 8) while SDS-PAGE can be used for visualization of recombinant protein expression (Figure 9).

Real-Time PCR

A more recent development in PCR is measurement of concentration of PCR products in real-time as the reaction proceeds PCR. It enables the viral load in a sample to be measured. Real-time PCR uses a beam of light of a specified wavelength with the capacity to illuminate sample in a thermal cycler with a capacity to detect the fluorescence emitted by the excited fluorophore. Real-time RT-PCR is a highly sensitive method that can be used for the laboratory detection of viral RNA from a variety of biological samples such as blood, tissue etc. However, in real-time RT-PCR reaction, viral RNA is reverse transcribed into cDNA and subsequently amplified and detected. The primers and probe combination for each real-time RT-PCR reaction are specific and designed to amplify and detect a targeted region of the viral genome (Vishwaradhya et al., 2013).

Multiplex PCR

In conventional PCR single set of primer is used at a time. However in multiplex PCR, several primer sets are used to allow amplification of multiple templates within a single reaction. It has been used for diagnosis of bluetongue virus, feline herpes virus, porcine epidemic diarrhea virus and transmissible gastroenteritis virus. Multiplex PCR enables the presence of nucleic acids from several pathogens to be detected in single reaction. However, care must be taken to avoid interference between different primer pairs or templates. It is also time and cost-efficient method and has been successfully used in several areas of nucleic acid diagnostics such as gene deletion analysis (Satish et al., 2011). The multiplex PCR was developed for molecular diagnosis of Indian FMDV serotypes O, A, Asia1 and C has demonstrated 100% efficiency in FMDV diagnosis with better diagnostic efficiency than ELISA (Giridharan et al., 2005). Recently, segment 6 based multiplex PCR for simultaneous detection of Indian isolates of BTV serotype 1, 2, 10, 16 and 23 has been standardized (Surendar and Reddy, 2012).

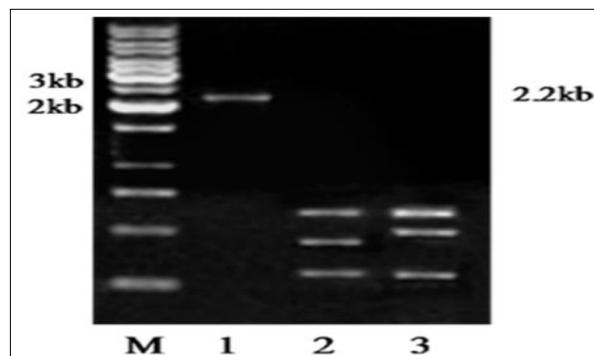


Figure 10: Restriction profile of 2.2 kb PCR product VP2 gene with *Rsa*I. Lane M: 1 kb marker (MBI Fermentas), 1: Uncut CPV 2.2 kb product, 2: R.E. digested pattern 1 of vaccine strain, 3: R.E. digested pattern 2 of field strain

Multiplex Real-Time PCR

The multiplex real-time PCR utilizes two to four fluorogenic oligoprobes for the discrimination of multiple amplicon. So far only a few truly multiplexed real-time PCR assays have been validated successfully. The use of non-fluorescent quenchers and the continuous development of better light sources in the machines are now in use. Now

this technique is in use for detection of viral diseases (Read et al., 2011).

Restriction Fragment Length Polymorphism (RFLP)

The RFLP technique differentiates pathogens by analysis of restriction patterns derived from cleavage of their nucleic acid using specific restriction endonuclease enzyme. Isolation of sufficient DNA for RFLP analysis is time-consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2–3 hours to the levels required for RFLP analysis. Thus, more samples can be analyzed in a shorter time. RFLP was used to differentiate the Canine Parvovirus2 (CPV-2) antigenic variants (Savi et al., 2009) (Figure 10).

DNA Microarrays

DNA microarrays can be used for the detection of unique DNA (or RNA) sequences. Thus it can be useful in the diseases diagnosis. Microarrays showed advantages over other traditional methods in automation in process, high sensitivity, rapid detection and low cost. In this technique the known DNA is immobilized on small solid supports such as glass slides, nylon membranes or silicon chips. The unknown DNA in the liquid phase is labeled to make probe. DNA or RNA is extracted from various biological samples, and subjected to RT-PCR and/or PCR amplification and labeled with a fluorescent dye. This labeled DNA is then hybridized with the microarray. The specific patterns of fluorescence are detected by a microarray reader which allows the identification of specific gene sequences. DNA microarrays have a potential to diagnose multiple viral pathogens including BTV (Jack et al., 2009).

Nucleic Acid Sequencing

Nucleic acid sequencing has provided an opportunity to study viral epidemiology by providing information about geographical origin of viral isolate. The determination of the nucleic acid sequences may provide information on where the virus came from. The application of nucleic acid sequencing is beyond just diagnosis and characterization of viruses. For example, VP2 and VP5 proteins of BTV are highly variable and determine the serotype specificity (Kumar et al., 2013; Ranjan et al., 2013). The nucleotide sequence and the phylogenetic analysis of these genes can provide a rapid approach for characterization of circulating BTV strains in particular geographical region.

Although, individual gene sequencing of viruses of veterinary importance is quite common, however, the continuing evolution of virus under selection pressure have forced researcher to go for full genome sequencing. Similarly to identify reassortment in viruses having segmented genome, complete genome sequence becomes essential. The full genome sequence of BTV isolates makes it possible to track any changes or reassortment events that occur in the virus isolates. In India full genome sequencing has been carried out for BTV serotype 16 (Minakshi et al., 2012).

NUCLEIC ACID SEQUENCING METHODS

First Generation Sequencing

First generation sequencing technologies include the Maxam-Gilbert method, discovered by Allan M. Maxam and Walter Gilbert (chemical method), and the Sanger method (or dideoxy method), discovered by Frederick Sanger. However, the Sanger method was more commonly

Table 2: Comparison of different sequencing technologies

Sequencing Technologies Parameters	Ion Torrent (Ion semi-conductor)	Roche 454 (Pyrosequencing)	Illumina (Sequencing by synthesis)	SOLiD (Sequencing by ligation)	Pacific Bio (Single molecule real-time sequencing)	Helicos (True Single Molecule Sequencing)	Sanger method (Chain termination)
Sequencing chemistry	Detection of released H ⁺	Pyrosequencing	Reversible terminators	Ligation	Fluorescently labelled dNTPs	Reversible terminators	Di- deoxy Chain termination
Adapter used	Adapters	Adapters	Adapters	Adapters	Hairpin adapters	Poly(A) adapter	N/A
Amplification method	Emulsion PCR	Emulsion PCR	Bridge amplification in situ	Emulsion PCR	Linear amplification	No amplification	Sequencing PCR
Separation method	Ion Spheres and high density array	Microbeads and 'picotitre' plate	Glass slide hybridization	Beads on glass slide	Captured by DNA polymerase in microcell	Flow-cell hybridization	Electrophoresis
Read length	200 -400 bp	700 bp	50 to 250 bp	50-75 bp	1000 bp	25 bp	400 to 900 bp
Reads per run	up to 5 million	1 million	up to 3 billion	1.2 to 1.4 billion	35-75 thousand	1 billion	Not available
Maximum data output per run	1 Gb	700 Mb	600 Gb	20 Gb	Not available	35 Gb	Not available
Accuracy	98%	99.9%	98%	99.9%	99%	99%	99.9%
Time per run	2 hours	24 hours	1 to 10 days	1 to 2 weeks	30 minutes to 2 hours	5-10 days	20 minutes to 3 hours
Cost per 1 million bases (in US\$)	\$1	\$10	\$0.05 to \$0.15	\$0.13	\$2	Not available	\$2400
Advantages	<ul style="list-style-type: none"> • Equipment relatively less expensive • Fast reaction 	<ul style="list-style-type: none"> • Long read size • Fast reaction 	<ul style="list-style-type: none"> • High sequence yield 	<ul style="list-style-type: none"> • Low cost per base of sequencing 	<ul style="list-style-type: none"> • Longest read length • Less time consuming 	<ul style="list-style-type: none"> • No PCR induced bias and errors • Tolerates degraded samples 	<ul style="list-style-type: none"> • Long individual reads. • Applied in many sequence based research
Disadvantages	<ul style="list-style-type: none"> • Homopolymer error 	<ul style="list-style-type: none"> • Homopolymer error • Runs relatively expensive 	<ul style="list-style-type: none"> • Needs high DNA concentration • Very expensive equipment. 	<ul style="list-style-type: none"> • Slower than other sequencing methods. 	<ul style="list-style-type: none"> • Low yield at high accuracy • Equipment very expensive. 	<ul style="list-style-type: none"> • Time to sequence a single nucleotide is high • High error rate 	<ul style="list-style-type: none"> • Higher cost per base of sequencing • Impractical for whole genome sequencing projects

used. Fundamental to this method is use of di-deoxy nucleotides, which lack a 3' hydroxyl group, along with the normal deoxy nucleotides. Here DNA chains are synthesized on a template strand. However, when one of the four possible di deoxy nucleotides added, the chain growth is stopped and prevents the further addition of coming nucleotides. In this way a population of nested, truncated DNA molecules are produced that represent each of the sites of that particular nucleotide in the template DNA. The truncated DNA molecules are the allowed for electrophoresis and DNA molecules are separated according to size on an electrophoresis gel. Thus, correct nucleotide sequence is deduced. In 1986, the laboratory of Leroy Hood at Caltech, in collaboration with Applied Biosystems (ABI), developed the first automatic DNA sequencer (Smith et al., 1986) which is now commonly used. The segment 6 and 3 of Indian isolates of BTV has been sequenced using ABI 3130xl genetic analyser (Ranjan et al., 2012; Minakshi et al., 2011b).

Second or Next Generation methods

Although first generation sequencing machines are commonly used, but they are of low through put type. To sequence whole genome second or next generation machines are developed. They are high through put sequencing methods enable many DNA fragments (sometimes on the order of millions of fragments) to be sequenced at one time and are much faster than first-generation machines. Four platforms for next generation sequencing are currently in worldwide use: the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, Ion Torrent/Personal Genome Machine (PGM) and the Applied Biosystems SOLiD™ System. Recently, another two massively parallel systems were introduced: the Helicos, Heliscope™ and Pacific Biosciences, SMRT instruments. These platforms are quite diverse in sequencing biochemistry array generation, read length generated, maximum data output per run, cost per base of sequencing etc (Table 2). However, their work flows are similar. The sequencing process starts with library preparation which is done by random fragmentation of DNA molecule, followed by *in-vitro* ligation of adaptor sequences to fragmented DNA molecules. The library sequences are then amplified through many different approaches such as emulsion PCR or bridge PCR depending upon type of machine used. The full genome sequence of one of the Indian isolate of BTV serotype 16 has been done using Ion Torrent –PGM (Minakshi et al., 2012).

CONCLUSION

Isolation of pathogen from various field samples will remain as gold standard for viral disease diagnosis. However, rapid detection and molecular characterization of the viral agent in clinical samples on the basis of nucleic acid sequence are very powerful tools for clinical and epidemiological studies. Other modern tests such as micro array, real-time PCR, next generation sequencing etc. are demand of modern era for rapid and reliable diagnosis. Moreover, very less is known about the molecular basis of pathogenesis and virulence of viral pathogens. There are still many situations where live virus in cell culture or animal hosts is inoculated for study of pathogenesis and virulence of virus. In addition, the speed and multiplexing capacity of PCR-based technologies have limitations not to detect live virus. While nucleic acid based diagnostic tests will have clear

significance as rapid tests, virus isolation will remain an important component of viral disease diagnosis of domestic as well as wild animals.

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