PCR FOR EQUINE INFECTIOUS ANEMIA VIRUS DETECTION IN WHOLE BLOOD FROM NATURALLY INFECTED HORSES IN COLOMBIA

PCR PARA DETECCIÓN DEL VIRUS DE LA ANEMIA INFECCIOSA EQUINA EN SANGRE TOTAL DE ANIMALES INFECTADOS NATURALMENTE EN COLOMBIA

Esperanza Trujillo[1], Alejandra Collazos[2] and Mauricio Camargo[3]

Abstract

The equine infectious anemia virus is associated with a variety of clinical symptoms that include anorexia, weight loss, pyrexia, and anemia. Serological tests and reverse transcriptase nested polymerase chain reaction (RT-nPCR) have been used to detect EIAV in infected horses. In the present study, the use of non-nested PCR for the detection of EIAV *gag* gene sequences in peripheral blood cells DNA from horses bred in farms around Medellín (Colombia) is reported. EIAV DNA was detected by PCR in blood cells from fifteen horses, which had been found positive in an agar gel immunodiffusion test, despite the absence of clinical symptoms of expression. An amplified product of about 1.300 bp was obtained with a round of PCR without requiring nested PCR or RT-PCR.

Key words: equine infectious anemia virus, horses, polymerase chain reaction.

Resumen

El virus de la anemia infecciosa equina (EIAV) está asociado con una variedad de síntomas clínicos que incluyen anorexia, bajo peso, fiebre y anemia. Pruebas serológicas y reacción en cadena de la polimerasa con transcriptasa reversa (RT-PCR) han sido utilizadas para detectar el virus en caballos infectados. En este estudio reportamos el uso de la PCR para detección de una secuencia del gen *gag* del virus en DNA de células de sangre periférica, de quince caballos provenientes de algunos criaderos de Medellín (Colombia), los cuales habían sido positivos en el test de inmunodifusión en gel de agar, y además no expresaban los síntomas de la anemia. Un producto amplificado de 1.300 pb se obtuvo con una ronda de PCR sin requerir de PCR anidado o RT-PCR.

Palabras clave: virus de la anemia infecciosa equina, caballos, reacción en cadena de la polimerasa.

INTRODUCTION

Equine infectious anemia (EIA) is a viral disease that occurs worldwide. It is produced by a retrovirus belonging to the subfamily *Lentivirinae*, which was first identified in 1904 (Valle and Carre, 1904). The equine infectious anemia virus (EIAV) can affect horses, donkeys and mules. The horse cells infected by the virus are peripheral blood monocytes and mainly tissue macrophages (Clements and Zink,1966; Gendelman *et al.*, 1985; McGuire *et al.*, 1971). The disease is usually spread by horse flies biting an infected horse, then biting a healthy horse. It can also be transmitted by the use of non-sterile needles and blood contaminated surgical instruments (Williams *et al.*, 1981). There is no treatment known to cure or eliminate the virus.sterile needles and blood contaminated surgical instruments (Williams *et al.*, 1981). There is no treatment known to cure or eliminate the virus.

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[1] Institute of Biology, University of Antioquia, AA 1226, Medellín, Colombia. E-mail: etbravo@epm.net.co.
[2] Institute of Biology, University of Antioquia, AA 1226, Medellín, Colombia. E-mail: alcofa@yahoo.com.
[3] Institute of Biology, University of Antioquia, AA 1226, Medellín, Colombia. E-mail: mcamargo@epm.net.co.

Equine infectious anemia has a wide variety of pathological manifestations (McGuire *et al.*, 1990) and three forms can be differentiated: acute, chronic, and inapparent. The acute form of EIA occurs rapidly, although a high fever is usually the only noticeable symptom (Kono, 1969). Usually, it takes one week to one month to show up. Because the onset of acute EIA is so rapid, the horse will not have made antibodies to the virus, even though it is already exhibiting some symptoms (McConnell and Katada, 1981; Issel and Cook, 1993). The chronic form of EIA involves recurring bouts of fever, weight loss, depression, small hemorrhages on the mucous membranes, swelling and anemia (Sellon *et al.*, 1994). Horses with inapparent EIA never show signs of having EIA (Montelaro *et al.*, 1993; Sellon *et al.*, 1994). The variation on different stages of disease depends on the sequence heterogeneity in viral regulatory elements, co-existence of varying genotypes, animal age and health, and immune competence (Salinovich *et al.*, 1986; Carpenter *et al.*, 1987; Hussai *et al.*, 1987; Perryman *et al.*, 1988; Kim and Casey, 1992).

EIAV diagnosis is commonly done by the agar gel immunodiffusion (AGID) Coggins test (Coggins *et al.*, 1972), which is the mandatory EIAV test in most countries. Also, an enzyme-linked immunosorbent assay (ELISA)-based test (Centaur, Inc., 1993; Matsushita *et al.*, 1989) has been developed. The AGID test will typically be positive during the acute stage of the disease (Toma, 1980; McConnell and Katada, 1981; Issel and Adams, 1982; Issel and Cook, 1993). If horses survive this stage, they progress to the chronic form of the infection (Sellon *et al.*, 1994) and produce a test positive in AGID. In the inapparent form of the disease, more sensitive techniques such as PCR are required to detect viral nucleic acids (Kim and Casey, 1994; Langemeier *et al.*, 1996). In 1996, Langemeier *et al.* compared the RT-nPCR assay with agar gel immunodiffusion test and conventional virus isolation for the detection of early infection in experimentally infected horses. In this work, samples from each pony were tested after infection and showed to be negative for AGID or immunoblot analysis in early infection, but were positive for RT-nPCR, indicating the test sensibility. However, the RT-nPCR has a limitation. It is necessary that the horse has a number greater than 5.000 copies of circulating viral RNA for each 2 ml of plasma, which implies that the animal is in the clinical phase of the disease (Langemeier *et al.*, 1996).

Recently, Oaks *et al.* (1998) tested the presence of virus in infected horses with laboratory strains of virus. They analyzed different tissues, including peripheral blood mononuclear cells (PBMC), lung, spleen, node, bone marrow and liver from animals with subclinically infection. They found that not all tissues contained sufficient proviral DNA to be detected with PCR, although all tissues tested (except PBMC) were positive by nested-PCR. By contrast, animals with chronic disease have amounts of proviral DNA that can be detected by PCR (Oaks *et al.*, 1998).

In Colombia (South America), EIAV is probably spread through insects (tábano, *Tabanus fuscicostatus*) and the laboratory diagnosis of disease is currently done by the AGID test. The purpose of this study was to modify and apply a PCR assay able to detect the EIAV in whole blood of farm-horses without clinic manifestations of the disease or inapparent carriers of EIAV.

MATERIALS AND METHODS

Horses and clinical parameters. Thirty farm-horses from the province of Antioquia were evaluated to detect the presence of the equine anemia infectious virus by PCR and nested-PCR. Among the 30 horses, fifteen were tested positive in AGID and did not have clinical symptoms of the disease. The others were negative by the AGID test. One horse, which showed acute clinical symptoms of the disease and was positive by the AGID test, was used as the positive control. The horses ranged in age from five to twenty years; sixteen were mares and fourteen were geldings.

DNA extraction. Seven ml of horse whole blood+ EDTA, obtained by jugular venipuncture, was used for

DNA isolation. The DNA was extracted by salting out and isopropanol precipitation.

Briefly, 40 ml of lisis buffer I (0.3 M sucrose, 10 mM Tris/pH 7.5, 5 mM MgCl, 1% Triton X-100) was added to 7 ml of blood, mixed and centrifuged at 2.800 g for 7 min. Supernatant was removed and 4.0 ml of lisis buffer II (0.075 M NaCl, 0.024 M Na-EDTA) was added, followed by 62.5 µl of SDS (20%, w/v) and 2.0 ml of 5 M sodium perchlorate and mixed with vortex; 2.0 ml of 6 M NaCl were added to the samples and mixed with vortex and centrifuged at 2.800 g for 7 min. Supernatant was removed and 4.0 ml of isopropanol were added and mixed. Samples were washed once with 70% ethanol and disolved in 200 µl TE.

PCR amplification. PCR amplification of an EIAV $_{Wyo}$ *gag* gene sequence, was performed using the procedure described by Oaks *et al.* (1998), with some modifications. The 25 µl reaction mixture contained 15 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2 µg genomic DNA, 50 µg of each primer, and 2.5 U *Taq* DNA polimerase (Promega). Oligonucleotide primers (IDT, Coralville, Iowa, USA) used for the amplification were 297 (5´CTAGTTTGTCTGTTCGAGATCC3´) and p26-2 (5´ATGTTTGTGCTGCCTTTAGTGG 3´) as described previously (Langemeier *et al.*, 1996; Oaks *et al.*, 1998).

The amplification profile consisted of one cycle at 95 °C for 6 min, followed by 40 cycles at 94 °C for 30 sec, 51 °C for 30 sec, 72 °C for 30 sec, and finally held at 72 °C for 7 min. PCR products were analyzed by electrophoresis in 0.8% agarose-TBE buffer (0.090M tris-borate, 0.002M EDTA, pH 8.0) and visualized by ethidium bromide staining. The size of the expected DNA fragment was determined by comparison with a 1Kb DNA ladder (Promega).

PCR specificity and sensitivity. PCR specificity was evaluated by the amplification of human, porcine, and bovine DNA controls, in the same conditions as described above. PCR sensitivity was tested by amplifying different amounts (1.5, 2, 2.5, 3 and 3.5 µg) of DNA from blood cells. The PCR products were also analyzed by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Diagnosis in non-infected and naturally infected horses. PCR tests were performed by using thirty-one blood samples collected from different local ranch horses, that were divided into two groups: The first group consisted of 15 AGID positive animals, whose infection was probably acquired by contact with other infected animals. These animals did not show clinical symptoms of the disease (table 1). When PCR test was performed, all samples from this group showed an amplified product of approximately 1.300 pb, i.e., within the expected size (figure 1). The PCR amplified product from the DNA sample of a AGID positive horse with acute EIA, used as control, was similar in both size and intensity to those from the first group DNA samples. The remaining fifteen horses belonging to the second group, which were EIA negative in the AGID test, were also negative in the EIAV PCR assay.

Table 1. EIAV DNA detected by PCR (whole blood)

No. of animals	Age range (years)	AGID test	PCR[a]
1[b]	10	+	+
15	3-14	+	+
15	4-9	—	—
Total = 31			

[a] (+) PCR positive according to the results in ethidium bromide-stained agarosa gels; (—), negative result.

[b] Positive control.

Optimization of PCR parameters. In all PCR positive animals, a robust amplification product of 1.300 bp was always observed together with a weaker band of approximately 1.000 bp (figure 1). The influence of annealing temperature on the appearance of the 1.000 bp band was investigated at four different temperatures (51 °C, 52 °C,

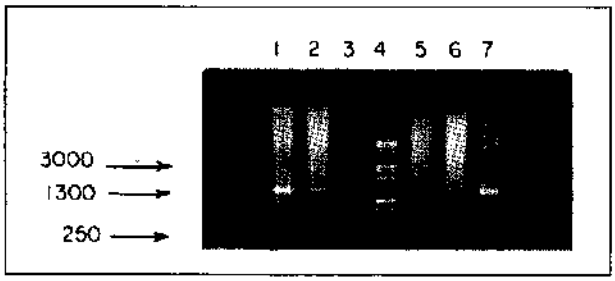

Figure 1. PCR amplification of EIAV DNA. Lane 1, positive control. Lanes 2, 3, 5 and 7, horses with inapparent infection. Lane 6, non-infected animal. Lane 4, 1.000 bp ladder

53 °C, and 54 °C). The PCR reactions and analysis were done as described in Materials and Methods. According to these results, the optimum temperature to avoid the minor PCR product was 53 °C (data not shown).

PCR specificity and sensitivity. Specificity was analyzed using non-horse DNA samples (human, porcine, and bovine). Simple round PCR products were not observed (data not shown).

Different PCR sensitivity tests were performed. It was noticed that the best resolution was achieved when 2 μg of DNA/per reaction were used. On samples with 1.5, 2.5 and 3 μg of DNA/per reaction the amplified band was not observed nor was tenuous. On samples of 3.5 μg of DNA/per reaction, the amplified product was not obtained.

DISCUSSION AND CONCLUSIONS

Serodiagnostic assays evaluate presence of a host immune response and not specifically the presence of plasma viremia. This imposes serious limitations, such as the inability to detect the virus during either early stages of infection, due to the fact that infected animals have immature humoral responses (Pearson and Beevar, 1971; McConnell and Katada, 1981; Issel and Cook, 1993) or the low amount of anti-EIAV antibodies during these phases can not be detected. This is wrongly interpreted as a negative result on the AGID test (Toma, 1980; McConnell and Katada, 1981; Issel and Adams, 1982).

Another method used to detect EIAV is RT-PCR. This test has limitations because it needs high viremia, and nested-PCR is sometimes necessary (Oaks *et al.*, 1998). Besides RT-PCR is a slow and laborious method. In our laboratory, we were unable to amplify viral sequences by RT-PCR from AGID positive horse samples with inapparent EIAV, in spite the fact that the RNA isolation and the RT-nPCR procedures were carried out as described by the authors (Chomczynski and Sacchi, 1987; Langemeier *et al.*, 1996). This discrepancy can be explained as result of low viremia at the moment that the horses were tested.

In this report we present a PCR method to detect equine anemia infectious virus (EIAV) in DNA samples from horses with inapparente infection (table 1, figure 1). This pilot study indicates that the methodology applied can detect proviral DNA in whole blood cells, avoiding the separation of monocytes/macrophages for DNA extraction. Therefore, there is a decrease on the time invested and the total cost for the application of the test. Another relevant aspect of this work is the fact that it is the first time that this kind of study has been done in Colombia (South America). In recent studies (Beisel *et al.*, 1993; Zheng *et al.*, 1997; Oaks *et al.*, 1998; Smith *et al.*, 1998) horses were infected with laboratory strains of EIAV to detect proviral DNA. In our work, we used blood samples from animals bred in some ranches.

An amplified product of approximately 1.300 pb was obtained from subclinically infected horses with only a PCR assay, without nested-PCR, indicating that there is sufficient amount of proviral DNA to be detected. A second band of 1.000 bp was obtained, that may correspond to another sequence of the proviral EIAV or an amplification of non-specific targets. It is also possible that a second reaction co-amplification was taking place; however, this second band disappeared with an annealing temperature of 53 °C.

It was found that the PCR method done in our laboratory is specific for the EIAV virus since PCR

for human and bovine DNA did not reveal an amplified product. The specificity was determined by using the same amounts of bovine, porcine, and human DNA as used in horse samples. An excellent sensitivity was established and evaluated with different amounts of total DNA. However, a relative high amount of genomic DNA should be used to obtain EAIV sequences amplification, probably due to the low copy number of viral sequences per cell and/or the low number of infected cells. In fact, the recent report by Oaks *et al.* (1998), uses 2-3 mg of genomic DNA per 100 µl reaction. Our results reveal that for the PCR test, 2 µg of DNA per 25 µl reaction can be used.

In conclusion, our work reveals that: (i) PCR in DNA from blood cells of horses bred in ranches, is an easy and fast diagnosis tool to detect EIAV. (ii) This method can detect the virus on equines with inapparent EIA. (iii) DNA samples from whole blood may be used. (iv) A decrease on the time invested and the total cost on the application of the test is obtained.

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