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Characterisation of Gamma Herpesviruses in the Horse by PCR

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A polymerase chain reaction (PCR) based on a combination of oligonucleotide primers selected using the octamer frequency disparity method with primers specific for EHV-5 (described by other authors) recognized all of a series of gamma herpesvirus field isolates. This PCR produced only three fragments: (1) one EHV-2-specific; (2) one EHV-5-specific; and (3) a fragment that occurred alone or in combination with the other two. Cloning and sequencing of four different isolates yielding only the last PCR product showed that this corresponds to a deletion/insertion mutant of EHV-2. The fact that this mutant was also plaque-purified from a culture producing all three PCR fragments demonstrated that the virus producing this fragment was distinct from the other two and that this specific DNA fragment was not an artefact due to PCR amplification. These data show that equine gamma herpesviruses are genetically more heterogeneous than previously assumed. The PCR failed to directly detect gamma herpesviruses from the DNA extracted from the same starting material used for the isolation of gamma herpesvirus by cocultivation with indicator cells. This demonstrates that the most reliable method for detection of equine gamma herpesviruses is the cocultivation with indicator cells. © 1997 Academic Press

INTRODUCTION

Two gamma herpesviruses have been described in the horse (Telford *et al.*, 1993). Equine herpesvirus 2 (EHV-2) was isolated from a horse with upper respiratory tract disease by Plummer and co-workers (Plummer and Waterson, 1963; Plummer *et al.*, 1969). EHV-5 was first described by Agius and co-workers (Agius *et al.*, 1992) and its pathogenetic potential is not completely understood because of the frequent occurrence with EHV-2. EHV-2 is widely distributed in horses in a hidden form. It can be activated and obtained as complete virus by cocultivation of blood leukocytes with equine or rabbit indicator cells. EHV-2 was isolated in 86% of horses in Scotland (Roeder and Scott, 1975), 89% in Iowa (Kemeny and Pearson, 1970), and 77% in Zurich (Schlocker *et al.*, 1995). In a recent study EHV-2 was isolated from 97% of abattoir horses in the United Kingdom. The highest frequency of isolation was obtained for bronchial lymph nodes (85%) (Edington *et al.*, 1994). EHV-2 has not been considered a pathogen because of its wide distribution in the equine population. Experimental infections, though, showed that pharyngitis could be induced in foals with virus shedding through nasal discharge for up to 118 days. No respiratory tract disease developed in adult horses after experimental inoculation (Blakeslee *et al.*, 1975). EHV-2 was more frequently isolated from tracheal aspirates of foals with respiratory diseases than from healthy foals (Murray *et al.*, 1996).

The biological, immunopathological, and epidemiological properties of the gamma herpesviruses in the horse are not completely understood A prerequisite for accurate studies in the future is the possibility of distinguishing the different virus types present in the horse and of identifying the truly gamma herpesvirus-negative horses. Our study represents a contribution to this goal.

MATERIALS AND METHODS

Horses

For this study 33 horses aged between 8 and 15 years (18 geldings and 15 mares) were recruited. Bronchoalveolar lavage (BAL) was performed on 22 of these horses.

EHV-2 stock preparation

Five hundred microliters of EHV-2 (Plummer strain, ATTC No. VR-701) suspension was added to 2 ml of Eagle's minimal essential medium with Earle's salts (EMEM; BioConcept, Allschwil, Switzerland) and used to infect a monolayer of equine embryonic lung fibroblasts (EHL, embrionic horse lung) in a 150-cm^2 cell culture vessel. After 1 h at 37° the suspension was removed and the monolayer was washed once with 30 ml of PBS; 40 ml of EMEM with 2% fetal calf serum (FCS) was then added to the cells. After 5 days the cytopathic effect was recognized and at day 6 the cell culture flask was frozen

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF014391–AF014395.

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at −20°. After two additional freeze–thaw cycles the suspension was filled in 1-ml vials and frozen at −70°.

Bronchoalveolar lavage

Horses were sedated with a combination of xylazine and methadone. A video bronchoscope was introduced trough the precleaned and topically anesthethised nostril and advanced until it wedged in a bronchus. During the advancement of the bronchoscope the bronchial surface was continuously anesthetised with a solution containing 1% lidocaine and 0.9% sodium chloride injected through a sterile catheter passed through the biopsy channel. Two hundred fifty milliliters of prewarmed sterile physiological saline solution was infused through the biopsy channel into the bronchus and immediately reaspirated with a suction pump into a sterile flask. The infusion/aspiration procedure was repeated three more times in the same lung segment.

Virus isolation

Mononuclear cells from 10 ml blood were isolated by density gradient centrifugation using standard methods and added to a monolayer of EHL in 25-cm^2 flasks in a volume of 10 ml EMEM with 10% FCS. Bronchoalveolar lavage cells were collected by centrifugation of the BAL fluid at 250 *g* for 15 min at 4° and washed once with PBS and 2×10^7 cells were resuspended in a volume of 20 ml EMEM with 10% FCS and added to two monolayers of EHL in 25-cm^2 flasks. The medium was removed weekly together with nonadherent and dead cells and replaced with fresh medium. When the first signs of infection were recognizable (rounding up and detachment of fibroblasts from the bottom) the medium was changed, and after 2 to 3 days the medium containing the infected cells was frozen at −20°. Cultures which remained negative for 8 weeks were considered negative.

Positive controls consisted of monolayers infected with aliquots of reference virus. Negative controls were obtained by scraping noninfected EHL monolayers from 25-cm^2 flasks with a rubber policeman and collecting the suspension.

Polymerase chain reaction

Sample preparation for PCR. Each 1-ml aliquot of the thawed samples was put into a 1.5-ml microcentrifuge vial and centrifuged at 14,000 *g* for 10 min at 4°. The supernatants were discarded and 100 μl of a solution containing 20 mM Tris, 0.1% Triton X-100, and 12 μg/ml Proteinase K was added to the pellets. After 1 h incubation at 56° and 5 min boiling, the vials were centrifuged at 14,000 *g* for 5 min at 4° and the supernatants were used as targets in PCR.

PCR conditions. PCR was run in a volume of 20 μl with 50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl, 0.1% gelatine, 200 mM each dNTP, 12.5 pmol primer, 1 U of *Taq* polymerase (Catalys AG, Wallisellen, Switzerland), and 5 μl of the samples. Thirty-five cycles of amplification were performed under following conditions: denaturation, 1 min at 94°C; annealing, 1 min at 60°C; extension, 1 min at 72°C. Five microliters of the product was loaded on 1.9% agarose gels containing 0.5 μg ethidium bromide per milliliter.

Primers. Primers and target sequence were chosen in order to achieve maximal specificity and ideal amplification conditions using the octamer frequency disparity method by screening the sequence of EHV-2 strain 86/67 (Telford *et al.*, 1995) using the Macintosh program PC-Rare (Griffais *et al.*, 1991). The target sequence is not conserved among herpesviruses (forward primer, 5′-TACATCCTCAAAATCCTGTGAATCGTCCGA-3′; reverse primer, 5′-CGCCGCTGTCCAAAACACCATGTAGCTACG-3′). The octamers at their 3′ ends are the rare octamers (frequency in the human genome $< 0.26 \times 10^{-6}$) chosen by the program. The target sequence is situated in a noncoding region between positions 40,661 and 41,266 of the EHV-2 sequence, and the expected product length is 604 bp.

The second EHV-2-specific primer set and the EHV-5-specific set used were designed by Reubel *et al.* (1995) and are called EHV-2R and EHV-5 oligonucleotide primers. The expected product lengths are 257 and 251 bp, respectively.

In a first phase a separate PCR amplification for each primer set was run. In a second phase a combination of our primer with the EHV-5 primer was used to detect EHV-2 and EHV-5 simultaneously and a parallel PCR was run with the EHV-2R primers.

Capsid PCR. The primers were designed for amplification of a target in the region coding for the major capsid protein of EHV-2 (forward primer, 5′-TAGAGAACAGACCCTACCCC-3′; reverse primer: 5′-GCCCCTATGTACTCTGTGAC-3′), which generated fragments of an expected size of 451 bp.

IL 10-like gene PCR. A PCR was performed to amplify the region of EHV-2 coding for the interleukin 10-like gene (forward primer, 5′-ATGAAGATGTCAAACTCGC-3′; reverse primer, 5′-AGGGTAAAGACCTTCTTTCAG-3′). The expected product length was 350 bp.

Sequencing of PCR products

The amplified products were purified from the agarose gel using the QiaQuick gel extraction kit (Qiagen AG, Basel, Switzerland) and cloned in the LigATor AT-cloning Vector pTAg (R&D Systems Europe Ltd., Abington, UK) according to the manufacturer's instructions. The selected positive plasmids were purified using the Qiagen Plasmid Mini kit (Qiagen AG), and sent for bidirectional sequencing to a private company with a certified sequencing error rate lower than 0.0078% (Microsynth GmBH, Balgach, Switzerland). Our internal reference

TABLE 1

Classification of Gamma Herpesvirus Isolates from Different Horses by PCR

Horse No.	Isolation from[a]	EHV-2 600 bp	EHV-2B 431 bp	EHV-5 251 bp	EHV-2R 257 bp
EHV-2 ATCC		+	−	−	−
EHV-2B (4)[b]		−	+	−	−
1	BAL	−	+	−	−
2	PBMC	+	−	−	−
3	PBMC	+	+	−	+
	BAL	+	+	+	+
5	PBMC	−	+	−	−
7	PBMC	+	−	−	−
8	PBMC	−	+	−	−
	BAL	+	−	−	+
10	PBMC	−	+	−	−
11	PBMC	−	+	−	−
12	PBMC	+	−	−	+
13	PBMC	+	−	−	+
14	PBMC	+	−	−	−
15	PBMC	+	−	−	+
16	PBMC	+	−	−	+
18	PBMC	+	+	−	+
29	PBMC	+	+	−	+
21	PBMC	+	−	−	
22	PBMC	+	+	+	+
23	PBMC	−	+	+	−
26	PBMC	+	−	+	−
	BAL	+	−	+	+
28	PBMC	+	−	−	−
	BAL	+	−	−	−
29	PBMC	+	−	−	+
31	PBMC	+	−	−	−
	BAL	−	+	+	−
32	PBMC	+	−	−	−
	BAL	−	+	+	−
33	BAL	+	−	−	+

[a] PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage cells.
[b] Plaque cloned EHV-2 producing in PCR a band of 431 bp.

strain was sequenced three times from three different cultures in both orientations. The sequences have been deposited in GenBank under Accession Nos. AF014391–AF014395.

RESULTS

Frequency of gamma herpesvirus isolation from blood and BAL

Twenty-four of 33 horses were virus positive (72%; Table 2). In Table 1 the isolation of gamma herpesviruses from BAL cells and PBMC of the 24 virus positive horses are documented. Virus was isolated from the blood of 22 horses (66.6%). BAL was performed in 22 of the 33 horses, and 8 (36.4%) were positive for gamma herpesvirus isolation. From the 22 blood-positive horses 6 were also positive in the BAL. Two horses were positive in the BAL but negative in the blood (Nos. 1 and 33).

Differentiation between gamma herpesviruses and identification of an EHV-2 variant

The results obtained are summarized in Table 2. Direct detection of gamma herpesviruses using DNA of PBMC purified from known positive horses was negative in 3 of 3 horses (Nos. 3, 8, 20). Thus the utility of PCR was restricted to the identification of virus type and variants in virus-positive material from cocultures. EHV-2 primers recognized EHV-2 (ATCC), producing a fragment of the correct length of 604 bp (Fig. 1, 1A). In our hands the EHV-2-R primers did not recognize the EHV-2 Plummer reference virus strain, but in 16 of the 34 isolates a product of the expected length of 257 bp was produced (Fig. 1, 2D). The PCR with our EHV-2 primers produced in some isolates a 431-bp fragment in addition to the characteristic 604-bp band (Figs. 1B and 2, 1B). The virus originating this shorter product was cloned by plaque purification from an isolate positive for all three products (horse 22). Subsequently five field isolates were found that were positive for the 431-bp band exclusively. These five isolates were not recognized by the EHV-2R primers. EHV-5 produced in EHV-5 positive isolates a product of the expected length of 217 bp (Fig. 1, 3C, 3D). With a combination of the EHV-2 and the EHV-5 primers all 34 virus isolates were identified. The PCR using capsid primers and interleukin 10-like gene primers recognized the EHV-2 reference and our variant but not EHV-5.

TABLE 2

Frequency of the Gamma Herpesviruses Isolates

	Isolates	Horses
N	34	33
Positive by virus isolation	34	24
Positive from PBMC	—	22
Positive from BAL	—	8/22[a]
PCR positive using EHV-2 and EHV-5 primers	34	24
PCR positive using EHV-2R and EHV-5 primers	21	15
PCR positive using EHV-2R but negative using EHV-2 primers	0	0
PCR positive with EHV-2R primers	16	12
PCR positive with EHV-2 primers (EHV-2/2B positive)	33	24
EHV-2 positive	25	19
EHV-5 positive	8	6
EHV-2B positive	16	12
EHV-2/5 positive	4	5
EHV-2/2B positive	7	7
EHV-5/2B positive	5	5
EHV-2/5/2B positive	2	4[b]

[a] BAL was performed in 22 of the 33 horses.
[b] In 2 horses the three viruses were isolated from distinct cultures.

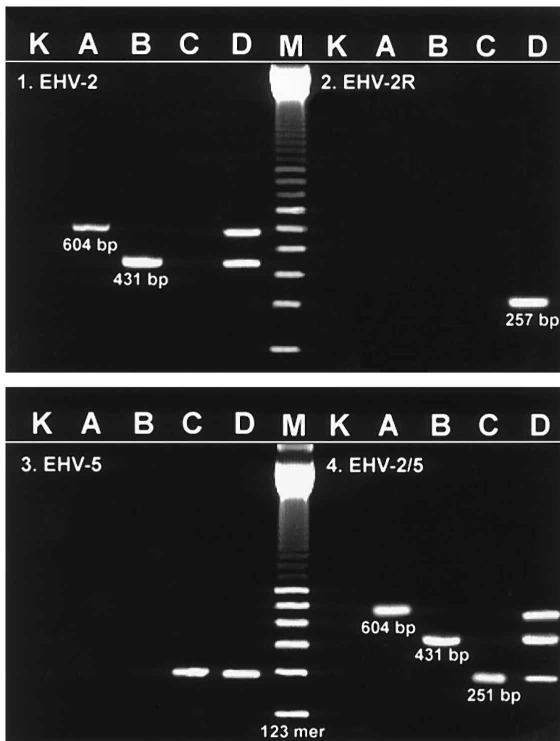

FIG. 1. PCR amplification using different oligonucleotide primers indicated in sectors 1–4 and different gamma herpesvirus targets: K, uninfected equine lung fibroblasts; A, EHV-2 ATCC reference strain; B, plaque cloned EHV-2 variant B(4), producing a shorter product; C, EHV-5 field isolate; D, field isolate from horse 22, containing all three gamma herpesvirus types; M, 123-bp DNA ladder (GIBCO BRL, Life Technologies AG, Basel, Switzerland).

The identified variant is a deletion/insertion mutant of EHV-2

The nucleotide sequence of the 431-bp amplification product was determined after cloning in a sequencing vector and was compared with the known sequences in the EMBL/GenBank data banks. The best score of sequence similarity was found within the EHV-2 sequence in the region expected to be amplified from the reference target. The sequence of the 431-bp PCR product obtained by amplification of our plaque-purified EHV-2 variant (No. 4) was aligned to the EHV-2 strain 86/67 sequence deposited in the GenBank data library under Accession No. U20824 (Telford *et al.*, 1995), introducing gaps to obtain an optimal alignment (bestfit). The sequence of the variant EHV-2 contains a deletion of 211 bp and two insertions of 37 and 4 bp, respectively (Fig. 3). The PCR product of four further EHV-2 variants directly amplified from positive cocultures from different horses at different times were cloned in the pTAg vector and sequenced bidirectionally using the M13 sequencing primers. These sequences showed a high degree of similarity with the plaque-purified reference variant strain No. 4. The alignment is shown in Fig. 2. We called this variant EHV-2B. The capsid PCR and the IL 10-like gene PCR with EHV-2 ATCC and EHV-2B produced fragments of the same length.

Frequency of the different gamma herpesvirus types

Repeated isolations from horses infected with more than one virus type always produced the same virus types, but sometimes one or two types failed to replicate in one or more cocultures. Virus isolation from the BAL cells was less efficient and required a longer incubation period than from blood (4–8 weeks vs 2–4 weeks). BAL cells and PBMC obtained from one horse on the same day differed sometimes in the gamma herpesvirus types they bore. EHV-2 was isolated more often from blood, whereas isolates from BAL contained the three virus types at a similar frequency (Table 3).

DISCUSSION

The pathogenic effect of equine gamma herpesviruses is still unclear. Gamma herpesviruses can be isolated from healthy horses but conjunctivitis, upper respiratory tract diseases, lower respiratory tract disease in foals, poor performance, malaise (Browning and Studdert, 1988; Belak *et al.*, 1980; Palfi *et al.*, 1978), and chronic lung disease (Schlocker *et al.*, 1995) have been associated with infection. The possibility of distinguishing between different virus types by PCR allows us to investigate whether pathogenicity or tropism are associated with one or more particular types/subtypes different from those isolated from healthy horses.

Considering that approximately 75% of the horse population is infected with gamma herpesviruses (Schlocker *et al.*, 1995; Kemeny and Pearson, 1970; Roeder and Scott, 1975), the reliability of the test used is very important in order to identify the true virus negative horses and to compare them with the infected population.

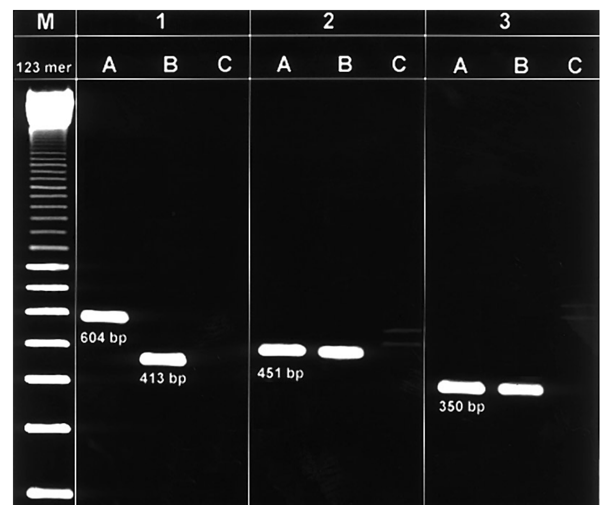

FIG. 2. PCR amplification of EHV-2 ATCC reference strain (A), EHV-2B (B), and EHV-5 (C) using different primers: 1, EHV-2 primers; 2, EHV-2 capsid primers; and 3, EHV-2 interleukin 10-like gene primers.

FIG. 3. Sequence alignment of the PCR products obtained from our plaque cloned variant EHV-2B No. 4 (4) and from 4 primary EHV-2B isolates (55, 283, 293, 355) to the EHV-2 strain 86/67 reference sequence (Telford *et al.*, 1995).

The low frequency of gamma herpesvirus-infected horses determined by direct PCR by others (Reubel *et al.*, 1995) contrasts with the high frequency of gamma herpesvirus isolations in present and other investigations and may be explained by different factors: (1) a geographically variable incidence of the infection; (2) the number of infected cells in peripheral blood may be below the detection limit of PCR (between 0.05 and 25 infectious cells per 10^6 PBMC) (Gleeson and Coggins, 1985); and (3) the described primers are specific for a subpopulation of gamma herpesviruses (in our study only 16 of 34 EHV-2- or EHV-5-positive isolates were positive with the EHV-2R primers and 21 of 34 were positive with the EHV-2R or EHV-5 primers). We conclude that the most reliable method to detect gamma herpesvirus-infected horses is the cocultivation of PBMC with equine fibroblasts. Thereafter PCR may be used for the unequivocal identification of the different types. In only some cases were positive results obtained with the PCR after 1 week of cocultivation (data not shown), whereas the infection was morphologically recognized after 2 weeks. Thus the strategy of an early detection of a gamma herpesvirus infection by PCR after 1 week of cocultivation is much less sensitive than the detection of virus after cocultivation and morphological identification.

TABLE 3

Relation between Viral Source and Gamma Herpesvirus Type

Source	*N*	EHV-2	EHV-2B	EHV-5
PBMC	24	19	10	3
BAL	10	6	5	5

The lower frequency of virus isolation from BAL cells contrasted with the high frequency of isolation from lung lymph nodes found by other authors (Edington *et al.*, 1994). This was probably due not to the absence of virus from BAL cells but to the activation of latent microbial spores present in lung macrophages during cocultivation. During cocultivation of BAL cells 11 of the 22 cultures were contaminated by fungal growth before the

time necessary to consider an isolation attempt as negative was over. Our results do not show an exclusive tropism of one virus type for blood or BAL cells but the predominant virus isolated from PBMC was EHV-2. In the BAL cells the three viruses were isolated with the same frequency, indicating a tendency of EHV-2 to be more hemotropic and EHV-5 and 2B to be more pneumotropic. The confirmation of this hypothesis is made difficult by the fact that fungal contamination due to phagocytosed fungal spores reduced substantially the isolation efficiency from BAL samples (antifungal agents were not added because of the observed inhibition of viral replication as a consequence of a toxic effect on the cells).

Our study demonstrates the importance of a reliable diagnostic procedure for the identification of gamma herpesvirus carriers in the horse population and proposes a combination of different PCR oligonucleotide primers in order to identify all the isolates that can be obtained after cocultivation of equine blood or BAL cells with indicator cell cultures. This primer combination recognized all our isolates, produced fragments of three different lengths, and revealed the existence of a frequent gamma herpesvirus deletion/insertion mutant distinct from EHV-2 and EHV-5. Small differences in the sequences of herpesviruses may be responsible for different biological properties. For example, differences in the amplification products of equine herpesvirus types 1 (EHV-1) and 4 made it possible to distinguish them in order to evaluate the risk of abortion and paralysis associated with EHV-1 (Wagner *et al.,* 1992; Kirisawa *et al.,* 1993). The isolation of a mutant of EHV-2 and the fact that the EHV-2R primers recognized only one part of our field isolates indicates that gamma herpesviruses are genetically more heterogeneous than previously known.

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