Detection of equid herpesvirus 1 DNA by Polymerase Chain Reaction after experimental inoculation of horses with a Brazilian A4/72 strain

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Abstract

Seven conventional adult horses were inoculated intranasally with a Brazilian A4/72 strain of equid herpesvirus-1 (EHV-1). In the first ten days after the inoculation, they showed signs of a mild, self-limiting upper respiratory tract infection. In spite of the presence of neutralizing antibodies before the trial, seroconversion was observed in some horses. The virus was not isolated from nasal swabs and peripheral blood leukocytes (PBL) of any of the horses. However, the EHV-1 was detected through the polymerase chain reaction (PCR) from PBL of all horses in the experiment within the third to the eighth day after the inoculation that illustrated the viremia. In addition, the PCR assay also detected the virus in bronchoalveolar lavage fluid samples starting on the ninth day after the experimental infection in most of horses. For that reason, as a diagnostic tool, the PCR assay showed higher sensitivity and specificity than the conventional laboratorial methods in detection of EHV-1.

Key words:
Horses.
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Diagnosis.
Polymerase chain reaction.
PCR.

Introduction

Equid herpesvirus-1 (EHV-1) and 4 (EHV-4), both serologically cross-related alpha herpesviruses, are important causes of upper respiratory tract (URT) disease in horses, known as equine rhinopneumonitis (ER). Furthermore, the EHV-1, as an etiologic agent, has also been related to other different forms of disease, such as abortion in pregnant mares, perinatal foal death, and myeloencephalopathy.[1,2]

EHV-1 is believed to be ubiquitous in horse populations worldwide and also disseminated in Brazil.[2,3] Additionally, the EHV-1 infection, which has had devastating effects on breeding farms and in performance horses, may result in substantial financial losses to the equine industry.[1] Therefore, the diagnostic tools for ER must be rapid and sensitive so that early intervention policies are adopted in order to reduce the spread of the virus in horse populations.[4]

At present, the conventional laboratorial methods available in Brazil for the diagnosis of ER include virus isolation (VI) with confirmation of its identity and serological tests.[5,6] However, these methods have many limitations, as viral antigens or virus-specific antibodies cannot be detected, thus making the presence of viral genome the only evidence of EHV-1 infection. Because of this, the polymerase chain reaction (PCR) techniques have been applied for the detection of EHV-1 DNA from clinical and cell culture samples. This technique is rapid, sensitive and does not depend on the presence of a viable virus. Also, local antibodies do not interfere with this nucleic acid amplification method. Moreover, the nested PCR test, utilizing an inner set of primers in a second round of amplification, has been adopted, thus resulting in an increase of the sensitivity and the specificity for the identification of the

EHV-1 DNA sequences without subsequent specification with restriction endonuclease digestion or Southern blot hybridization of the amplified products.[3,7,8]

EHV-1 shedding in nasal secretions as well as its spreading in respiratory and lymphoid tissues after experimental infection in horses is well established.[9,10] However, to our knowledge, there have been few published reports concerning EHV-1 distribution detected by PCR in the bronchoalveolar lavage fluid (BALF) cells and peripheral blood leucocytes (PBL) samples immediately after experimental virus inoculation of horses. Published data available are limited and refer only to detection of latent EHV-1 infection.[11,12] The main purpose of this experiment was to study the distribution of the EHV-1 in these cell populations using this diagnostic tool before and after the experimental viral inoculation of horses. In addition, we also described the clinical, hematological and serological responses of horses to the EHV-1 challenge.

Materials and Method

The Brazilian A4/72 strain of EHV-1 (Instituto Biológico, São Paulo, SP, Brazil), originally obtained from an aborted equine fetus, was used in the 15th passage level in Vero cells for all experiments.

Seven conventional horses (six geldings and one mare), aged 8-16 years, were inoculated intranasally with $10^{6.6}$ $TCID_{50}$/mL of EHV-1. For a minimum period of three weeks prior to the experimental challenge, serological monitoring ruled out any possibility of concurrent field EHV-1 infection (Table 1). All these horses were kept in separate stalls that did not allow aerosol circulation among animals in the study. They were bedded on shavings and fed on pelleted concentrates and dust-free hay. The experimental animal procedures were approved by the ethical committee of the Faculdade de Medicina Veterinária da Universidade de São Paulo (protocol 240/2002) and the facilities and environment conditions followed the guidelines of the C.C.A.C.[13].

Rectal temperature and clinical manifestations were recorded daily, in the morning and evening, for 10 days after EHV-1 inoculation. Endoscopic examinations of URT were conducted once before inoculation and during the immediate post-challenge period by using a 180-cm fiberoptic endoscope (CF-LB2, Olympus,

Table 1 - Serum virus neutralizing (VN) antibody titers of horses before and after inoculation with EHV-1 strain A4/72. (*n* = 7) - São Paulo - 2005

	Horse						
D.p.i.	H1	H2	H3	H4	H5	H6	H7
-21	1.2	0.0	1.2	1.2	1.5	1.5	1.2
0[a]	1.0[b]	0.0	1.2	1.0	1.5	1.5	1.0
+07	1.0	0.0	1.0	1.0	1.8	1.8	1.0
+14	1.5	0.7	1.0	1.2	1.8	2.7	1.8
+21	1.2	0.7	1.2	1.5	2.1	2.1	1.2
+28	1.0	1.0	1.2	1.2	1.8	1.8	1.2

D.p.i.: days post-inoculation; [a]Horses were infected on day 0; [b]Virus neutralizing (VN) antibody titers are given as the reciprocal log $_{10}$

Tokyo, Japan). Blood samples (with 2mg EDTA/mL) were collected daily for 10 days for total and differential WBC counts that were measured by standard procedures.

Bronchoalveolar lavage (BAL). BAL was performed using silicone catheter (Bivona Inc., Gary, IN, USA) as previously described by Mori et al.[14]. Repeated BALs were performed with at least seven days of interval: –7, +2, +9, +16, +23 and +30 days post-inoculation (dpi). The cytological slides were prepared as described.[14]

Isolations of virus were attempted from nasal swabs taken on the first, second, third, fourth, fifth, seventh and ninth dpi and from PBL collected from 20 mL of whole blood (with 2 mg EDTA/mL) daily for 10 dpi. These specimens were placed in the Eagle's minimal essential medium (MEM) plus 10% of fetal bovine serum and after that inoculated in Vero culture cells. When these cells exhibited cytopathic effect (CPE), the identification of isolates could be performed as described by Saxegaard.[15]

Serum was taken before viral inoculation and, afterwards, periodically every seven days for four weeks for the detection of neutralizing antibodies against EHV-1 (expressed as $\log_{10}$ values) using the microtitration method, as previously described[5]. Seroconversions were defined as increases of four-fold (or 0.6 $\log_{10}$) of VN antibody titers between acute and convalescent serum samples.[16]

DNA, isolated from PBL and BALF samples, was extracted following a method described elsewhere.[17] The primer pairs were selected based on the nucleotide sequences of the glycoprotein B (gB) region of EHV-1 and EHV-4 genomes described by Kirisawa et al.[18] and were predicted to yield PCR products for outer (FC2-RC) and inner (FC2-R1 and FC2-R4) primers (Invitrogen Brasil Ltda, São Paulo, SP, Brazil) as shown in table 2.

The amplification conditions were performed in a reaction mixture of total volume 50μl containing 100ng of DNA sample, 0.5μM of each primer (outer primers), 0.2mM of each dNTP mixture, 2.5 units of *Taq* DNA polymerase (Invitrogen Brasil Ltda, São Paulo, SP, Brazil), 1´ PCR buffer (20mM of Tris-HCl pH 8.4, 50mM of KCl), 1.5mM of $MgCl_2$ and milliQ water QS. Amplification was carried out in a thermal cycler (PTC-200, Bio-Rad Laboratories Inc., Waltham, MA, USA) under the following conditions: DNA template was initially denatured at 94°C for 5-min followed by 35 cycles of 1-min denaturation at 94°C, 1 min primer annealing at 60°C and 1-min extension at 72°C. Lastly, the reaction was completed in a 7-min final extension step at 72°C. For the semi-nested PCR, 1μl of the primary amplification template was added to 49μl of a reaction mixture containing the inner primers, and the thermal cycles program was repeated. Both positive viral DNA (EHV-1 strain A4/72) and negative (water) controls were run for all PCR assays. The PCR products were loaded onto 1% agarose gel and electrophoresis carried out at 100V for 70-min in TAE buffer. The gel was stained with

Table 2 - Nucleotide sequences of primers used for EHV-1 and EHV-4 differentiation and PCR fragment size[18]

Primers	Sequence (5'-3')	Specificity	Fragment size (bp)
FC2, forward	CTTGTGAGATCTAACCGCAC	EHV-1 and -4	
RC, reverse	GGGTATAGAGCTTTCATGGG	EHV-1 and -4	1181
R1, reverse[a]	GCGTTATAGCTATCACGTCC	EHV-1	460
R4, reverse[a]	CCTGCATAATGACAGCAGTG	EHV-4	943

[a]Nested primers

0.5μg/mL ethidium bromide and DNA bands were visualized on UV transilluminator. Negative amplifications were repeated at least once in order to detect the presence of PCR inhibitors.

Precautions were adopted in order to avoid cross-contamination due to manipulation of PCR products such as: physical separation of DNA extraction, pre-PCR and post-PCR amplifications; exclusive positive displacement pipettes and aerosol filtered pipette tips.

To determine the analytical sensitivity, EHV-1 strain A4/72 with 10^{-3}/25µl $TCID_{50}$ was serially 10-fold diluted. The 460bp second round product was detected at a dilution of 10^{-9}, the highest dilution returning a positive semi-nested PCR result.

Data analysis (serum VN antibody titers and BALF differential cell counts) was performed using a statistical software Graph Pad InStat version 3.01, 32 bit for Windows 95/NT. Each variable was tested for normality applying the Kolmogorov and Smirnov method. One way analysis of variance (ANOVA) was used to compare means, for data normally distributed. If data did not seem to be normally distributed, Kruskal-Wallis test was used. The level of statistical significance was set at $P<0.05$.

Results

On the day following the viral challenge, all horses simultaneously showed some clinical manifestations of disease: bilateral serous ocular and nasal discharges, hyperemia of the conjunctival and ocular mucous membranes and enlargement of submandibular lymph nodes. The intensity of these signs was at its peak between days 3 and 5; afterwards it was minimal on day 8 and lastly disappeared on day 10. Between days 3 and 5, endoscopic findings indicated increased amounts of mucus in the pharynx and larynx. Nevertheless, there was no increase in rectal temperature nor evidence of more severe complications of EHV-1 infection (i.e. respiratory distress or systemic illness). The total and differential WBC counts during the whole trial remained within the normal reference range described for the specie.

As it can be seen in table 3, there was a predominance of macrophages (31 – 90%) and lymphocytes (10 – 50.5%) in the BALF samples obtained from healthy horses (-7 dpi). Neutrophils comprised less than 10% in the BALF samples of all healthy horses except for H5 and H6, which exhibited elevated numbers of these cells before inoculation. After challenge, there was no significance difference in any of the BALF cytological counts. However, during the convalescence phase of viral infection, the horses H3, H4 and H7 showed a marked rise in neutrophil values.

No virus was recovered from PBL and nasal swab samples at any time after the inoculation. The pre-inoculation and post-inoculation serum VN antibody titers are given in table 1. There was no statistically significant difference in VN antibody levels from horses infected with EHV-1. Nevertheless, the horses H2, H5, H6 and H7 seroconverted to EHV-1 by 14 and 21 dpi. The horses H1 and H4 also showed also a slight rise of post-challenge serum antibodies that was insufficient to be named seroconversion.

According to table 4, the EHV-1 DNA was detected through the PCR from PBL samples of all horses within the third to the eighth day after the inoculation. In addition, as shown in table 5, the PCR assay detected the DNA virus in BALF samples starting on the ninth day after the experimental infection in horses H2, H6 and H7. Horse H4 was positive from day 2, while horse H5 was positive only after 23 days. However, no amplification was detected for EHV-4 from any PBL and BALF samples.

Discussion

In the first ten days after the inoculation, all the animals showed signs of a mild, transient and self-limiting URT infection. These findings were very similar

Table 3 - Differential cell counts of leukocytes recovered by bronchoalveolar lavage fluid (BALF) samples of horses before and after inoculation with EHV-1 strain A4/72. (*n* = 7) - São Paulo - 2005

Horse	Leukocytes (%)	Days post-inoculation					
		-7	+2	+9	+16	+23	+30
H1	Mϕ	45.7	65.0	35.3	90.0	58.3	36.3
	L	43.0	32.7	48.7	9.0	34.0	50.3
	N	8.7	2.3	15.3	1.0	6.3	12.3
H2	Mϕ	74.3	89.7	71.7	76.0	59.3	65.3
	L	17.7	9.3	24.7	20.3	34.7	30.7
	N	7.0	1.0	3.7	2.7	4.3	3.0
H3	Mϕ	90.0	87.3	68.7	76.3	65.0	45.3
	L	10.0	11.0	25.3	22.7	25.3	15.0
	N	0.0	1.7	5.7	0.7	9.7	38.0
H4	Mϕ	59.0	57.0	45.5	16.0	45.0	53.7
	L	40.0	42.0	52.0	24.0	40.0	40.0
	N	1.0	1.0	2.5	59.0	13.5	4.7
H5	Mϕ	47.5	55.5	64.0	55.0	51.5	64.4
	L	31.0	35.0	23.0	28.0	28.0	24.4
	N	21.5	9.0	11.5	16.0	19.0	9.7
H6	Mϕ	60.5	44.0	59.0	46.5	58.0	62.0
	L	25.0	46.0	29.0	48.5	27.0	29.7
	N	14.0	8.0	12.0	4.5	15.0	8.4
H7	Mϕ	31.0	41.0	26.0	47.5	31.0	52.0
	L	50.5	42.0	62.0	44.0	46.0	18.0
	N	2.5	14.0	11.0	6.5	18.0	22.5

Mϕ: macrophages; L: lymphocytes; N: neutrophils

Table 4 - Results of the semi-nested PCR assay to detected EHV-1 DNA performed on peripheral blood leucocytes (PBL) samples - São Paulo - 2005

Horse	Days post-inoculation													
	0	+01	+02	+03	+04	+05	+06	+07	+08	+09	+10	+16	+23	+30
H1	-	-	+	+	+	+	+	+	+	-	-	-	-	-
H2	-	-	+	+	-	+	+	+	+	-	-	-	-	-
H3	-	+	-	+	-	+	+	+	-	-	+	-	-	-
H4	-	-	-	+	+	+	+	+	+	-	-	-	-	+
H5	-	-	-	+	-	+	-	+	+	+	+	-	-	-
H6	-	-	-	+	+	+	+	+	-	+	-	+	-	-
H7	-	-	-	-	+	+	-	+	+	+	-	+	-	-
Total (%)[a]	0	14.3	28.6	85.7	57.1	100	71.4	100	71.4	42.9	28.6	28.6	0	14.3

"+" refers to positive PCR; "-" refers to negative PCR; [a]Percentage of positive cases

to the results obtained from horses after experimental EHV-1 re-infections of the respiratory tract.[1,19,20] It seems evident that the presence of local immunity tends to reduce the viral multiplication of URT, and consequently the damage to this tissue.[21,22]

Based on hematologic results, no animal developed transient blood leukopenia, involving lymphocytes as well as neutrophils. Moreover, other hematological changes such as reactive lymphocytes, considered an indicator of active virus infection, were not observed in the present study either. This work supports the earlier observations that horses exposed to infection on numerous occasions showed less severe illness.[1,19]

From table 3 it may be inferred that in the majority of cases the fluctuation of leukocyte populations in the bronchoalveolar compartment cells was not related to an EHV-1 infection. Unlike what was described by Kydd, Hannant and Mumford[23], no horse showed a transient BALF neutrophilia during the acute viral infection phase. This absence of leukocyte recruitment to the affected areas in the peripheral circulation and bronchoalveolar compartment may be associated with reduced signs of EHV-1 infection. In addition, we could not find other cytological changes considered consistent with viral respiratory disease, such as lymphocyte responses in BALF samples, contrasting with other published studies.[14,24]

In this trial, the BALF neutrophil numbers of two healthy horses (H5 and H6) were higher than those described previously.[23] When the value of neutrophils in BALF of healthy horses is higher than 10% it can be related to several factors, such as stabling[25] and aging (>6 years) .[26]

Results of this study also suggested that the pulmonary neutrophilic influx observed in BALF samples of horses H3, H4 and H7 during the convalescence viral infection phase may be induced by sequential lavages. According to Traub-Dargatz et al.[27] and Sweeney et al.[28], the constituents of saline solution used for lavage and BAL technique may contribute to lung inflammation.

It can be seen in table 1 that seroconversion was observed in horses H5, H6 and H7 even in the presence of high levels of VN antibodies before the trial. On the other hand, the results of horses H1, H3 and H4 were similar to those described by other investigators, in which reported responses of serum VN to EHV-1 re-infections have usually been absent.[21,29]

In this study, there were no viruses recovered by VI from either nasal swabs or PBL of any horses after inoculation with EHV-1. This failure of VI was probably due to the low level of infectious virus[19]. The duration and amount of virus can be reduced in animals with high levels of circulating VN antibody as a result of previous exposure.[20,21]

Despite serum VN antibodies were not detected in horse H2 before experimental infection (Table 1), EHV-1 could not be isolated from nasal swab, suggesting that protective immunity against clinical disease and viral shedding depend on several factors, beside the humoral response. A*dditional mechanisms,* such as $CD8^+$ cytotoxic T lymphocytes, are responsible for the protective immunity against EHV-1 infection and essential for suppression of viral replication.[14, 20]

On the other hand, as it can be observed in table 4, the EHV-1 DNA was detected from PBL in the majority of the horses within the third to the eighth day after inoculation. It seems that all horses sampled in this study were viremic after the challenge and it can also be inferred that the PCR was a more sensitive diagnostic tool than VI in detecting virus from PBL samples.

PCR patterns from PBL samples showed an intermittent EHV-1 viremia in horses H2, H3, H5, H6 and H7, suggesting a moderate level of viral activity. This oscillation in EHV-1 amount could lead a low virus load, below of semi-nested PCR's detection limit.

According to table 5, it appears that there was the dissemination of the EHV-1 throughout the respiratory tract after virus exposure in horses H2, H4, H5, H6 and H7.

Table 5 - Results of the semi-nested PCR assay to detected EHV-1 DNA performed on bronchoalveolar lavage fluid (BALF) samples - São Paulo - 2005

	Days post-inoculation					
Horse	-07	+02	+09	+16	+23	+30
H1	-	-	-	-	-	-
H2	-	-	+	+	-	+
H3	-	-	-	-	-	-
H4	-	+	+	+	+	+
H5	-	-	-	-	+	+
H6	-	-	+	+	+	+
H7	-	-	+	+	+	+
Total (%)[a]	0	14.3	57.1	57.1	57.1	71.4

"+" refers to positive PCR; "-" refers to negative PCR; [a]Percentage of positive cases

In contrast, previous reports indicated that the detection of EHV-1 was less extensive in lavaged bronchoalveolar cells than in lymphoid tissues[11,12] and PBL[11]. Because of that, the detection of positive PCR product in the BALF following the EHV-1 challenge suggests that the animals in this study become infected.

Nevertheless, whether EHV-1 survives in the convalescent animal as a persistent (active virus replication) or latent virus infection is not known either from BALF or PBL samples. It is known that the differentiation between active and latent infection may be important for management control and treatment of EHV-1 infected horses. And as the conventional PCR technique used in this work does not allow the differentiation between these two states of EHV-1 in positive samples, further studies should be carried out, such as the quantitative real-time PCR assay[30] and the identification of latency associated transcripts (LATs) expression in RNA by RT-PCR[31,32].

Despite the significance of EHV-4 as respiratory pathogen in horse populations worldwide[1,2], all clinical samples in present work were negative for EHV-4 detection by PCR. As observed by Carvalho et al.[3], the role of EHV-4 in Brazilian horses remains to be investigated.

Based on the results obtained from this study, it can be affirmed that the PCR is a highly effective technique in detecting the EHV-1. It may be used in circumstances where traditional methods are not efficient due to the fact that it provides an enhanced diagnostic procedure for underdiagnosed diseases.

Detecção do DNA do herpesvírus eqüino 1 pela reação em cadeia pela polimerase em cavalos inoculados com a estirpe brasileira A4/72

Resumo

Sete cavalos adultos de status sanitário convencional foram inoculados por via intranasal com a estirpe brasileira A4/72 do herpesvírus eqüino tipo 1 (EHV-1). Nos primeiros dez dias após a inoculação viral, todos os cavalos apresentaram manifestações de infecção respiratória leve e restrita às vias aéreas anteriores. Apesar de possuírem títulos de anticorpos neutralizantes antes da inoculação, alguns cavalos apresentaram soroconversão após o desafio viral. O EHV-1 não foi isolado a partir das secreções nasais e leucócitos sanguíneos periféricos (PBL) de nenhum animal. Entretanto, o DNA viral foi detectado pela

Palavras-chave:
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reação em cadeia pela polimerase (PCR) nos PBL entre o terceiro e o oitavo dias pós-inoculação (d.p.i.) em todos os animais, indicando a ocorrência de viremia. Além disso, a prova de PCR detectou o vírus nas amostras do lavado broncoalveolar a partir do nono d.p.i. na maioria dos animais. Com base nos resultados obtidos, foi possível concluir que a PCR é uma técnica com alta sensibilidade e especificidade para o diagnóstico do EHV-1, capaz de detectar a presença do DNA viral mesmo quando não ocorre a constatação do agente pelos métodos tradicionais.

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