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Review

STANDARD AND MOLECULAR METHODS IN THE DIAGNOSTICS OF INFECTIONS CAUSED BY EQUINE HERPESVIRUSES 1 AND 4

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Abstract

Background. Equine herpesvirus type 1 (EHV-1) is responsible for respiratory disease in young animals, abortion in pregnant mares and neurological disease, whilst equine herpesvirus 4 (EHV-4) is mainly the causative agent of respiratory disorders and rarely causes abortion. These viruses are considered as one of the most clinically and economically important pathogens of horses and can be detected in a range of tissues.

Scope and Approach. Serological methods are used to detect the presence and titre of specific antibodies to EHV-1 and EHV-4 in the sera of examined horses and are useful in epizootiological studies. Commercially available ELISA kits are able to differentiate specific EHV-1 and EHV-4 antibodies. EHV-1 and EHV-4 can both be isolated using susceptible cells such as primary horse cell cultures and other non-equine cells with visible cytopathic effect. Since standard diagnostic methods can be time consuming and arduous, the scope of many studies has been to develop and confirm the sensitivity and specificity of molecular diagnostic methods.

Key Findings and Conclusions. Polymerase chain reaction (PCR) has proved to be a good screening method for the presence of latent infections of horses caused by these viruses, also making possible the rapid identification and differentiation of EHV-1 and-EHV-4 in the examined samples. Real-time PCR is a sensitive, specific and quantitative method that enables the determination of viral kinetics in infected horses. Genome sequencing can be used to discover mutations in the genomes of EHV-1 and EHV-4 as well as to track the spread of their different strains globally.

Key Words: EHV-1, EHV-4, PCR, serology, sequencing, virus isolation

INTRODUCTION

At first, before 1981, equine herpesvirus type 1 (EHV-1) and equine herpesvirus type 4 (EHV-4) were considered as two subtypes of the same virus commonly named "equine rhinopneumonitis virus" and "equine abortion virus" (Crabb and

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Studdert 1996). Information based on genome sequencing confirmed that EHV-1 and EHV-4 are actually two closely related, but different viruses, with an amino acid sequence identity ranging between 54.9% and 96.4% (Telford et al., 1998). To date, EHV-1 and EHV-4 are both classified as members of the genus *Varicellovirus* in the *Alphaherpesviridae* family (Patel and Heldens, 2005; Davison et al., 2009). Antigenic cross reactivity of these two viruses is a consequence of their genetic relatedness and it often hinders the use of serological methods for differentiation purposes (Allen and Bryans, 1986; Carvalho et al., 2000; Kydd et al., 2006). EHV-1 is responsible for respiratory disease in young animals, abortion in pregnant mares and neurological disease, whilst EHV-4 is mainly the causative agent of respiratory disorders and rarely causes abortion. The first isolation and identification of EHV-1 in Serbia was performed by Mihajlović et al. (1987). Both EHV-1 and EHV-4 are endemic in horse populations worldwide (Campbell and Studdert 1983; Allen and Bryans 1986; Reed and Toribio, 2004; Marenzoni et al., 2008). These viruses may cause latent infection in lymphoid tissue and the sensory and trigeminal ganglions. The influence of stress, transport, co-mingling of horses as well as intercurrent disease can lead to virus reactivation in asymptomatic carrier horses and consequently to the spread of the disease in the equine population (Campbell and Studdert 1983; Welch et al., 1992; Slater et al., 1994; Kydd et al. 1994; Allen, 2006; Pusterla et al., 2010). EHV-1 and EHV-4 are considered as one of the most clinically and economically important pathogens of horses. Extensive outbreaks of abortion, perinatal/neonatal foal death as well as neurological and respiratory disorders are a significant cause of economic loss to the equine industry and thus effective diagnosis, control and prevention of equine herpes viral disease remains necessary (Allen and Bryans, 1986; Yasunaga et al., 1998; Gilkerson et al., 1999; Studdert et al., 2003). These viruses can be detected in a range of tissues including lung, kidney, liver, spleen, trigeminal ganglia, submandibular lymph nodes including nasal swabs, placenta and blood of live horses (Borchers et al., 1997; Dunowska et al., 2002; Hornyák et al., 2006; Diallo et al., 2007; Ataseven et al., 2009; Pusterla et al., 2010; Pusterla et al., 2012; Turan et al., 2012). A variety of methods can be utilized for detection and differentiation of EHV-1 and EHV-4 in clinical samples, including virus isolation, serology, polymerase chain reaction (PCR), real-time PCR and sequencing (Yasunaga et al., 1998; Diallo et al., 2007; Ataseven et al., 2009; Turan et al., 2012; OIE, 2015).

Serological testing has proved to be a reliable tool for presumptive evidence of infection and/or vaccination status in animals (Milić et al., 1997; Milić et al., 1998; Milić et al., 1999; Yasunaga et al., 2000; Milić et al., 2001; Kydd et al. 2006; Milić et al. 2009; Nišavić et al., 2016). Furthermore, the use of serology is necessary in epizootiological studies that form a base for eradication programs of numerous infectious diseases of animals (Lazić et al., 1993; Yasunaga et al., 1998; Ataseven et al., 2009; Petrović et al., 2015; Lazić et al., 2016). Increase in antibody titre (4-fold or higher) detected by virus-neutralization test (VNT) or complement fixation test (CFT) in blood serum samples collected 7–21 days apart, provide evidence of recent EHV infection (Kydd

et al. 2006). In the study performed by Senthil and Parameswaran (2014) a total of 162 horse sera samples were subjected to VNT using Madin–Darby bovine kidney cell line (MDBK) adapted EHV-1 antigen and 53 (32.7 %) were found to be positive for EHV-1 specific antibodies. This test was used to detect the seroprevalence as well as the highest antibody titre in seropositive animals. Dunowska et al. (2002) reported that out of the 82 sera tested by VNT, 59 (70 %) showed neutralization antibodies to EHV-1 among horses in New Zealand. However, a disadvantage of VNT and CFT is the inability of discrimination between antibodies to EHV-1 and EHV-4 (Carvalho et al., 2000; Hartley et al., 2005; Kydd et al.2006; OIE, 2015). A type-specific ELISA using fusion proteins expressing variable regions of glycoprotein G (gG) has been developed by Crabb and Studdert (1993), providing for the first time the opportunity to differentiate antibodies to these viruses; the type specificity of this test has been confirmed in many studies (Crabb et al., 1995; Glikerson et al., 1997; Yasunaga et al, 1998; Yasunaga et al., 2000). Commercially available ELISA kits e.g. Svanovir EHV-1/EHV-4-Ab ELISA are able to differentiate specific EHV-1 and EHV-4 antibodies (Studdert et al., 2003; Hartley et al., 2005; Gür and Yapici 2008; Ataseven et al., 2009).

EHV-1 and EHV-4 can both be isolated using primary equine fetal kidney cells or cell strains of equine fibroblasts derived from dermal (E-Derm) or lung tissue. It can be convenient to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, since equine-derived cell cultures should be used for successful isolation of EHV-4 (OIE, 2015). The study of Ploszay et al. (2013) describes the first successful isolation of EHV-4 from Polish horses on Vero cells; however, there was no correlation between the results of PCR and virus isolation since some samples that were PCR-positive yielded negative results in the isolation test. The authors concluded that the right choice of susceptible cells such as primary horse cell cultures as well as proper sample collection, transportation and handling, might be important when isolating EHV-4. EHV-1 can be isolated on other cell types e.g. rabbit kidney (RK-13), baby hamster kidney (BHK-21), Madin–Darby bovine kidney (MDBK), pig kidney (PK-15) and Vero cells. Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera (Dunowska et al., 2002; OIE, 2015).

Despite being considered "gold standard", standard virological methods are often very arduous and time consuming, particularly when examining numerous samples, which led to the requirement for the implementation of molecular methods in standard laboratory procedures in the diagnostics of many animal viruses (Carvalho et al., 2000; Milić et al., 2010; Nišavić et al., 2010; Ohta et al., 2011; Nišavić et al., 2016). Furthermore, given its sensitivity, PCR is a suitable screening method for latent EHV-1 and EHV-4 infection in horses (Welch et al., 1992; Kirisawa et al., 1993; Borchers et al., 1997; Allen, 2006, Pusterla et al., 2012). Hornyák et al. (2006) compared the sensitivity of PCR and virus isolation for the detection of EHV-1 in organ samples of aborted foals. Despite the fact that the results of virus isolation and PCR correlated with each other, PCR proved to be more sensitive detecting the virus in 27 samples in contrast to virus isolation which was successful for 25 samples.

Ohta et al. (2011) evaluated the usefulness of PCR for diagnosis of respiratory disease induced by EHV-1. The authors used PCR primers for glycoprotein C (gC) previously described by Lawrence et al. (1994) and concluded that it can be introduced to routine practice in order to make an early diagnosis of EHV-1 infection. Pusterla et al. (2012) examined the prevalence of latent alphaherpesviruses in submandibular (SMLN) and bronchial lymph (BLN) nodes, as well as from the trigeminal ganglia (TG) of 70 racing thoroughbred horses. In this study, eighteen (25.7 %) and 58 (82.8%) horses were PCR positive for the gB gene of EHV-1 and EHV-4, respectively, in at least one of the three tissues sampled. The distribution of latent EHV-1 and EHV-4 infection varied in the examined samples, whilst the TG were found to be most commonly infected. Borchers et al. (1997) applied nested-PCR and *in situ* PCR to study the tropism of EHV-4 and their results proved that EHV-4 establishes latency in the TG. Kirisawa et al. (1993) developed a PCR system that could differentiate EHV-1 and EHV-4 types rapidly and applied this technique on specimens from aborted fetuses. The authors designed primers for PCR from aligned nucleotide sequences of glycoprotein B genes of EHV-1 and EHV-4 to amplify specific regions for EHV-1 or EHV-4. By using type specific primer mixture, amplified fragments were identified as EHV-1 or EHV-4 in a one-step reaction in spite of the high degree of sequence homology. Another advantage of this technique was the possibility of rapid detection of mixed infections in horses and it has been applied by numerous authors (Wang et al., 2007; Hebia-Fellah et al., 2009; Ploszay et al., 2013). The objective of the study of Hebia-Fellah et al. (2009) was to assess the possible risks for the horizontal transmission of EHV-1 and EHV-4 via semen in an equine population. Examination of the semen involved the detection of viral DNA using specific PCR as previously described by Kirisawa et al. (1993). EHV-4 viral DNA was not detected in any of the semen samples, whilst EHV-1 DNA was identified in 51 of the 390 samples (13 %). The results obtained indicate that EHV-1 may be transmitted via semen at mating or by artificial insemination. Wang et al. (2007) developed a nested multiplex PCR as a rapid (<12 h), sensitive test for the simultaneous identification of equine herpesviruses (EHV-1, EHV-4, EHV-2 and EHV-5) in clinical samples from horses without clinical signs of respiratory disease. Multiplex PCR was verified as a sensitive and specific technique for the detection of EHV in clinical samples as well as cell culture. The detection of up to three EHVs in the same sample was enabled by a combination of multiplex PCR and cell culture or direct multiplex PCR. Similarly, Ataseven et al. (2009) pointed out that virus isolation in conjunction with nested multiplex PCR assay would lead to a higher virus identification rate in multiple respiratory infections caused by EHV-1 and EHV-4. Virus isolation and detection was performed on nasal swab samples and buffy coat cells from horses showing signs of respiratory disease. Two samples that tested positive for EHV-1 and EHV-4 specific DNA by multiplex nested PCR gave no EHV-specific cytopathic effects in cell culture. In the study of Carvalho et al. (2000) a multiplex PCR procedure was developed for differentiation of strains and field isolates of EHV-1 and EHV-4. Specific oligonucleotide primers were designed to amplify the thymidine kinase (TK) gene region of EHV-1 and EHV-4 and the obtained results

confirmed that the genome structures of EHV-1 and EHV-4 within the chosen TK gene region are highly conserved. This PCR assay proved to be sensitive and specific for the diagnosis of field isolates of EHV-1 and -4 and it was proposed for use as a rapid method for screening large numbers of horses in epidemiological surveys.

In addition to successful identification of various animal viruses in clinical samples, the application of molecular methods enables the molecular characterization of the viruses, i.e., genotyping which is significant regarding surveillance purposes (Milić et al., 2012; Turan et al., 2012; Milić et al., 2015; Ataseven et al., 2016). Real-time PCR technology is a rapid, sensitive, specific and quantitative method for the diagnosis of infectious diseases that grants the possibility of differentiating between diverse viral states (Hussey et al., 2006; Diallo et al., 2007; Slater, 2007; Pusterla et al., 2009; Milić et al., 2010). The OIE Reference Laboratories use quantitative real-time PCR assays that target heterologous sequences of major glycoprotein genes to distinguish between EHV-1 and EHV-4 (OIE, 2015). A multiplex real time PCR targeting *gB* gene of EHV-1 and EHV-4 was presented by Diallo et al. (2007). The authors have demonstrated that a multiplex real-time PCR can be successfully used to detect and differentiate the closely related EHV-1 and EHV-4. The test can be used as a one-tube reaction and proved to be rapid, sensitive and specific. Ko et al. (2013) examined the prevalence of equine respiratory virus infections among a suspected population of race horses in Republic of Korea. The nasal swabs of 1,574 horses were examined by real-time PCR using primers described by Diallo et al. (2007). The results of this investigation implied that equine respiratory viruses are endemic at Seoul Race Park whilst the molecular prevalence rates of EHV-1 and EHV-4 were 5.6 % and 7.9 %, respectively. Hussey et al. (2006) developed and validated a real-time PCR diagnostic assay for detection of EHV-1 in equine nasal and blood samples and compared its performance with virus isolation on cell culture. The target sequence for real-time PCR was a region of the EHV-1 gB gene that is sufficiently different from the EHV-4 gB gene. The authors concluded that the potential for rapid diagnosis, high throughput, as well as sensitivity and quantitative nature of the real-time PCR test all make it highly suitable to replace virus isolation for both surveillance and diagnostic and research purposes. The results published by Pusterla et al. (2009) showed that the viral load, strain and state of EHV-1 in the blood and nasopharyngeal secretions of adult horses can sucessfully be determined using real-time PCR. The authors proposed this molecular approach to help assess the risk of exposure to other horses and permit determination of viral kinetics in infected horses. Turan et al. (2012) conducted molecular and pathological investigations of EHV-1 and EHV-4 infections in horses in Turkey in order to investigate abortion storms that occurred in the Marmara region using real-time PCR. The method used for the real-time PCR was similar to those reported by Diallo et al. (2007). Nucleotide sequencing of positive PCR products was performed in order to establish the phylogenetic relationship between the EHV strains. The alignment indicated that the Turkish EHV-1 isolates were homogenous, although one was grouped with other EHV-1 strains from the United Kingdom, Germany and

from the United States. Turkish EHV-1 isolates were grouped in a clade of their own, but were closely related to a European strain of EHV-1. The EHV-4 strain clustered with EHV-4 strains from the United Kingdom and Australia based on their sequence similarities. Ataseven et al. (2016) performed the partial characterization of Turkish (TR) EHV-1 field strains based on *gB* gene using a novel designed primer set for EHV-1. The results of the molecular investigation of Turkish EHV-1 strains contributed to the monitoring of EHV-1 infection in Turkey and showed that these strains were closely related to the European EHV-1 strains.

CONCLUSION

EHV-1 and EHV-4 are one of the most important viral pathogens of horses that cause major economic loss to the equine industry around the world. Therefore, control and prevention of equine herpes viral disease based on proper and timely diagnosis remains vital. Serological methods are used to detect the presence and titre of specific antibodies to EHV-1 and EHV-4 in the sera of examined horses, enabling an overview of the population in epizootiological studies. The inability of discrimination between antibodies to EHV-1 or EHV-4 by VNT and CFT are overcome by the use of type-specific ELISA using fusion proteins expressing variable regions of the glycoprotein G protein of these two viruses. EHV-1 and EHV-4 can both be isolated using susceptible cells such as primary horse cell cultures as well as some other non-equine cells with visible cytopathic effect (CPE) and may further be confirmed by PCR or by immunofluorescence with specific antisera. Since these standard methods are time consuming and laborious, especially when examining numerous samples, many authors have developed and confirmed the sensitivity and specificity of molecular diagnostic methods (PCR, real-time PCR, sequencing). Apart from making possible the rapid identification and differentiation of EHV-1 and-EHV-4 in the examined samples, PCR has proved to be a good screening method for the presence of latent infections of horses caused by these viruses. Real-time PCR is a sensitive, specific and quantitative method that permits the determination of viral kinetics in infected horses, whilst the application of genome sequencing is significant for molecular epidemiology since it enables the detection of mutations in the genomes of EHV-1 and EHV-4, as well as the tracking of the spread of these different strains globally.

Authors' contributions

Nenad Milić: participated in the design of the study (ES), conceived of the study, and participated in its design and coordination and helped to draft the manuscript (FG).

Andrea Radalj: participated in the design of the study (ES), conceived of the study, and participated in its design and coordination and helped to draft the manuscript (FG).

Jakov Nišavić: participated in the design of the study (ES), conceived of the study, and participated in its design and coordination and helped to draft the manuscript (FG).

All authors read and approved the final manuscript.

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Declaration of conflicting interests

The authors hereby declare that they have no competing interests.

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STANDARDNE I MOLEKULARNE METODE DIJAGNOSTIKE INFEKCIJA IZAZVANIH KONJSKIM HERPESVIRUSIMA 1 I 4

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Kratak sadržaj

Uvod. Konjski herpesvirus 1 (EHV-1) izaziva respiratorne infekcije mladih konja, promene u CNS-u i pojavu abortusa kod gravidnih kobila. Infekcije izazvane konjskim herpesvirusom 4 (EHV-4) se manifestuju ispoljavanjem respiratornih simptoma oboljenja kod životinja, a ređe pojavom abortusa. Infekcije izazvane prethodno navedenim virusima prisutne su u populaciji konja širom sveta.

Cilj i pristup. U cilju laboratorijske dijagnostike, odnosno izrade odgovarajućih epizootioloških studija o prisustvu virusa EHV-1 i EHV-4 u populaciji konja na određenom prostoru, primenjuju se serološke metode kao što su ELISA ili test virus neutralizacije. Primenom navedenih metoda se vrši otkrivanje prisustva i titra specifičnih antitela protiv prethodno navedenih virusa u uzorcima krvnih seruma konja. Ovde treba napomenuti da su danas dostupni i komercijalni kitovi za izvođenje metode ELISA koji omogućavaju diferencijaciju specifičnih EHV-1 i EHV-4 antitela. Konjski herpesvirus 1 i konjski herpesvirus 4 se mogu izolovati na kulturama ćelija poreklom od konja, kao i na drugim ćelijskim linijama, a daju karakterističan citopatogeni efekat. Pored navedenih klasičnih virusoloških metoda, danas su u upotrebi i molekularne metode dijagnostike kao što su PCR ili real-time PCR.

Ključni nalazi i zaključak. Lančana reakcija polimeraze (PCR) predstavlja pogodnu *screening* metodu za brzo i pouzdano otkrivanje prisustva virusa EHV-1 i EHV-4 u ispitivanim uzorcima poreklom od konja. Real-time PCR je osetljiva, specifična i kvantitativna metoda čijom primenom se takođe može utvrditi prisustvo navedenih virusa u organizmu konja. Metodom sekvenciranja genoma virusa EHV-1 i EHV-4 se mogu utvrditi sličnosti ili razlike između pojedinih sojeva virusa, odnosno može se izvršiti molekularna karakterizacija sojeva virusa EHV-1 i EHV-4 dominantno prisutnih u populaciji konja na određenoj teritoriji.

Ključne reči: EHV-1, EHV-4, PCR, serologija, sekvenciranje, izolacija virusa