Animal Viral Diseases Diagnosis in Qatar: Current Situation and Future Prospects

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ABSTRACT

Diagnosis and control of viruses that cause diseases in domestic and wild animals and human, pose continuous challenges to veterinary and medical sciences. Many techniques have for a long time been used to diagnose individual infectious viral agents by their clinical signs, symptoms and histopathological changes produced in the susceptible host. But it is not always feasible to recognize or differentiate the diseases on the basis of its clinical signs and pathology, it consequently requires use of other more specific procedures to clearly identify and confirm the pathogens. These procedures involve both conventional as well as state of the art techniques. Several traditional techniques such as isolation in cell culture, serology for both antibody and antigen detection are routinely used to identify viral pathogens. But, the development of rapid molecular techniques for detection of infectious agents in animals as well as in environment have nowadays become an essential part of any modern diagnostic laboratory. These molecular diagnostic technologies are more advantageous as they offer more sensitive, less time consuming, and provide accurate diagnosis and provide deep understanding of present and emerging diseases. In addition, these novel tools can detect the presence of pathogen before the appearance of clinical disease. Herein, we discuss some aspects of the current and contemplated diagnostic tools and their potential applications for diagnosis of viral diseases of both domestic and wild animals in Qatar.

Keywords: Animal diseases, antibody detection, antigen detection, diagnostic tools, molecular techniques, Qatar, virus isolation.

I. INTRODUCTION

The veterinary laboratory of the State of Qatar is highly interested in establishing up to date diagnostic capabilities in the field of animal diseases diagnosis, especially viral diseases to guard against introduction, through animal trade, of exotic animal diseases, especially viral diseases, that may have serious economic and public health consequences. Currently, there is a small special unit for diagnosing animal viral diseases, that uses few of both conventional and molecular techniques. Nonetheless, new diagnostic methods are now being introduced and/or developed and established that were not used in the past, to help diagnose and control viral diseases.

Viral diseases are significant threats to human and animal health. Up to date only few successful antiviral drugs are available for treatment of viral infections. Thus, early and quick detection and identification of viral pathogens are essential [1]. Viral diseases diagnosis is important for deciding the management and control strategies, and to determine the prevalence of viruses and their different forms such as serotypes or genotypes. Several animal viral diseases like bluetongue of ruminants, camels, peste des ruminants of sheep and goats, Newcastle disease of poultry, foot and mouth disease of ruminants have constantly been causing significant economic problems in the field in Qatar and elsewhere [1].

Various sensitive and rapid diagnostic methods such as molecular techniques like polymerase chain reaction (PCR), probe hybridization, microarray, nucleic acid sequencing etc and Enzyme immunoassays have been widely used in diagnosis of animal viral diseases [2]. In this paper, the methods for viral disease diagnosis used currently, and some methods that might be introduced and used in the future in Qatar are surveyed and the possibility for their application are discussed.

II. GENERAL METHODS FOR VIRAL DISEASES DIAGNOSIS

In general, various viral disease diagnostic methods essentially depend on:
A. Clinical Presentation

Which include gross pathology, histopathology, epidemiology, and knowledge of diseases circulating in the area or in exporting countries are very helpful in diagnosis of diseases. In conjunction with electron microscopy these methods can provide hints to specify culprit viruses on the basis of tissues infected, structure, shape, and size, and their specific replication site within the cell. Despite existence of other techniques that are useful for identifying virus exposure or presence, careful histopathology examination is usually decisive for identification of viral disease.

B. Antibodies Detection (Serology)

Specific antibodies for a particular virus may be detected in a diseased, convalescent or healthy animal serum. However the sensitivity of most of the available serologic tests do not allow detection of the relatively small amounts of antibodies produced during the early stage of infection or disease and diagnosis by serology need to be carried out at least one week (IgM) or two weeks after the onset of the disease. For some qualitative serological tests two sera samples are usually collected, one in the early stage of the disease (acute serum) and one at two or more weeks later (convalescent serum) and a fourfold increase in the titer of the antibodies in the second serum is considered diagnostic for the particular virus under investigation. Serological testing is thus considered a retrospective or it might be considered presumptive in case of IgM detection.

Serological tests include a variety of tests such as serum neutralization (NT), Haemagglutination inhibition (HAI), Immunofluorescence (IF), Enzyme linked Immunosorbent assay (ELISA), Agar gel diffusion test (AGID) etc. and some of these tests will be detailed in the antigen detection section below.

In addition other very important uses of serological tests are to

1) Determine whether an animal has ever been exposed to a particular virus.
2) Establish a link between an infectious agent and the clinical symptoms manifested in the animals.
3) Determine responses to vaccination.
4) Monitor virus eradication programs.
5) Certify animals for trade and movement.

C. Virus Antigen Detection

Several direct antigen detection systems are available including virus isolation, but gel diffusion, ELISA, immunohistochemistry, immunochromatography, electron microscopy etc. They are increasingly being used for virus diagnosis.

1) Virus Isolation

Virus isolation is one of the most effective and standard methods for viral disease diagnosis. Except in few instances, it is highly sensitive and provide maximum chance for subsequent viral characterization. Suckling mice, and other larger experimental animals, cell cultures, and chicken embryo, inoculation may be used for virus isolation. Various cell lines have been established for viral isolation such as (to mention a few) FMD in BHK–21 cell line and primary bovine kidney cell, Vero for camel pox virus, NDV and PPRV, BHK–21 for BTV, and MDCK for influenza virus. The cell lines may be primary (Monkey Kidney), semi–continuous cells (Human embryonic kidney) and continuous cultures (HeLa, Vero cell line). Following isolation, confirmation of the presence of virus may be carried out using neutralization, immunofluorescence, immunohistochemistry, electron microscopy or molecular techniques [3, 4].

2) Antigen Detection by Serological Tests

Several serological based 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 particularly 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. However, enzyme immunoassays (EIAs) provide much better sensitivity, specificity and reproducibility than classical techniques such as CFT and HAI. But Haemagglutination Inhibition (HAI) test have been preferentially used to detect some viruses such as influenza, parainfluenza, flaviviruses etc. The Agar gel immunodiffusion (AGID) test and dot immunobinding assays were widely used for pox viruses [1].

a) Haemagglutination (HA) Tests

Some viruses can bind to red blood cells (RBCs). Such viruses bind to sialic acid residues on the RBCs and a single virion can bind to several different RBCs, or an RBC can be bound by multiple virions to form a large web or network, of cell and virus that can be easily visualized by naked eye. The HA test is fast and low-cost and does not require either advanced instrumentation or considerable training. It is carried out using serial dilutions of a virus sample. An aliquot of each dilution is then added to RBCs in test tube or a microtiter plate well. Negative control well that contains RBCs and saline and another known positive control well that contain reference sample of virus are also prepared. The samples are gently mixed and allowed to sit at room temperature. In the negative wells the RBCs will settle down to form a tight button at the bottom of the tube. In positive wells the RBCs and virions will bind to each other to form a lattice of cells on the bottom of the tube [5].

b) Haemagglutination Inhibition Assay (HIA)

This assay is used either to detect antibody to a virus or to identify a suspect virus. The HIA is performed by mixing virus samples with dilutions of serum. Antibody is allowed time to fasten the virus and then RBCs suspension is added to the mix. Viruses that have bound to antibody will be unable to attach to RBCs. Thus in the HIA, presence of hemagglutination is a negative result and the absence of hemagglutination is a positive result. If a hemagglutinating virus is the known reagent, the HI assay can be used to detect antibody. If the hemagglutinating virus is unknown, it can be identified by using a group of known antibodies [6, 7].

c) Virus Neutralization Assay

This assay is used in conjunction with an infectivity assay, such as the plaque assay. This assay detects antibody that has the potential of preventing virus replication (or in other words, antibody that can neutralize virus infection). Virus neutralization is a specialized type of immunossay because it does not reveal all antigen-antibody reactions, it only uncovers antibody that can inhibit virus replication. This is important because related groups of viruses may share...
common antigens that can be evident in other tests e.g. CF or AGID, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually defined by virus neutralization. For example, there are 25-29 BTV serotypes based on neutralization assay [8], [9].

d) Immunohistochemistry

The basic principal of immunohistochemistry is that a tissue section is first incubated with enzyme-tagged antibodies followed by addition of a colorless substrate to the sample. If enzyme-labelled antibodies are present, the substrate is cleaved to produce a colored precipitate. This is a pretty powerful technique as it allows examination of individual virus-infected cells in a tissue section. Patient samples (biopsies of affected tissues) are oftentimes preserved in formaldehyde or are stored frozen at ultra-cold temperatures. When samples are well preserved, they can be investigated for the presence of viral antigen even after years or even decades. Examples of viruses detected by immunohistochemistry methods is rabies virus [10].

e) Agar Gel Immunodiffusion (AGID)

Demonstration of anti-virus specific antibodies in serum or other samples can be accomplished by simple diffusion in agar of serum and viral antigen. A visible precipitin line is formed where the diffusing virus-specific antibodies and viral antigens meet and form complexes,. This test has a rather low sensitivity and requires relatively large amounts of antigen; however, it has the merit of being a rapid (2 days) and is easy to perform. It is used for example to detect Inectious Bursal Disease Virus (IBDV) [11].

f) Immunofluorescence (IF)

IF assay is usually used for the rapid detection of virus specific antigen in clinical samples or cell culture, as well as the virus-specific antibody. The technique is mainly based on reaction between fluorescein–tagged antibody and virus specific antigens in specimens (cells). The cells containing virus specific antigen will fluoresce under UV light illumination. Virus specific antibody can be detected using indirect IF whereas virus specific antigen is detected using direct or indirect IF [12].

g) Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is currently the most widely used form of immunoassay for the speedy detection of viral antigens and antibodies and commercial antigen detection ELISA kits are used for diagnosis of several viral diseases such as FMD, PPR, WNV, BTV, WNV etc. Peptide based ELISAs are also available for diagnosis of various viruses like infectious bursal disease virus, PPRV, FMDV, and IBDV [13]-[15].

D. Molecular Biology Approaches

In this section we will briefly describe some of the techniques that we intend to introduce into our laboratory.

Various molecular biology techniques have increasingly been used for direct detection of viral nucleic acid (genome) in the specimen for specific viral diagnosis. In these techniques, viral nucleic acid is extracted from the sample which can then be amplified using thermocyclers or vectors. The amplified DNA can then be probed by DNA hybridization or other techniques or sequenced. Most commonly, DNA is amplified by various kinds of polymerase chain reaction (PCR) using specific primers for diagnosis. The amplified PCR products can then be sequenced for confirmatory diagnosis [16]-[19].

1) Conventional Polymerase Chain Reaction (PCR)

PCR is a technique for in vitro amplification of DNA by a thermo stable DNA polymerase PCR reaction that uses short oligonucleotide primer sequences, dNTPs. DNA template and thermo stable DNA polymerase enzyme. PCR reaction is carried out in three steps viz. denaturation, annealing and extension repeatedly for 30–40 cycles in a thermal cycler and is thus used for detection of virus specific genome. The PCR amplified products can be used for e.g. sequence determination, as a confirmatory diagnostic test, and for detection of phylogenetic variations and detection of new strains of pathogens [20]. PCR has been developed for the detection of a myriad of viruses including herpes viruses, pox viruses etc.

2) Reverse Transcription Polymerase Chain Reaction (RT–PCR)

In this technique, 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 primers in PCR. In molecular virology RT–PCR is employed for diagnosis of RNA viruses such as FMD, PPR, Rotavirus and BTV etc. [21]-[23]. It is an ideal scheme for use in the surveillance and monitoring of many viral infections.

3) Real–time PCR

RT-PCR is a recent development in PCR for measurement of concentration of PCR products in real–time as the reaction proceeds. It enables the viral concentration in a sample to be measured. In this technique the viral RNA is also first reverse transcribed into cDNA and subsequently amplified and detected. The primers and probe combination for each real-time RT–PCR reaction are specific to amplify and detect a specific region of the viral nucleic acid [24].

4) Multiplex PCR

Unlike conventional PCR where a single set of primer is used at a time, in multiplex PCR several primer sets will be used to allow amplification of several NA templates during a single reaction; to put it another way, this test enables the presence of nucleic acids genomes from several pathogens to be detected in a single reaction. However, great care must be implemented to avoid interference between different primer pairs or templates. Multiplex PCR is also time and cost–efficient method and has been highly beneficial in Rota and Reoviruses detection as well as in other several areas of nucleic acid diagnostics such as gene deletion analysis [25].

5) Multiplex Real–Time PCR

The multiplex real–time PCR makes use of two to four fluorogenic oligoprobes for the discrimination of multiple amplicons. Howeve only a few truly multiplexed real–time PCR assays have so far been established and validated. The use of non–fluorescent quenchers and the continuous development of better machines light sources are are being sought to enhance the usefulness of this technique [26].

6) Loop–Mediated Isothermal Amplification (LAMP)

The development of easy to carry equipments for PCR and
Real-time PCR has made possible molecular diagnosis of animal viruses in the field. This approach, however, requires costly and delicate instruments and relies on precision thermocycling. Thus other modalities, like loop-mediated amplification (LAMP), were developed, which allow the tests to be conducted under field conditions using inexpensive devices. In LAMP, specific nucleotide sequences are amplified at a constant temperature and as such does not require a thermocycler. LAMP DNA amplification depends on the use of an autocycling strand displacement reaction employing a set of two specially designed inner primers and two outer primers and a DNA polymerase with high strand displacement activity [27]. The reaction is accomplished in less than an hour using a standard water bath or heating block and the results can be visualized with the naked eye [28]. The technique has been developed for detection of several viruses.

7) Polyacrylamide Gel Electrophoresis (PAGE) Analysis

The RNA–PAGE is used as a confirmatory tool for the identification of segmented genome dsRNA viruses such as rota viruses and bluetongue viruses. Viral dsRNA are extracted either from cell culture grown samples or directly from various biological samples such as feces, blood, semen or vector. A highly sensitive silver staining method for BTV has recently been developed and used for diagnosis [31]. The characteristic genomes of Rota virus or BTV are visualized after silver staining [29]-[32].

8) Restriction Fragment Length Polymorphism (RFLP)

The RFLP technique identify pathogens by analysis of restriction patterns derived as a result of specific restriction endonuclease enzymes cleavage of their nucleic acid. PCR can be used to amplify very tiny amounts of DNA, to obtain the required amounts of nucleic acids needed for RFLP analysis thus allowing more samples to be analyzed [33].

9) DNA Microarrays (Microchips)

DNA microarrays may be used for the detection of individual DNA (or RNA) sequences. Thus this technique may be useful in the diseases diagnosis because it can detect multiple pathogens simultaneously. Microarrays has the advantage over other methods in being amenable to automation, has high sensitivity, very short time of detection, and relatively low cost. In microarray a 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 act as probe. This labeled DNA is subsequently hybridized with the microarray and the peculiar patterns of fluorescence are detected using a microarray reader which allows the identification of distinct gene sequences [34].

10) Nucleic Acid Sequencing

Nucleic acid sequencing has enabled the study of viral epidemiology by providing information about geographical origin of viral isolate. The determination of the nucleic acid sequences may provide data on where the virus originated from. The nucleotide sequence and the phylogenetic analysis of the viral genes is a rapid method for virus characterization e.g. virus strains circulating in particular geographical regions and detection of reassortant viruses [22], [23]. Two methods are used for nucleic acid sequencing:

• First generation sequencing

First generation sequencing include the Maxam–Gilbert method, discovered by Allan M. Maxam and Walter Gilbert (chemical method), and the Sanger method (or dyeoxy method). In 1986, the first automatic DNA sequencer was developed [35] and is now widely used.

• Second or next generation methods (NGS)

First generation sequencing machines are commonly used, however they are of low throughput and they are usually used to sequence small parts of the genome. To sequence whole genome the next generation machines were developed. They are high throughput sequencing methods that allow numerous DNA fragments (sometimes millions of fragments) to be sequenced at the same time and are much faster than first-generation machines. Four platforms for next generation sequencing are currently in use: the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, Ion Torrent/Personal Genome Machine (PGM) and the Applied Biosystems SOLiD System [1].

Despite all the advances in virus detection and identification techniques, isolation of pathogen from various field samples will remain as the gold standard for viral disease diagnosis. However, speedy detection, molecular identification and characterization and new cultivation methods of the viral agent from clinical samples have tremendously increased the power of virus isolation.

III. CURRENT CONVENTIONAL APPROACHES USED FOR ANIMAL VIRAL DISEASES DIAGNOSIS IN QATAR

This section will provide an overview of the most commonly used techniques in diagnostic virology in the country.

A. Classical Virology Methods Currently used in Qatar for Animal Viral Diseases

1) Virus Isolation

The techniques currently used include the isolation of live virus in embryonated eggs and different cell cultures. These techniques, which were only recently introduced, will be used to isolate viruses such as FMD, Pox viruses, Newcastle disease virus and other poultry diseases viruses and a wide array of other viruses.

2) Hemagglutination assay (HA)

Hemagglutination test is used for diagnosis common poultry viral diseases such as Avian Influenza, Infectious Bursal Disease, Newcastle disease Infectious laryngotracheitis virus and Marek's Disease.

3) Hemagglutination Inhibition Assay (HI)

HI test is used for diagnosis viral diseases such as Avian Influenza, Avian Encephalomyelitis, Infectious Bursal Disease and Infectious Bronchitis.

4) Agar Gel Immunodiffusion (AGID)

The test has the traits of being a rapid (2 day) and easy to perform. The test is used to detect Camel poxvirus, sheep poxvirus and PPRV.

5) Enzyme Linked Immunosorbent Assay (ELISA)

Both antibodies and antigen detection commercially available ELISA kits are one of the most important tests currently used in the case of imported and exported animals,
as well as in diagnosis. The most important diseases that are examined by ELISA are: FMD, PPR, RVF, WNV, BTV etc.

IV. MOLECULAR APPROACHES

Molecular biology techniques such as PCR and real time PCR have been used for direct detection of viral genome (nucleic acid) in clinical specimens. These tests have already been used to diagnose FMD, Pox viruses, and PPR.

Methods planned to be introduced in the near future

We aspire to establish future methods in the virology lab, in order to upgrade our capabilities of viral diseases diagnosis as well as in research. Such techniques that we think will be most beneficial in our laboratory will include:

A. Neutralization Test

Neutralization test is to detect neutralizing antibodies against variety of viruses. A fourfold increase or decrease in antibody titer can be diagnostic. The test can also identify unknown viruses by using known neutralizing sera. The test will be used for FMD and BTV diagnosis and serotyping.

B. Immunofluorescence

It is used in antigen testing, especially for rabies and PPRV antigens. It is also used to test antibodies for some other viruses such as Influenza viruses, BTV etc.

C. Immunohistochemistry

For in situ detection of viruses in tissue samples by using enzyme labeled antibodies and their substrates to stain viruses in tissue samples such as poultry viruses.

D. Multiplex PCR

This technique can simultaneously detect multiple viruses so as to help testing for more than one virus at the same time. The test is also beneficial in cases of mixed viral infections. The test will be used for detection of FMD virus serotypes and BTV serotypes or multiple co-infections.

E. Multiplex Real–time PCR

Is more accurate, sensitive and quicker than the normal multiplex assay but with the same advantage of detecting multiple viruses at the same time and will be used to detect multiple RNA viruses infections. The test will be used for detection of FMD virus serotypes, herpes viruses 1, 4 that are common in horses in Qatar and to detect common respiratory viruses in several animal species.

F. Loop-Mediated Isothermal Amplification (LAMP)

As stated above LAMP is a quick and sensitive test that does not require sophisticated or expensive equipments and can be used to diagnose almost all viral diseases for which the proper primers are made. Detection can be used in a high throughpt system [35]. The test is to be used for FMDV, PPRV, BTV, RVFV and WNV etc.

G. Polyacrylamide Gel Electrophoresis (PAGE)

Page analysis can be used to visualize virus nucleic acids such as those of BTV, Reovirus and Rota viruses using silver staining or other labeling techniques.

H. Restriction fragment length polymorphism (RFLP)

RFLP can differentiate pathogens by their nucleic acids digestion pattern with endonucleases. It will be used to test FMD, PPR, Newcastle virus etc.

I. DNA Microarrays

Microarray can detect unique nucleic acid and are useful for viral diagnosis. The technique can detect multiple viruses at the same time. DNA microarrays will be used to diagnose multiple viral pathogens including BTV.

V. CONCLUSION

Isolation of pathogen from various field samples will continue as the gold standard for diagnosis of viral disease as the speed and multiplexing capacity of PCR–based technologies have their constraints in not being able to detect live viruses. However, speedy detection and molecular identification and characterization of the viral agent in clinical samples on the basis of nucleic acid sequence are very powerful methods for clinical and epidemiological studies. Other up-to-date tests such as real–time PCR, LAMP, microarray, next generation sequencing etc. are tools of the modern age for rapid and reliable diagnosis and epidemiological studies. Furthermore, these tools can help study of viral in cell culture or animal hosts to detect live virus. The introduction of the techniques mentioned above will be gradual and will not be an easy task. However, such introduction will greatly increase the diagnostic and research capacity of our laboratory and upgrading will continue as long as new technologies are arrived at and as our diagnostic and research needs increase in both veterinary and public health domains.

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CONFlict OF interest

Authors declare that they do not have any conflict of interest.

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