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EIAV Effectiveness Detection by Nested PCR Comparing two Different Samples: PBMCs and Bronchoalveolar Wash

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Abstract: Problem statement: Equine Infectious Anemia (EIA) is a chronic, relapsing infectious disease of horses caused by Equine Infectious Anemia Virus (EIAV). The objective of this experiment was compared EIAV effectiveness detection in Peripheral Blood Mononuclear Cells (PBMCs) and Bronchoalveolar wash (BAW) from naturally infected horses. **Approach:** Fifty seven DNA samples isolated from PBMCs and BAW of naturally infected animals were used for a nested PCR amplifying a 408 bp gag gene fragment region. Equine Dermal cells (ED) infected and non infected by EIAV was used as nested PCR negative and positive control. Horses' blood samples were firstly tested as positive in Agar Gel Immunodiffusion Assay (AGID). **Results:** Results demonstrated 89% (51/57) of gag gene amplification in PBMCs samples and only 47% (27/57) of gag gene amplification in BAW samples. **Conclusion:** The nested PCR assay used in the present study detected more EIAV positive samples in PBMC than in BAW, indicating PBMCs as a reliable source for EIAV diagnosis.

Key words: Diagnosis, EIA, EIAV, nPCR and PBMCs

INTRODUCTION

Equine Infectious Anemia (EIA) is a horse disease caused by Equine Infectious Anemia Virus (EIAV), a retrovirus transmitted by bloodsucking insects. The control of EIA is made through the identification of infected animals by the serological tests ELISA and IDGA (Alvarez *et al*., 2007), but many factors contribute to inconsistent results with these tests that require confirmation using more specific techniques (Alvarez *et al*., 2007). Thus, the nested PCR technique is a sensitive diagnostic test for the detection of the virus in recent infections in which the immune response is still being built and those cases of neonatal infections, besides elucidating the inconclusive serological results (Issel and Cook, 1993; Langemeier *et al*., 1996). The main targets of EIAV replication *in vivo* are cells from the monocyte-macrophage lineage and the main tissue targets are the macrophages from spleen, liver, lymph nodes and lungs (Harrold *et al*., 2000). Preliminary data indicate that infection of blood monocytes by EIAV causes a non-productive infection and that differentiation of the infected monocytes into macrophages is necessary to trigger viral replication (Maury, 1994; Sellon *et al*., 1996) and macrophages are the major cell type found in bronchoalveolar wash samples from equids (Ainsworth *et al*., 2002; 2003; Dyer *et al*., 1983). Additionally, some studies have found that the latent infection of mononuclear cells in blood is an important mechanism for viral persistence and dissemination (Gendelman *et al*., 1985; 1986).

Therefore, the aim of this study was compared the efficiency of the nested PCR technique to amplify EIAV gag gene sequence in DNA samples from PBMCs and BAW isolated from naturally infected horses.

MATERIALS AND METHODS

A nested Polymerase Chain Reaction (nPCR) amplifying a region of the gag gene of Equine Infectious Anemia Virus (EIAV) was developed for comparing the virus detection effectiveness in Peripheral Blood Mononuclear Cells (PBMC) and Bronchoalveolar Wash (BAW) samples from 57 naturally infected horses accredited by the Ministry of Agriculture in Minas Gerais state, Brazil. Blood samples from all animals were positive for EIAV in Agar Gel Immunodiffusion Assay (AGID) and Equine Dermal cells (ED) infected and non infected by

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EIAV was used as negative and positive control, respectively. Genomic DNA was isolated from BAW and PBMCs samples using QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The specific primers pair (5'-cgacatccgtaaggacctgt-3') and (5'-gtggacaatgaggccagaat-3') were designed to amplified β-actin reference gene sequence fragment with 191 base pairs and for EIAV gag gene amplification we used the flanking primers 636 (5'-ccattgctggaagatgtaac-3') and 1399 (5'-tgcgttctgaatagtcagtg-3') and the internal primers 854 (5'-ggctggaaacagaaatttta-3') and 1262 (5'-aggttttccaatcatcact-3'), amplifying a 408 base pair fragment, using the protocol from the Platinum® Taq DNA Polymerase kit (Invitrogen). The actin and gag amplified fragments was observed under UV light, after agarose gel electrophoresis at 1% and stained by ethidium bromide. To confirm the analytical specificity of the amplicon, its sequence was determined.

RESULTS

Results demonstrated 89% (51/57) of gag gene amplification in PBMCs samples and only 47% (27/57) of gag gene amplification in BAW samples (Fig. 1 and 2). The reference gene β-actin were amplified in all samples.

The nested PCR assay developed in the present study detected more EIAV positive samples in PBMCs than in BAW, indicating PBMCs as a reliable source for EIAV diagnosis. To examine the agreement between the tests, Kappa statistical analysis was performed using the Win Episcope 2.0 software (Table 1). The results from the sequence analysis of the 408 bp fragment amplified by nPCR showed that it corresponded to the gag region of EIAV.

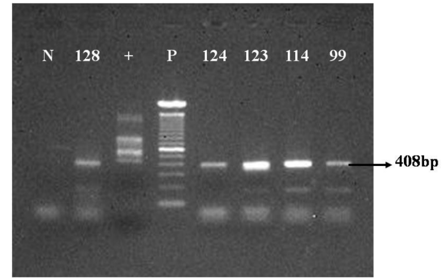

Fig. 1: Agarose gel 1% from gag nPCR. N: negative control; +: positive control; 128, 124, 123, 114 and 99: EIAV positive samples from BAW; P: DNA ladder (100 bp-Invitrogen)

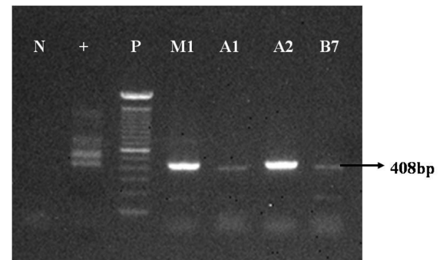

Fig. 2: Agarose gel 1% from gag nPCR. N: negative control; +: positive control; M1, A1, A2 and B7: EIAV positive samples from PBMCs; P: DNA ladder (100 bp-Invitrogen)

Table 1: Comparison results of gag nPCR in DNA samples from PBMCs and BAW with the respective value for kappa

		BAW nPCR gag		
		Positive	Negative	Total
PBMC	Positive	26	25	51
nPCR gag	Negative	1	5	6
Total		27	30	57
KAPPA	0,124			

DISCUSSION

According to these results PBMCs are more suitable for proviral DNA amplification by nPCR than BAW. In addition, six DNA samples from PBMCs showing positive serological results were negative in the nPCR and these negative results could be due to insufficient number of infected cells or to a low number of provirus copies per cell (Eaves *et al*., 1994; Reichel *et al*., 1998), as a consequence of the end of the viremic episodes. Additionally, Monocytes infected by EIAV appear cyclically in the bloodstream and their appearance is associated with viremic episodes (O'Rourke *et al*., 1991). Moreover, as it was not possible to determine the stage of the disease by the time the animals were killed, they could be in an asymptomatic stage with viremy in plasma and the provirus copy inside the PBMCs may not be detected (Oaks *et al*., 1998). On the other hand, the high number of negative results in BAW samples indicates that there may be a low number of provirus copies or insufficient numbers of cells containing the provirus. These results are contrary to those described earlier, that indicate tissue macrophages are the main cells involved in viral replication during the various phases of the disease and that less than 1% of viral load is found in peripheral monocytes (Harrold *et al*., 2000; Rice *et al*., 1989). Besides, the viral copies may be in a transition process from blood monocytes to tissue macrophages where occurs viral replication (Oaks *et al*., 1998; Sellon *et al*., 1992). Such transition may compromise the efficiency of the nPCR assay to detect EIAV sequences in DNA samples from BAW.

CONCLUSION

According to PBMC and BAW gag PCR results, it can be concluded that PBMCs samples are more suitable and reliable source for EIAV diagnosis by nested PCR comparing with BAW samples from the same animals.

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