Bulgarian Journal of Veterinary Medicine (2010), **13**, No 1, 18–22

DETECTION OF PROVIRAL SEQUENCES OF EQUINE INFECTIOUS ANEMIA VIRUS IN PERIPHERAL BLOOD CELLS OF HORSES IN IRAN

H. MOMTAZ[1] & S. NEJAT[2]

[1]Department of Microbiology, [2]Department of Clinical Sciences; Faculty of Veterinary Medicine, Islamic Azad University of Shahrekord branch, Shahrekord, Iran

Summary

Momtaz, H. & S. Nejat, 2010. Detection of proviral sequences of equine infectious anemia virus in peripheral blood cells of horses in Iran**.** *Bulg. J. Vet. Med.*, **13**, No 1, 18–22.

Equine infectious anemia virus (EIAV), a member of the genus *Lentivirus* of the family *Retroviridae*, causes a chronic infection with world-wide spread in the equids; it has been evidenced in horses, donkeys and mules. Whole blood and serum samples (n=310) from horses over one year old from different regions of Iran were examined. Samples were initially checked in the agar gel immunodiffusion (AGID) test and 9 cases (2.90%) had antibody against EIAV. Then, positive serum samples and 301 apparently healthy horses (negative in the AGID test) were examined by nested PCR to detect proviral DNA of EIA. PCR results showed that all positive sera and 2 of AGID-negative sera were positive.

Key words: agar gel immunodiffusion, equine infectious anemia, provirus, nested PCR

INTRODUCTION

Equine infectious anemia (swamp fever) is a contagious viral disease of horses that has been described more than 70 years ago. It is characterized by intermittent fever, haemolytic anaemia, icterus, depression, chronic loss of weight and swelling of the legs, brisket and lower abdomen (Oaks *et al*., 1999; Ataseven & Arslan, 2005; Radostits *et al*., 2007). In the acute form, animals are extremely sick and may die. Horses that have the chronic form, or recover from the acute or subacute form to become chronic, usually have intermittent attacks with intervals of one week to a month or more between attacks (Spyrou *et al.,* 2003). Some horses die during these recurrent episodes. A latent or subclinical form of EIA may also develop in equines. These animals appear normal but are carriers of the virus in their blood. Carrier animals are potential sources of infection for susceptible healthy horses (Montelaro *et al.,* 1993; Cook *et al*., 1996). The virus persists for the entire life of infected animals despite a vigorous lifelong humoral response which is usually produced within 45 days after infection, as demonstrated by the presence of EIAV serum antibodies (Hammond *et al.,* 1999; Howe *et al*., 2002; Paré & Simard, 2004). Currently, the agar gel immunodiffusion assay, (AGID, Coggins test), is widely accepted and used for the sero-detection of EIAV group specific antigen p26 (Coggins *et al*., 1972). Besides serological assays, sensitive diagnostic tests are also required for the direct detection of EIAV in infected animals. A PCR assay for the detection of

EIAV proviral DNA in blood cells is likely to be sensitive and specific not only in identifying subclinical EIAV infected horses, but also recently infected horses in the process of mounting an immune response and infected foals with colostral anti-EIAV antibodies which interfere in serological assays (Coggins & Auchnie, 1977; Issel & Cook, 1993). In this report, a study on EIA in naturally infected horses is presented. The nested PCR assay developed in the present study detected more EIAV positive animals, was found as specific as the AGID assay and offers great potential as a diagnostic test for the detection of EIAV infections in horses.

MATERIALS AND METHODS

Samples

Blood and serum samples were collected randomly from male (n=150) and female (n=160) horses (from different breeds) older than one year of age from 4 different geographic regions in Iran (north, south, west and east) between May and October 2006.

Preparation of samples

The blood samples were collected into clot activator vacuum tubes and centrifuged at 800×*g* for 10 min. The separated sera were heat inactivated at 56 °C for 30 min before testing. EDTA-blood samples (10 mL) were centrifuged at 18 °C for 35 min at 1,400×*g*. Buffy coat cells were resuspended in 4 volumes of sterile 0.2% NaCl to lyse erythrocytes. After 1 min, 7.2% NaCl was added to reconstitute isotonicity. The cells were washed in phosphate-buffered saline and stored at −20 °C (Müller-Doblies *et al.,* 1998). DNA was isolated from peripheral blood lymphocytes using Genomic DNA purification kit (Fermentas) and the optical density was measured at 260 nm (Laird *et al.,* 1991).

Serological test

Serum samples were tested for antibodies against the p26 protein of EIAV by the commercial Diasystem EIA agar gel immunodiffusion kit (IDEXX laboratories, Maine, USA), according to the manufacturer's instructions (Alvarez *et al.,* 2007).

Nested PCR assay

Isolated DNA from buffy coat was used for PCR. The oligonucleotide primers used for nested PCR were from the *gag* gene coding for the capsid protein sequence of EIAV. Their sequences were as those described by Nagarajan & Simard (2001):

P1: 5-GTAATTGGGCGCTAAGTCTAG-3
P2: 5-CCTCTAATAAATCTTGCTGTC-3
P3: 5-GGCTGGAAACAGAAACTTTA-3
P5: 5-CCAGTGGAGCATTCGGTAA-3

For replication of mentioned gene segment, we used Mastercycler gradient PCR machine (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The first amplification was done by using a pair primer *P1* and *P2* to amplify a 610 bp fragment. The second amplification was performed to amplify 260 bp fragment, using a pair primer *P3* and *P5*. The cycle conditions for the first step amplification consisted of initial denaturation at 94°C for 5 min followed by 34 cycles of 94 °C for 60 s, 58 °C for 50 s, 72 °C for 60 s and a final extension of 72 °C for 8 min. Nested PCR amplification was carried out with 5 µL of the primary PCR product as template. Both first and second round amplification reactions contained 1.5 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphate,

1 μM of each primer, 1U of Taq DNA polymerase in a final volume of 50 μL. The thermal conditions for the second round amplification consisted of 35 cycles of 94 °C for 60 s, 59 °C for 50 s, 72 °C for 55 s and a final extension of 72 °C for 5 min (Sambrook & Russell, 2001). Twenty μL of final PCR and nPCR products were run on a 1.5% agarose gel containing ethidium bromide in 1X TBE buffer along with 100 bp DNA ladder (Fermentas).

RESULTS

In AGID test, 9 out of 310 samples had antiviral antibodies. These 9 and another 301 samples which were proved negative in the AGID test, were then tested by PCR assay. The results were shown in Table 1 and Fig. 1. Two samples from the second set evaluated as EIAV-negative in AGID, were positive in the PCR assay.

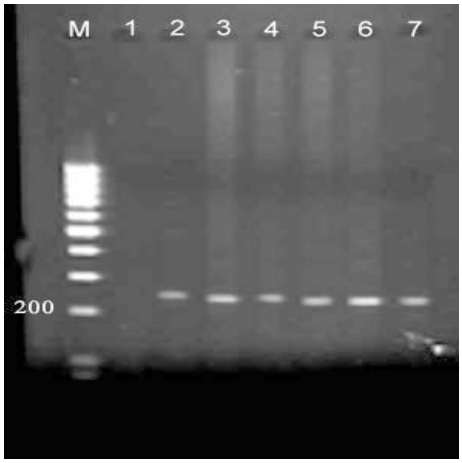

Fig. 1. Nested PCR products of the samples. Column M = 100 bp DNA Ladder, Column 1 = negative control, Columns 2, 3, 4, 5, 6, 7 = samples.

Table 1. Comparison of agar gel immunodiffusion test (AGID) with nPCR for detection of EIAV

Test	nPCR negative	nPCR positive	Total
AGID positive	0	9	9
AGID negative	299	2	301
Total tested sera	299	11	310

According to these results, positive samples in nPCR assay contained 260 bp DNA fragment of provirus of EIA virus.

DISCUSSION

The equine infectious anemia virus (EIAV) often results in lifelong subclinical infection following early episodes of clinical disease (Radostits *et al*., 2007). In reality, antigenic drift in gp45 and gp90 allowed the mutant virus to escape from the surveillance of the immune system, to proliferate and produce clinical signs (Oaks *et al*., 1999; Alvarez *et al.,* 2007). The EIA agent is a non-oncogenic retrovirus that possesses an RNA-directed DNA polymerase that infects macrophages throughout the body and integrates into the host genome (Ataseven & Arslan, 2005). This viral disease elicits brisk humoral and cellular immune responses. Clinical signs are associated with recrudescence of viraemia, which results in immunologically mediated damage to red blood cells and a variety of other tissues. The disease consists of a series of recurrent episodes that are thought to be the result of sequential production and release of antigenically novel strains of the virus that temporarily escape the host's immunosurveillance system. The absence of clinical signs in the chronic form is

thought to be caused by the host's immune response against antigenic epitopes common to all EIA viruses. In this study, it is noteworthy that two animals which were seronegative in the AGID assay were found to have EIAV provirus DNA in their peripheral blood leukocyte fractions (Table 1). These data suggest a greater sensitivity of PCR assay over serological tests. These results were not surprising considering that the majority of blood samples were collected from horses reared in EIAV-infected premises. It is quite possible that those horses were in early stage of infection and in the process of mounting an immune response. Transient levels of antibodies were also reported to be associated with a latent carrier state and restricted viral replication. In those circumstances, serological diagnostic tests could give false negative results, as reported elsewhere (Langemeier *et al.,* 1996). Since horses infected with EIAV remain infected for life and the inapparent carrier stage of infection is maintained (Hammond *et al.,* 1999), false negative animals could serve as reservoir of infection for uninfected animals over extended periods of time. The PCR assay developed in this study, offers the potential for equivalent specificity and most probably greater sensitivity than the gel immunodiffusion assay in effectively detecting EIAV-infected horses. As such, nPCR assay should be of great value in identifying EIAV-infected animals in control programmes or to confirm EIAV status of seropositive animals.

In one study, agreements between serological and molecular methods were considered. In Canada, the percentage of agreement between AGID and nPCR assay for diagnosis of EIAV was 94% (Nagarajan & Simard, 2001) and in our study 99.35%. In conclusion, the EIAV-nested PCR assay described in this study, using primers derived from the *gag* gene of EIAV provides a rapid, sensitive and specific diagnostic method for detection of EIAV in naturally infected horses. This nPCR assay can play a major role in detecting seronegative latent carrier horses or horses in early stage of infection and can be useful in the diagnosis of EIAV in foals, where serological tests are not suitable due to the interference of maternal antibodies.

ACKNOWLEDGEMENTS

We thank Dr. F. Hemmatzadeh, Dr. H. Keyvanfar for their cooperation. This study was funded by the Islamic Azad University of Shahrekord in Iran.

REFERENCES

Alvarez, I., G. Gutierrez, A. Vissani, S. Rodriguez, M. Barrandeguy & K. Trono, 2007. Standardization and validation of an agar gel immunodiffusion test for the diagnosis of equine infectious anemia using a recombinant p26 antigen. *Veterinary Microbiology,* **121**, 344–351.

Ataseven, V. S. & H. H. Arslan, 2005. Equine infectious anemia in mules, donkeys, and horses: Epidemiologic studies in the different geographic regions of Turkey. *Journal of Equine Veterinary Science*, **25**, 439–441.

Coggins, L. & J. A. Auchnie, 1977. Control of equine infectious anemia in horses in Hong Kong. *Journal of the American Veterinary Medical Association,* **170**, 1299–1301.

Coggins, L., N. L. Norcross & S. R. Nusbaum, 1972. Diagnosis of equine infectious anemia by immunodiffusion test. *American Journal of Veterinary Research,* **33**, 11–18.

Cook, R. F., C. J. Issel & R. C. Montelaro,

1996. Equine infectious anemia. In: *Virus Infections of Equines,* ed M. J. Studdert, Elsevier, Amesterdam, pp. 297–323.

Hammond, S. A., S. J. Cook, L. D. Falo, C. J. Issel & R.C. Montelaro, 1999. A particulate viral protein vaccine reduces viral load and delays progression to disease in immunized ponies challenged with equine infectious anemia virus. *Virology*, **254**, 37–49.

Howe, L., C. Leroux, C. J. Issel & R. C. Montelaro, 2002. Equine infectious anemia virus envelope evolution *in vivo* during persistent infection progressively increases resistance to *in vitro* serum antibody neutralization as a dominant phenotype. *Journal of Virology,* **76**, 10588–10597.

Issel, C. J. & R. F. Cook, 1993. A review of techniques for the serologic diagnosis of equine infectious anemia. *Journal of Veterinary Diagnostic Investigation*, **5**, 137–141.

Laird, P. W., A. Zijderveld , K. Linders, M. A. Rudnicki, R. Jaenisch & A. Berns, 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Research*, **19**, 4293.

Langemeier, J. L., S. J. Cook, R. F. Cook, K. E. Rushlow, R. C. Montelaro & C. J. Issel, 1996. Detection of equine infectious anemia viral RNA in plasma samples from recently infected and long-term inapparent carrier animals by PCR. *Journal of Clinical Microbiology,* **34**, 1481–1487.

Montelaro, R. C., J. M. Ball & K. E. Rushlow, 1993. Equine retroviruses. In: *The Retroviridae*, ed. J. A. Levy, Plenum Press, New York , pp. 257–360.

Müller-Doblies, U. U., H. Li, B. Hauser, H. Adler & M. Ackermann, 1998. Field validation of laboratory tests for clinical diagnosis of sheep-associated malignant catarrhal fever. *Journal of Clinical Microbiology*, **36**, 2970–2972.

Nagarajan, M. M. & C. Simard, 2001. Detection of horses infected naturally with equine infectious anemia virus by nested polymerase chain reaction. *Journal of Virological Methods*, **94**, 97–109.

Oaks, J. L., C. Ulibarri & T. B. Crawford, 1999. Endothelial cell infection *in vivo* by equine infectious anaemia virus. *Journal of General Virology*, **80**, 2393–2397.

Paré, J. & C. Simard, 2004. Comparison of commercial enzyme-linked immunosorbent assays and agar gel immunodiffusion tests for the serodiagnosis of equine infectious anemia. *Canadian Journal of Veterinary Research*, **68**, 254–258.

Radostits, O. M., C. C. Gay, K. W. Hinchcliff & P. D. Constable, 2007. Veterinary Medicine, A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats, 10th edn, Saunders Elsevier.

Sambrook, J. & D. W. Russell, 2001. Molecular cloning: A Laboratory Manual, 3rd edn, Cold Spring Harbor Laboratory, New York.

Spyrou, V., M. Papanastassopoulou, V. Psychas, C. H. Billinis, M. Koumbati, J. Vlemmas & G. Koptopoulos, 2003. Equine infectious anemia in mules: Virus isolation and pathogenicity studies. *Veterinary Microbiology,* **95**, 49–59.

Paper received 11.05.2009; accepted for publication 11.09.2009

Correspondence:

Dr. Hassan Momtaz
Department of Microbiology,
Faculty of Veterinary Medicine,
Islamic Azad University
of Shahrekord branch,
P.O.Box 166, Shahrekord-Iran,
Tel/fax: 0098 381 3361083,
e-mail: hamomtaz@yahoo.com