EQUINE HERPES VIRUS 2 INFECTION IN HORSE POPULATIONS IN POLAND

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Summary. – The prevalence of Equine herpesvirus 2 (EHV-2) infections in the horse populations in Poland was investigated. Peripheral blood leukocytes (PBLs) of 139 horses were tested. The animals were divided into four groups: clinically healthy horses, horses suffering from respiratory disorders, mares with a recent abortion and horses with diagnosed ataxia. Thirty-four virus isolates were obtained from leukocytes of the tested animals by cocultivation with equine dermal cells and were identified as EHV-2 by PCR using primers for the gB gene of EHV-2 and/or primers for the sequence located upstream of the gene homologous to the equine interleukin 10 (IL-10) gene. These results indicate that EHV-2 is prevalent in horse populations in Poland. As the virus was most frequently isolated from horses with respiratory disorders its etiological importance may be considered.

Key words: Equine herpesvirus 2; horse populations; interleukin 10; prevalence; cocultivation; PCR

Introduction

EHV-2 (the species *Equid herpesvirus* 2, the genus Rhadinovirus, the subfamily Gammaherpesvirinae, the family *Herpesviridae*) (van Regenmortel *et al.,* 2000) infections commonly occur in various horse populations throughout the world. It has been reported that the prevalence of EHV-2 in PBLs of adult horses is up to 68% in Sweden and 71% in the United Kingdom (Nordengrahn *et al.*, 2002). The infection implicates various upper respiratory tract disorders, lymphoadenopathy, immunosuppresion, keratokonjunctivitis, general malaise and "poor performance" in racehorses (Blakeslee *et al.*, 1975; Borches *et al.,* 1997; Collison *et al*., 1994; Schlocker *et al.*, 1995; Studdert, 1996). It is also considered a predisposing factor for *Rhodococcus equi* infections in foals (Palfi *et al.*, 1978; Nordengrahn *et al.*, 1996). However, many aspects of EHV-2 pathogenicity are not fully defined.

EHV-2 shows considerable genetic heterogeneity based on restriction analysis and serum neutralization studies (Browning and Studdert, 1987; Turner *et al*., 1970a; Turner and Studdert, 1970b). It has been reported that several genetically different EHV-2 strains can be isolated from a given group of horses and even from individual animals (Browning *et al.*, 1987). It was not elucidated whether these horses were infected with different strains of virus or whether a strain mutated during replication in different tissues, thus leading to new strains (Borchers *et al.*, 1998). The correlation between EHV-2 genomic heterogeneity and pathogenicity (Borchers *et al.*, 1997) also remains a poorly understood issue. On the other hand, detection and accurate diagnosis of EHV-2 infection is difficult due to virus latency, strain heterogeneity and the fact that an individual animal may be infected with several strains of EHV-2 at the same time.

The aim of this study was to investigate the prevalence of EHV-2 infection among horses in Poland. This study evolved as a consequence of our previous investigation of latent Equine herpesvirus 1 (EHV-1) infection in 1996–1998 (Bańbura *et al.*, 2000), which had indicated the possibility of the prevalence of EHV-2 among horses in Poland. Therefore, we decided to employ leukocyte cocultivation and PCR to evaluate this possibility.

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Abbreviations: CPE = cytopathic effect; ED = equine dermal; EHV-1 = Equine herpesvirus 1; EHV-2 = Equine herpesvirus 2; IL-10 = interleukin 10; ORF = open reading frame; PBLs = peripheral blood leukocytes; vIL-10 = viral IL-10

Table 1. Results of PCR and cocultivation

Group[a]	No. of horses tested in 1996–1998	No. of virus isolates obtained (1996–1998)	No. of virus isolates identified by PCR (1996–1998)[b]		No. of horses tested in 2000–2002	No. of positive PBLs samples (2000–2002)[c]			No. of virus isolates obtained (2000–2002)	No. of virus isolates identified by PCR (2000–2002)[d] vIL-10
			gB	vIL-10		PCR	PCR and cocultivation	cocultivation		
1	77	15	8	15	20	7	2	1	3	3
2	–	–	–	–	35	7	10	3	13	13
3	1	1	–	1	3	2	–	–	–	–
4	1	1	–	1	2	–	1	1	1	1
Total[e]	79	17	8	17	60	16	13	4	17	17

[a]1 = clinically healthy horses; 2 = horses suffering from upper respiratory tract disorders, bronchitis or pneumonia; 3 = mares with recent abortion; 4 = horses with ataxia.

[b]All virus strains isolated in 1996–1998 were tested with the primers specific for EHV-2 gB and with the primers specific for the sequence located upstream of the ORF coding for vIL-10.

[c]PBLs isolated from horses in 2000–2002 were tested by both cocultivation and PCR using the primers specific for the sequence located upstream of the ORF coding for vIL-10.

[d]Isolates obtained in 2000–2002 were identified as EHV-2 only by PCR using the primers specific for the sequence located upstream of the ORF coding for vIL-10.

[e]Total number of horses tested in 1996–1998 and 2000–2002 was 139, total number of virus isolates was 34.

Materials and Methods

Horses. A hundred and thirty-nine horses of various breeds from various regions of Poland were included in this study. Arabian and thoroughbred horses, usually originating from studs, were on average 2 years of age. Draught horses usually originated from small farms, were kept individually and their average age was 8 years. On the basis of the clinical status, the horses were divided into 4 groups: clinically healthy animals (97); animals suffering from respiratory tract disorders (35), mares with recent abortion (4) and animals exhibiting ataxia (3).

Cocultivation. Five-ml blood samples were collected from each animal and diluted with 5 ml of 2x RPMI 1640 Medium (Gibco BRL) supplemented with 25,000 U of heparin (Polfa Tarchomin, Poland). PBLs (1 x 10^7), isolated from whole heparinized blood (Jacobsen *et al.,* 1982) were added to a monolayer culture of equine dermal (ED) cells (kindly provided by Dr. K. Borchers, Institut für Virologie der Freie Universität Berlin, Germany) and incubated at 37°C until cytopathic effect (CPE) appeared. If no CPE appeared by day 6 of incubation, the culture was repassaged. When no CPE developed after 3 passages, the sample was considered negative. Infected cultures were stained with hematoxylin-eosin to visualize syncytia and eosinophilic intra-nuclear inclusions. Virus isolates were obtained by standard plaque method.

Identification of EHV-2 isolates. The virus isolates were propagated in ED cells. To identify EHV-2, total DNA was extracted (Strauss, 1992) and subjected to PCR using gB-specific primers (Reubel *et al*., 1995) and/or primers for the sequence located upstream of the ORF coding for viral IL-10 (vIL-10) (Borchers *et al*., 1997). The PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide and visualized under UV-light. The DNA from ED cells infected with the 86/67 strain of EHV-2, kindly provided by Prof. M.J. Studdert, School of Veterinary Science, Melbourne, Australia, was used as positive control, while the DNA from uninfected cultures was used as negative control.

Identification of specific EHV-2 sequences in PBLs. PCR was also performed directly with DNA extracted from PBLs isolated from horses. DNA extraction and PCR techniques were the same as above.

Statistical analysis was performed using the chi-square test.

Results

Identification of EHV-2 isolates

Thirty-four virus isolates were obtained from 139 horses tested by cocultivation of PBLs with ED cells (Table 1). In the case of PBLs positive for the virus CPE appeared on the days 4–5 of cocultivation in the first passage or on the days 2–5 in the second or third passage. CPE was visible as plaques, syncytia and intra-nuclear inclusion bodies. All 34 virus isolates were identified as EHV-2 by cocultivation and PCR (Table 1). Eighteen isolates originated from 97 clinically healthy horses (18.5%). The remaining 16 isolates (38%) were obtained from 42 horses with various clinical pictures: respiratory disorders, abortion and ataxia. Thirteen of these isolates (37.1%) originated from animals suffering from respiratory tract diseases. Statistically significant differences ($P \leq 0.05$) were observed only between clinically healthy horses and horses with respiratory tract disorders.

The PCR with primers for the gB gene identified 8 of 17 tested isolates, while that with primers for the sequence located upstream of the ORF encoding vIL-10 identified all the 34 isolates (Table 1). The length of the product of the second round of the nested PCR varied among various isolates in about 100 bp (Fig. 1).

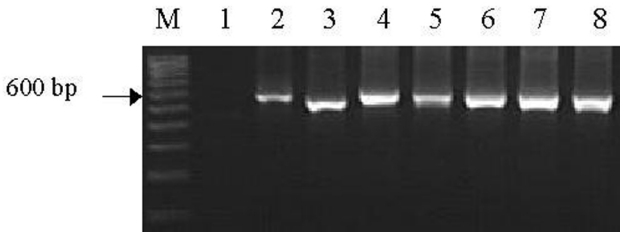

Fig. 1

Results of the nested PCR with primers specific for EHV-2 sequences located upstream of vIL-10

The results of the second round of the nested PCR. 100 bp DNA ladder (lane M). Negative control (lane 1). EHV-2 strain 86-67 (lane 2). EHV-2 isolates tested (lanes 3–8).

Identification of specific EHV-2 sequences in PBLs

Using the nested PCR we detected EHV-2-specific sequences in 29 of 60 (48.3%) PBLs samples tested (Table 1). Whereas 16 PBL samples were positive only by PCR 4 PBLs samples were positive by cocultivation only. As noted above, the product of the second round of the nested PCR varied among various isolates in about 100 bp.

Discussion

EHV-2 has been identified in horse populations worldwide, however, the prevalence of this virus in Poland is poorly defined. Our study provides a clear evidence for the presence of EHV-2 in various populations of horses in Poland. Similarly to other reports (Roeder and Scott, 1975; Borchers *et al.*, 1997) we isolated EHV-2 from healthy horses but more frequently from animals suffering from respiratory disorders. Although successful isolations were done much more frequently from horses with respiratory diseases, we cannot consider this virus the obvious etiological agent of the disease(s) of concern, because many other factors might have been involved. We just emphasize that an accurate differential diagnosis to exclude other infectious agents should be performed in each case. Furthermore, a proper interpretation of these results is difficult because a successful isolation of EHV-2 by cocultivation may represent, in fact, a detection of native virus or a virus reactivated from latency.

Among 60 PBLs samples tested in 2000–2002 twenty-nine (48.3%) were EHV-2 positive by PCR. These results confirm that EHV-2 infections are widespread in horse populations. Four samples were EHV-2 positive only by cocultivation. Such results could be due the fact that the amount of leukocyte DNA used in PCR was very small as compared to the number of cells used for cocultivation. It is also possible that the number of viral particles was too small even for the nested PCR. Drummer *et al*. (1996) have proved that an infected PBL contains 1 to 10 million of viral particles. Therefore only reactivation of a latent virus in a susceptible cell in culture results in successful virus detection.

A practical application of our findings presented here is obviously the diagnosis of EHV-2 infections by PCR. Primers for the gB gene of this virus (Reubel *et al.*, 1995) have been initially used for the identification of EHV-2 isolates in our laboratory in 1996–1998. However, only 8 of 17 isolates initially tested were identified as EHV-2. Similar results have been obtained by other authors (Franchini *et al.*, 1997). Out of 34 EHV-2 isolates obtained by Franchini *et al*. (1997) only 16 were identified by use of the primers specific for the gB gene (Reubel *et al*., 1995). Later on, Holloway *et al.* (2000) have identified amino acid variations in gB sequences. Therefore, in our studies carried out in 2000–2002, we decided to use another primer set designed to amplify the sequences located upstream of the ORF encoding the virus homolog of equine IL-10, vIL-10 (Borchers *et al*., 1997). Despite the fact that the product of second round of the nested PCR varied in size by about 100 bp, the primer set mentioned above was able to identify all the isolates tested.

The heterogeneity of EHV-2 appears an important feature of biology of this virus. We observed the genomic heterogeneity, variable growth rate and plaque size of various EHV-2 isolates in tissue culture. An intriguing problem is to find the correlations between plaque size, genetic heterogeneity, type of tissue from which the virus was isolated, and pathogenicity. The so far reported correlations are questionable (Borchers *et al*., 1997; Browning *et al*., 1987).

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