

Review Paper

DETECTION OF EQUINE HERPESVIRUS INFECTION: CONVENTIONAL VERSUS MOLECULAR APPROACHES

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ABSTRACT

Equine herpesvirus (EHV) are the highly contagious pathogens that infect both domestic and wild equine populations causing a major impact on equine industry worldwide. The methods for diagnosis of EHV have shown a vast improvement in the last decade. Although some conventional techniques are still applicable in certain cases, most of the clinical testing now focusing on rapid diagnosis by using the nucleic acid amplification-based techniques as major advances for the detection of EHV. The diagnosis of EHV does not only depend on clinical situation alone, but the suitability of diagnostic test is also vital for equine clinicians to make a decision regarding the specific treatments and control measures to be taken. Therefore, crucial understanding of the strengths and limitations of each assay are needed in order to interpret the results. Realizing the issue, this review intends to outline the clinical application of conventional approaches and the progress of the new molecular approaches. Relative advantages and limitations of each method have also been discussed.

Keywords: detection, equine herpesvirus, molecular, diagnosis.

INTRODUCTION

Over sixty years, EHV-1 and EHV-4 are among nine ubiquitous herpesviruses that pose the most serious health risks for domestic horses. The infections of these two viruses result mainly in upper respiratory tract disease, abortion and neurological disease. The diagnosis of EHV infection depends mostly on laboratory techniques in addition to clinical signs and historical disease. The clinical manifestations of EHV infection are similar to the infection of equine influenza, equine arteritis virus (EAV), equine adenovirus, equine protozoal myeloencephalitis, eastern equine encephalitis (EEE) and West Nile virus encephalitis (Powell, 1991). Therefore, the differential diagnosis of EHV infection is crucial for the prevention and control of the disease. A traditional technique of virus isolation formed a foundation of diagnostic virology. However, it has often constrained by expense, turnaround time and low sensitivity. Serological techniques are much more rapid, economical, easy to perform and suitable for screening purposes for EHV but most of these assays are unable to differentiate between infected and vaccinated animals and false-positive results are to be expected. Advance progresses in new diagnostic tools have provided a vast improvement in EHV detection with newer molecular-based assays such as real-time polymerase chain reaction (Pusterla *et al.*, 2017; Hafshejani *et al.*, 2015) and loop-mediated isothermal amplification (LAMP) (Kinoshita *et al.*, 2016) as the highly sensitive and specific in favoured

to previously time-consuming and laborious methods. Molecular tools for the detection of equine herpesvirus detection has been very used recently (Laabassi *et al.*, 2017; Badenhorst *et al.*, 2015). Equine practitioners should fully understand the advantages and the limitations of available tests beforehand in order to provide an accurate result interpretation. This review intends to demystify diagnostic approaches for equine herpesviruses detection from an insight of conventional to recent approaches. Further, advantages and limitations of these methods has also been discussed.

CONVENTIONAL TECHNIQUES

Virus Isolation: Virus culture and isolation remained as the gold standard for laboratory diagnosis of EHV infections. The technique demonstrates the typical cytopathic effect (CPE) in susceptible cell cultures inoculated with sample supernatant and use concurrently with the rapid test of polymerase chain reaction (PCR) or Immunoassays. Besides sample type and handling, one of the most important criteria to consider when isolating the virus is the type of cell culture. EHV-1 infects a variety of non-equine derived cell type such as fetal kidney cells, lung cells, dermal fibroblasts and endothelial cells from human, porcine, bovine, canine, feline, and rabbit (Trapp *et al.*, 2005). The common cell used to isolate EHV-1 are rabbit kidney (RK-13), baby hamster kidney (BHK-21), Madin-Darby bovine kidney (MDBK), and pig kidney (PK-15) (OIE, 2012). Unlike EHV-1, EHV-4 is mainly restricted to equine-derived cells such as equine dermis (ED), equine embryonic lung (EEL) and equine foetal

kidney (EFK) (Allen *et al.*, 2004). Despite of it all, some studies have shown that EHV-4 is capable of growing in the Vero cell (Dynton *et al.*, 2007; Ploszay *et al.*, 2013). Nevertheless, it is important to understand that different cell lines have different sensitivity which could cause false negative results (Balasuriya *et al.*, 2012) and thus, the most susceptible cell must be favoured especially if the infrequent cases of EHV-4 abortion are to be detected. The alphaherpesviruses, EHV-1 and EHV-4 produce CPE characterised by rounding and clumping of cells, the detachment of cells, and multinucleated syncytial cells within at least one to two days post-infection of high viral load sample (Crabb *et al.*, 1995; Lunn *et al.*, 2009). The presence of the virus in cell culture is confirmed with PCR.

Isolation of EHV-1 from nasal swabs or peripheral blood mononuclear cells (PBMC) from clinically infected horses could provide robust evidence of the viral infection. For EHM suspected case, it is recommended to collect the nasal swab sample during the early stages as the neurological signs start to appear at the end of viremia phase. The virus also can be detected in PBMC during the viremia phase before the horse start to develop the neurologic signs. As for suspected case of EHV-1 abortion, tissues samples such as placenta, lung, liver, thymus and spleen from the aborted foetus the virus isolation can be attempted for virus isolation. Periodically, the virus is also presence in the cerebrospinal fluid (CSF) along with neurological signs (7-16 dpi) but it is rarely collected since isolation is difficult (Kohn *et al.*, 2006).

The viability of the virus is required in the sample and it may be affected by factors such as sample collection and storage, timing of sample collection, post-mortem interval and sample transport (Bourgeois and Oaks, 2013). For nasal swab, it is recommended to take the sample during the initial febrile phase and instantaneously transport them in sterile cold transport medium (Pusterla *et al.*, 2008). Blood sample intended for buffy coat separation should not be frozen while tissue samples should be kept at 4°C instead of -20 °C until inoculated into cell culture. Samples that will not be processed immediately should be stored at -70°C (OIE, 2012). The result of virus isolation may be negative due to procedural mistakes that could have affected the viral envelope integrity; a crucial component for host infection (Goehring *et al.*, 2010). It is also due to intermittent of virus shedding and interference of local antibodies (Harless and Pusterla, 2006).

The reactivation of latent infection *in vitro* can be done by co-cultivation method and has been considered as gold standard for demonstration of latent virus. Latently infected tissues should be dispersed to yield infectious virus and the viable tissue cells will be cultured and serially passage for three times with susceptible cells. The sensitivity of co-cultivation method

can be increased for latently infected leukocytes by stimulation with T-cell mitogens (Slater, 2013).

In the diagnostic lab, virus isolation helps to determine the status of the infections (lytic or latent) from the DNA in PCR test. The failure to isolate the virus could be explained as a latent infection or the infected horse harbours the virus in an inactive form in the body. While rapidness and specificity are unquestionable advantages of PCR over virus isolation, the superiority of PCR sensitivity could be argued as the virus isolation may provide higher sensitivity than PCR if the viable virus is presence in the sample and suitable cell lines are used for cell culture. This is due to larger volume of the sample inoculate onto the cell (50 to 150ml) than that being used in PCR. McBreaty *et al.* (2013) were able to isolate EHV-4 from PCR-negative samples which suggest that the sensitivity of virus isolation has a potential to surpass the PCR. However, the likelihood of simultaneous detection and differentiation of various types of EHV and rapid diagnosis offered by PCR is considered substantial over virus isolation technique under certain circumstances. Besides time-consuming and laborious, virus isolation requires the presence of viable virus which can be challenging to achieve in the field. In summary, as the conventional technique of cell culture is considered insufficient to produce a reliable result, more advanced techniques should be concurrently utilised.

Serology-Based Assay: Serological testing for EHV antibodies in serum or plasma has been the key tool to gain retrospective diagnosis and forms a valuable part of longitudinal surveillance. Serology helps to provide information on EHV-1 exposure in a non-vaccinated horse which can be used to guide the management practices (Irwin *et al.*, 2007). Despite some limitations, most serologic assays are still reliable and useful for viral diagnosis, cost-effective and fairly easy to perform. Over the years, a variety of serology methods has been used in the detection of EHV antibodies including the most common test formats for equine virus diseases, virus neutralization (VN) and enzyme-linked immunosorbent assay (ELISA), which will be discussed in details with several other serologic tests.

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA is one of the most common serologic tests used as a screen test in detection of many infectious diseases. In ELISA, the antigen is bound to the wells of the plastic plate. The serum samples are added, and if EHV-1 or EHV-4 are present, they will bind to the antigens.

When unbound antibody are washed, a secondary antibody against antibody such as anti-equine immunoglobulin G (IgG) conjugated to an enzyme like horseradish peroxidase is added afterwards and becomes a complex with these antibodies. Unbound antibodies will be removed prior the addition of a substrate solution. The addition of colorimetric substrate produces a visible

colour change which indicates the positive reaction. The results can be interpreted visually or by microplate spectrophotometer.

Theoretically, detection of EHV-1 specific antibody would provide a more convenient way to identify latently infected horses. Earlier ELISA assays, like other serologic assays, did not discriminate between EHV-1 and EHV-4 because of the close antigenic similarity between these two strains. Through recent advance, a type-specific ELISA using fusion proteins expressing variable regions of glycoprotein G (gG) homologues has been developed and able to distinguish antibody to EHV-4 and EHV1 (Crabb *et al.*, 1995; Crabb and Studdert, 1993). It has been shown to be a valuable diagnostic tool to identify horses infected with EHV1 or EHV4 when acute and convalescent sera are available. The IgG ELISA has been used in the epidemiological studies of EHV infection (Gilkerson *et al.*, 1999; Yasunaga *et al.*, 1998) as well as in the management of outbreaks of EHV-1 myeloencephalitis and abortion (Drummer *et al.*, 1995; Studdert *et al.*, 2003). A commercial ELISA test kit has been developed using EHV-1/4 recombinant glycoprotein G for the detection and distinguishing EHV-1 and EHV-4 infections such as Svanovir® and has been used broadly in to detect EHV-1 and EHV-4 infections (Ataseven *et al.*, 2009; Brown *et al.*, 2007; Dynon *et al.*, 2007; Gür and Yapici, 2008).

However, a correlation between the virological and serological diagnosis of EHV-1 using ELISA gG was reported as relatively poor. It is assumed that EHV-1 specific antibody presumably maintained by latently infected horses do not recognise gG and some antibody are below the detection limits of this assay, explaining the low levels of EHV-1 antibody (Dunowska *et al.*, 2015).

Virus Neutralization: Virus neutralization (VN) test detects antibodies capable of neutralizing the infectivity of the virus. This technique involves a serial dilution of heat-inactivated serum followed by incubation with 100-200 TCID₅₀ virus which leads to neutralization of the virus with antibodies. The mixture is added into the cells to see any infection (Bourgeois and Oaks, 2013). The presence of the viral growth is examined microscopic for the evidence of cytopathic effect (CPE). In the case of the low amount of virus present in the cell or no CPE observed, immunofluorescence staining should be applied. By neutralizing the infectivity of the virus, antibody protects the cells against viral infection. The highest dilution that neutralized virus infectivity known as the titre (Murphy *et al.*, 1999).

The detection of specific antibody in serum required more than a single blood collection as it is insufficient for a positive diagnosis of current and active infection. The diagnosis of acute EHV-1 cases by VN is reliable if the sampling is conducted during the onset of the disease. Therefore, it is recommended to collect a

paired serum samples from in-contact horses since many of these animals seroconverts, showing indirect indication of EHV-1 infection (Pusterla *et al.*, 2009a). The acute phase sera sample should be taken after the beginning of clinical signs followed by convalescent phase sera taken two to four weeks after. Fourfold or greater increase in virus-specific antibody titre is needed to confirm the infection (OIE, 2012). According to American Association of Equine Practitioners, high titers in a single VN between 1:1024 to 1:2048 or greater are most probably demonstrate the recent infection rather than vaccination. Moreover, VN test is suitable to be used in prevalence surveys as it is capable of indicating the historical exposure of the infection (Slater, 2013).

Complement Fixation: The complement fixation (CF) is a method used to detect the presence of specific antibody such as IgM or antigen in the serum. CF test depends on formation of specific antibody-antigen complex in which during the reaction, complement will bind or fix to it. Sensitized sheep red blood cells are added to measure the complements that have bound in the reaction. If the complements have been fixed due to the antigen-antibody reaction, there will be no complements remain for the lysis of the sensitized sheep red blood cell and no haemolysis indicates the positive result.

CF, along with other serological assays is suitable for ascertaining antibodies against EHV-1 or EHV-4. CF antibodies have low vitality which is normally untraceable for three months when infection happen (Thomson *et al.*, 1976). Hence, it is visibly effective during the outbreak by utilizing two pairs of collected sera within a fortnight apart as it is capable in providing the most powerful serological evidence for early EHV-1 infection (Hussey *et al.*, 2006; McCartan *et al.*, 1995). Epitopes recognized by CF antibodies, however, can be cross-reactive with EHV-1 and EHV-4, leading to a false result of type differentiation (Crabb and Studdert, 1993).

Immunofluorescence: Immunofluorescence (IF) is a technique to detect specific target antigens in nasal or nasopharyngeal swab samples or in frozen (cryostat) sections from aborted fetal tissues (lung, liver, thymus and spleen) and placental tissue in the detection of EHV using the fluorescent-labelled antibodies. In IF techniques, the sections are mounted on microscope slides, fixed with acetone and incubated at 37°C treated with a proper dilution of the swine antibody specific for EHV-1 which formed chemically with fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). These marked antibodies attach (directly or indirectly) to the antigen of interest which enables the detection of antigen via fluorescence microscope (Robinson, 2009) (Figure 1). One of the advantages of IF is it is very rapid and simple test with acceptable sensitivity and specificity. Direct

immunofluorescence detection of EHV-1 or EHV-4 antigens in cryostat sections of tissues freshly dissected from aborted fetuses offers a rapid technique for the diagnostic laboratory to conduct an early diagnosis of EHV abortion (Allen *et al.*, 2004). Usually, IF is combined with histopathology for the confirmation of the viral antigen. In addition, it can demonstrate antigen which related to lesions or affected tissues which are the additional evidence that implies the connection to the lesion. The detection of viral protein is typically characteristic of active replication, instead of the latent infection. Therefore, detection of viral antigen in post-mortem fresh, frozen or fixed samples of lymph nodes and trigeminal ganglia during latent infection is theoretically producing negative results. The major disadvantage includes the visualization of the lesion from the poor morphology of frozen sections which can be technically challenging to read (Bourgeois, 2013).

Immunohistochemistry: Enzyme immunohistochemical staining such as immunoperoxidase has been established as a technique for detecting EHV-1 antigen paraffin-embedded tissue of aborted equine fetuses or neurologically affected horses. Furthermore, it also can be attempted on infected cell monolayers for both EHV-1 and EHV-4 detection (van Maanen *et al.*, 2000). The technique particularly useful for evaluation of morphological lesions associated with EHV-1.

Histopathology: Histopathology is a crucial method for confirming EHV infection in aborted fetuses and post-mortem samples collected from neurologically affected horses. The characteristic of the virus usually includes eosinophilic inclusion bodies in airway epithelial and hepatocytes from aborted fetuses (Rimstad and Evensen, 1993). Infection of the vascular endothelium causes necrotising vasculitis, thrombosis and ischemia damage of blood capillaries in the spinal cord or brain and endometrial blood vessels (Edington *et al.*, 1991; Jackson *et al.*, 1977; Smith *et al.*, 1992; Wilson, 1997).

In-Situ Hybridization (ISH): Viral DNA can be detected by in-situ hybridization (ISH). ISH in combination with polymerase chain reaction (PCR) have been applied to samples of foetal and placental tissue from EHV-1 abortions. This assay is able to expose viral nucleic acids in endothelial cells of endometrial arterioles (Mukaiya *et al.*, 2000; Smith and Borchers, 2001). DNA-DNA ISH has higher sensitivity than IHC and has the possibility to detect latent and active infected cells. However, experimental evidence suggests that the technique is lacking in sensitivity to detect low amount of virus genomes in latently infected cells and more sensitive RNA-DNA hybridization assay should be used (Slater, 2013).

Molecular approaches: Latency by alphaherpesviruses is seen as an important epidemiological plan to secure

vitality while expanding inside the natural host population (Whitley and Gnann, 1993). The latency site for EHV-1 and EHV-4 have been found in the lymphoid tissue debilitating the respiratory tract (Chesters *et al.*, 1997; Edington *et al.*, 1994; Slater *et al.*, 1994) and in the peripheral blood (Chesters *et al.*, 1997; Smith *et al.*, 1998; Welch *et al.*, 1992). Other studies claim latency is established mainly in the trigeminal ganglia (Borchers *et al.*, 1999; Slater *et al.*, 1994). During latency, the entire viral genome exists inside the host cells but only a few part is transcribing into detectable viral RNA, known as latency-associated transcripts (LATS). This features of EHV renders the conventional methods of detecting the virus in the latent form. Molecular-based approaches are evidently more sensitive and specific, which have provided a rapid and accurate detection and characterization of EHV. By targeting these LATS, latency can be detected by using reverse transcriptase-polymerase chain reaction (RT-PCR) or by real-time PCR. From the past decades, diagnosis by PCR including nested, multiplex PCR and quantitative PCR have form a vital part of the range of diagnostic tests currently available for EHV. The advent of the latest technology of loop-mediated isothermal amplification (LAMP) has great potential to emerge as a new approach for a rapid diagnostic tool for early detection and identification of EHV.

Conventional Polymerase Chain Reaction: PCR in detection of EHV-1 and EHV-4 is considered superior to virus isolation in terms of rapidness, sensitivity and specificity (Diallo *et al.*, 2007; Marenzoni *et al.*, 2008; Varrasso *et al.*, 2001). The assay is able to detect 10 to 100 copies of target viral (Borchers and Slater, 1993) and produce a positive result although the testing with virus isolation is negative due to the low magnitude of viral load (Lunn *et al.*, 2009). The sensitivity of PCR is suitable for consensus study involving tissue samples in previously characterized EHV as well as providing new sequence information for previously unreported EHV (Leon *et al.*, 2008).

Nested, semi-nested PCR (Borchers and Slater, 1993; Pusterla *et al.*, 2005; Varrasso *et al.*, 2001) and multiplex nested PCR (Ataseven *et al.*, 2009; Carvalho *et al.*, 2000; Wang *et al.*, 2007) for detection of EHV have been described. The nested PCR has higher sensitivity than standard PCR, but extreme sensitivity of nested PCR is prone to yield false positive results from a minute amount of previous amplicons contamination (Bourgeois and Oaks 2013). Therefore, the use of a nested PCR assay is not recommended for the routine laboratory diagnosis of EHV-1. Multiplex PCR could simultaneously detect multiple EHV isolates (Carvalho *et al.*, 2000; Wang *et al.*, 2007) and equally sensitive and specific for detection of EHV with reduced labour and reagents cost compared to single PCR assays (Edwards and Gibbs, 1994).

Reverse-transcription PCR (RT-PCR) assay also have been used to differentiate between the active and latent infections of EHV-1 by detection of transcripts of EHV-1 glycoprotein B (Allen *et al.*, 2008). Since EHV-1 is a DNA virus, this type of PCR is rarely used to detect lytic infections but more likely for detecting and quantifying latent EHV-1 in tissues and whole blood (Borchers *et al.*, 1999; Chesters *et al.*, 1997). The sensitivity of RT-PCR can be increased by using semi-nested, nested or three-step nested. However, due to the complexity and time consumption of this method, it is regularly used in research setting rather than in routine diagnosis.

Several PCR assays target different target genes of EHV; glycoprotein gene B (gB), C (gC), D (gD), H (gH) and thymidine kinase (TK) gene have been developed (Borchers and Slater, 1993; Carvalho *et al.*, 2000; Lawrence *et al.*, 1994; Varrasso *et al.*, 2001). Detection of EHV has been attempted in clinical or pathological specimens such neonatal foal tissues, blood leukocytes, nasal mucus, brain and spinal cord, inoculated cell cultures and paraffin-embedded archival tissues (Brown *et al.*, 2007; Kirisawa *et al.*, 1993; Lawrence *et al.*, 1994; Rimstad and Evensen, 1993; Sharma *et al.*, 1992; Studdert *et al.*, 2003). According to World Organisation for Animal Health (OIE), PCR methods are most useful for the confirmation of the virus in the samples of aborted fetuses, nasopharyngeal swabs and peripheral blood leukocytes of foals and yearlings for diagnosis of active EHV infection. In neurological diagnosis, PCR is important in examinations of the spinal cord, brain tissue and PBMC.

The EHV could shed intermittently in the body without showing any clinical signs and thus, collecting the samples from asymptomatic horses outside the outbreak for PCR testing is not recommended (Lunn *et al.*, 2009). Tested horse that has been exposed and latently infected could cause a confusion in clinical evaluation as it may continuously produce the positive result for a long time. The complexities of a test clarification could put the equine practitioner in a hard situation when taking the decision regarding the facilities confinements or the annulment of the competitions. Isolation and close monitoring for clinical signs development however should be done when the high risk asymptomatic horses were positive in nasal secretion by PCR during the outbreak. This is because, under such circumstances, the magnitude of virus during incubation period resembles with that subclinical stage (Pusterla *et al.*, 2009a).

The drawback of the non-quantitative PCR is the incapability to measure the viral load in the clinical sample has led to a newer molecular platform of quantitative PCR assay with promising advantages for EHV detection. In addition, the standard PCR is incapable of distinguishing between latent and lytic infection. Although extensive development for PCR

assays has been made, the lack of standardization of the laboratory and quality assurance procedures persist to be constant challenges.

Real Time PCR: Quantitative PCR or real-time PCR (qPCR) was developed in 1996 using a probe-based detection system and enables the PCR amplification in a closed-tube system (Wilhelm and Pingoud, 2003). The 5' nuclease activity of Taq polymerase will digest the probe during the primer extension, causing the release of quenched fluorescent signal which is measured continuously by sequence detector during each PCR amplification cycle (Heid *et al.*, 1996). The fluorescent signals are collect by a digital camera in real time and the data is stored in the attached computer (Pusterla *et al.*, 2006). Presently, variety fluorescent chemistries such as SYBR Green dye, Fröster resonance energy transfer (FRET) and Scorpion primers can be used as the alternatives to TaqMan with similar performance characteristics.

Compared to conventional PCR, qPCR provide a lower risk of cross-contamination due to its close and automatized amplification system. The high technology offered by this assay, however, requires some initial high capital costs for instruments such thermal cycler which most of the low resource setting laboratory could not afford.

In the recent study of EHV detection, qPCR is in favoured of virus isolation and other assays in terms of sensitivity, specificity and rapidity specificity and allow the quantification of virus load for EHV in clinical samples (Allen and Breathnach, 2006; Diallo *et al.*, 2007; Elia *et al.*, 2006; Hussey *et al.*, 2006; Marenzoni *et al.*, 2008; Perkins *et al.*, 2008; Pusterla *et al.*, 2009b). The ability to quantify viral load is valuable for a prominent characterization of disease phase, evaluation of risk exposure in susceptible horses and control measures and vaccination evaluation through measurement of viral load and viral shedding. qPCR has been used to demonstrate differences in the viral loads between disease stages in adult horses and amongst clinically and subclinically infected horses (Pusterla *et al.*, 2008). The viral loads of EHV-1 in nasal secretions are varying between horses. Previous assessments of the peak of viral DNA in the nasal secretion of EHV-1 infected horses using qPCR were approximately 4.2×10^4 (Pusterla *et al.* 2009) and 10 (Hussey *et al.* 2006) gB gene copies per 1 ng of template DNA. It is difficult to get the exact values due to different stages of disease progression and sampling method between the studies.

The advanced in quantitative real-time PCR enables the discrimination between two viral states. The differentiation between latent and lytic infection rely on identification of the viral genome (DNA) and viral transcripts (mRNA) for one of the structural genes. Detection of viral DNA without the viral mRNA

indicates the latent virus while detection of both viral DNA and mRNA demonstrate an active EHV infection. In a previous study, the expression of the structural glycoprotein B in nasal swabs of the infected foal was detected only during the first week of the 28 days study period after the onset of the clinical signs. There is no persistence of LAT expression was recorded within the period, suggesting that the lytic infection of EHV-4 can be marked either by high DNA load or by detection of the transcriptional activity of the glycoprotein B (Pusterla *et al.*, 2005).

A real-time PCR have been utilised in the variety of samples including nasal swabs, blood, tissues, lymph nodes, trigeminal ganglia, placenta, brains, lungs, liver and spleen (Allen *et al.*, 2006, 2007; Hussey *et al.*, 2006; Sarani *et al.*, 2013; Turan *et al.*, 2012). A study has suggested that the detection of EHV-1 using qPCR assay of nasal secretions is more sensitive than assay of buffy coat leukocytes (Brown *et al.*, 2007) but greater sensitivity in peripheral blood mononuclear cells was observed (Hussey *et al.*, 2006). Meanwhile, EHV-4 has a low load, lack of viral replication and short duration in peripheral blood leukocyte (PBL), suggesting that it is not suitable to use as a diagnostic material to detect acute EHV-4 respiratory infection (Pusterla *et al.*, 2005).

The real-time PCR could detect active nasal viral shedding or viremia but is generally fail to detect latent infection. As the positive real-time PCR indicate active infection, it is therefore categorized as potentially contagious infection, in which this characteristic is important for the diagnosis of EHV-1 (Hussey *et al.*, 2006).

The development of a real-time PCR assay using allelic discrimination E_2 to distinguish between neuropathogenic and non-neuropathogenic strains of EHV1 has been reported (Allen *et al.*, 2008). The following study managed to tackle the lack of sensitivity for routine diagnostic applications by using allelic discrimination E_1 in the open reading frame 30 (ORF30) which showed 10-fold-higher sensitivity than E_2 allelic discrimination (Smith *et al.*, 2012).

The target of the conserved regions in conventional real-time PCR can be too short and problematic. Moreover, variants with unidentified polymorphisms in the conserved regions could be undetected. By introducing the minor groove binder (MGB) probe technology, it allows the shorter probes to descry short conserved regions when developing assays to discover various strains. The post-amplification melt-curve analysis is allowed by 5'-MGB probe to confirm the real-time amplification results (Corpus, 2010).

Loop-mediated Isothermal Amplification (LAMP): LAMP is a nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and rapidity under isothermal condition (Parida *et al.*, 2008).

It requires four to six different primers that are designed specifically to recognize six to eight specific gene sequences. The DNA amplification is accomplished with the use of DNA polymerase with strand-displacing activity using only a water bath or heating block to incubate the reaction mixture at a constant temperature between 60-65°C (Notomi *et al.*, 2000). Exclusion of expensive instruments demands in LAMP amplification has changed the ways on how the researchers are able to conduct the testing outside the laboratory setting. A further prominent advantage of LAMP assays is the omission of DNA extraction steps which allows the reaction to be carried out directly from the samples, thus, reduce the turnaround time, cost and labour (Nemoto *et al.*, 2011).

The amplification of DNA can be seen with the naked eye in the form of turbidity (Figure 2C) or visual fluorescence (Figure 2D). Positive LAMP amplification can be indicated by the synthesis of a large amount of white precipitate magnesium pyrophosphate in the reaction which causes the turbidity, or by the colour change after the addition of DNA binding dye such as SYBR Green, calcein, ethidium bromide, PicoGreen or propidium iodide (Mori *et al.*, 2001). Gene amplification products also can be detected by agarose gel electrophoresis in which a ladder pattern of varying sizes of bands are produced (Figure 2B). Such pattern is due the cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Notomi *et al.*, 2000). Furthermore, LAMP assays also allow the quantification of the template DNA by real-time turbidimetry (Mori *et al.*, 2004) (Figure 2A). In 2006, visual detection of LAMP product in a sequences specific manner through precipitation by cationic polymers, polyethylenimine (PEI) has been reported (Mori *et al.*, 2006). Moreover, further improvement has been used to detect the magnesium pyrophosphate by-product based on colorimetry using hydroxynaphthol blue (HNB) indicator (Goto *et al.*, 2009). For the confirmation of the structure, the amplified product is digested using several restriction endonucleases such as BamHI, PvuI and PvuII which each one of them cut at different regions. Confirmation also can be done by cloning and sequencing (Notomi *et al.*, 2000).

Since the first report of LAMP in 2000, this molecular method has become a focus among the researchers due to the specific features, which favour its use in simplified testing systems that might be appropriate in a resource-limited setting in developing countries, where the lethal tropical diseases are endemic (Mori *et al.*, 2013). In 2010, the application of LAMP for the rapid detection of both EHV-1 and EHV-4 from nasal swab has been conducted (Nemoto *et al.*, 2010). The result using LAMP has shown to be in good agreement with PCR for EHV-1 and EHV-4. Despite all the

advantages, the main limitation of the LAMP is the primer designing of four to six primers which are crucial for a successful amplification. Hybridization of the four main primers to the target DNA is considered critical for the LAMP efficiency. Furthermore, LAMP is unsuitable to be used for the detection of unknown or unsequenced targets. In addition, the other important thing to consider using this assay is the size of the target DNA. While PCR could amplify the region of target DNA up to 2kb, LAMP

is only limited within the range of less than 300 base pair because one rate limiting step for amplification in this method is strand displacement DNA synthesis (Notomi *et al.*, 2000). However, due to the use of simple rapid and efficient method of LAMP, the assay could be considered as an alternative to previous methods of detecting the EHV where it would enormously support control programs and management during outbreak which could limit the spread of the disease.

Table 1. Summarized the comparison of conventional and molecular methods for detection of equine herpesvirus.

Diagnostic test	Rapidity (Yes/No)	Required Sample	Advantages	Disadvantage
Immunofluorescence	Yes	Airway swabs, Frozen tissue, fresh blood	Simple, fair sensitivity; high specificity; suitable for screening test for tissue and swabs	False negative occur; interpretation of results are challenging; required skilled personnel
Virus Isolation	No	Fresh blood PBMC / buffy coat, tissue samples, airway swabs, lavages	Highly specific and sensitive; can differentiate between EHV's.	False negative occur; laborious, time-consuming; required skilled personnel to perform; prone to contamination
Serology: ELISA	Yes	Serum (clotted blood)	Sensitive and specific; only ELISA allow differentiation between EHV-1/EHV-4	False positive occur due to previous vaccination and maternal antibodies
VN	No	Paired sera taken 10-14 days apart for demonstration of increasing antibody titers	specific IgG; provide retrospective evidence of infection	
CF				
Histopathology	No	Fixed tissue sections	Specific and sensitive method to confirm infection in post-mortem samples.	Contamination of tissue sections during several stages of tissue processing, cutting and staining; required skilled personnel to perform.
Polymerase Chain Reaction (PCR)	Yes	Airway swabs, blood, frozen or fixed tissue samples, lavages	Highly specific and sensitive; able to differentiate between EHV's	False negatives occur; cross contamination; non-quantitative; unable to differentiate latent and active infection
Real-Time PCR	Yes	Airway swabs, blood, frozen or fixed tissue samples, lavages	Highly specific and sensitive; allow estimation of virus load; detection of multiple classes of genes; distinguish lytic and latent infection	Require expensive equipment
Loop Mediated Isothermal Amplification	Yes	Airway swabs, blood	Highly specific and sensitive; less sensitivity to inhibitory substances present in samples; simple and cost effective, require no expensive instruments.	Complicated primer design (requirement for four to six primers); inadequate for the detection of unknown or unsequenced targets.

CN, complement fixation; ELISA, enzyme-linked immunosorbent assay; VN, virus neutralization; PBMC, peripheral blood mononuclear cells

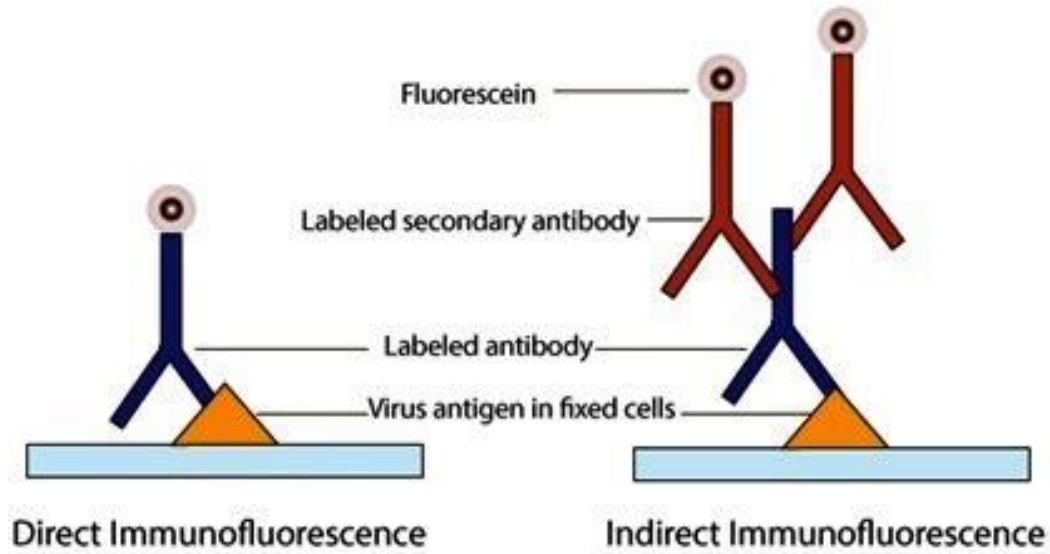
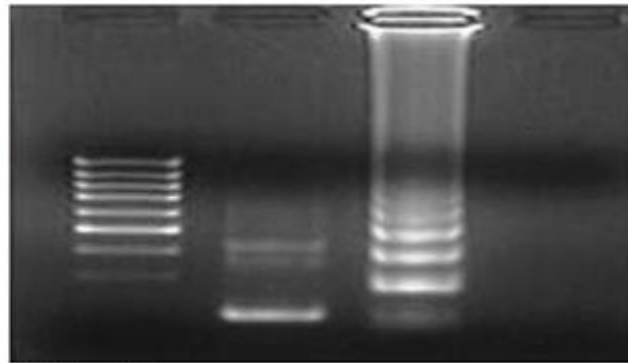


Fig.1. Schematic representation of direct and indirect immunofluorescence

A. Real Time Monitoring



B. Agarose Gel Analysis



C. Visual Turbidity



D. Visual fluorescence

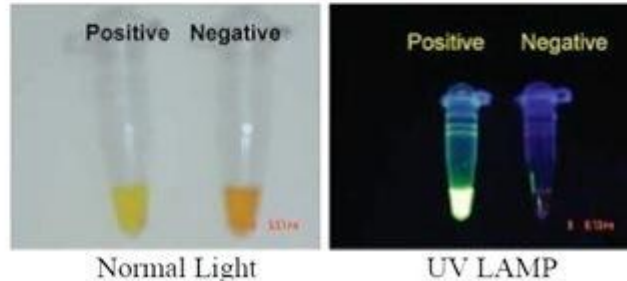


Fig.2. Monitoring of LAMP amplification. (A) The turbidity of magnesium pyrophosphate, a by-product of the reaction, can be detected real-time by a real-time turbidimeter. (B) Agarose gel analysis revealing the typical electrophoresis pattern of LAMP amplified product, which is not a single band but a ladder pattern because the LAMP method forms amplified products of various sizes consisting of alternately inverted repeats of the target sequence on the same strand. (C) Visual turbidity in the form of white precipitate as observed in positive control due to accumulation of magnesium pyrophosphate in proportion to the accumulated amplified products. (D) When the tube containing the amplified products incorporating a fluorescent intercalating dye is illuminated with a UV lamp, the fluorescence intensity increases. Reproduced from (Parida *et al.* 2008), with permission from the publisher (Wiley).

Conclusion: Table 1 summarized the comparison of conventional and molecular methods for detection of equine herpesvirus comprising the advantages and disadvantages as each of the tests that have been discussed. The conventional approaches have been well-developed and used widely for testing and still applicable to be used despite their limitations. Molecular assays have become the powerful tools for virus diagnostic but when new applications for molecular testing are being introduced, the questions arise whether they can supplant the traditional methods in term rapidity, sensitivity and cost of the test. The newer molecular methods show a promising technology but extensive evaluation is needed in order to be fully implemented in a routine diagnostic.

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