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Latent Herpesvirus Infection in Red Deer: Characterization of a Specific Deer Herpervirus Including Comparison of Genomic Restriction Fragment Patterns

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Rønsholt, L., L. Siig Christensen and V. Bitsch: Latent herpesvirus infection in red deer: Characterization of a specific deer herpesvirus including comparison of genomic restriction fragment patterns. Acta vet. scand. 1987, 28, 23–31. – Glucocorticoid treatment of imported red deer (Cervus elaphus), seropositive to Infectious Bovine Rhinotracheitis (IBR) virus, reactivated a latent herpesvirus infection, which was transmitted to a seronegative deer with a fatal outcome. However the virus did not spread to cattle housed in close contact with the infected deer, and serological indication og infection in the cattle was observed only on direct nasal installation of virus. The virus isolate had characteristics in common with other Alpha herpesviruses and especially the Bovid Herpesvirus type 1 (BHV-1) but distinguished itself from the latter by its host specificity, serological reaction and genomic restriction fragment pattern (RFP). The host specific red deer herpesvirus was tentatively designated Cervid Herpesvirus type 1 (CHV-1). It was concluded that CHV-1 seropositive deer can be a threat to red deer farming, while in cattle the infection may only cause minor inconvenience through interference with the serological IBR diagnosis.

red deer herpesvirus; CHV-1; latent infection; genomic restriction fragment pattern; RFP; cattle.

Introduction

Antobodies against Bovid herpesvirus 1 (BHV-1, IBR/IPV virus) are often found in deer (*Chow & Davis* 1964) and especially in the red deer Cervus elaphus (*Lawman et al.* 1978). Recently, these seroreactions were observed in animals with clinical symptoms of ocular disease, such as conjunctivitis, corneal lesions besides affection of the upper respiratory tract (*Inglish et al.* 1983, *Nettleton et al.* 1986, *Reid et al.* 1986). Only very young just weaned animals seemed to be affected, and the clinical symptoms resembled those of the earlier reported milder outbreaks of IBR among cattle. However, the virus isolated differed serologically from the BHV-1 virus, and it was suggested that the deer herpes virus is a distinct pathogen of deer (*Reid et al.* 1986). Due to a great interest in deer farming, there has been an increasing international trade with red deer. Animals seropositive to herpesvirus could carry a latent infection, which might be transmitted to cattle and thereby interfere

with the serological diagnosis of IBR. It was the intention of the present investigation to reactivate such a possible latent herpesvirus infection in seropositive red deer, to characterize the topical virus and follow the result of an exposure of cattle to the virus.

Materials and methods

Animals, clinical surveillance and sampling procedures

Three newly imported one-year-old red deer hinds were housed indoor without any restraint together with 2 one-year-old heifers in a pen covering an area of 18 m^2. Before installation 2 of the hinds (I & II, Tab. 1) had very low neutralizing antibody titers against BHV-1 virus, while the third one (III) and the two heifers (A & B) were seronegative. From the day after arrival (Day 1, Fig. 1) and the following 11 days all 3 deer were treated intramuscularly with 50 mg of a synthetic glucocorticoid (Prednisolon, DAK). Clinical observations including body temperatures were recorded daily. For the purpose of virus isolation, secretions from the nose and vagina were collected 8 times from each animal with intervals of 1 or 2 days during the first 2 weeks.

Sampling was done by means of tampons, which after application in the nasal cavity and the vagina for 15 min were soaked in cell culture medium (2 ml) supplemented with antibiotics (10 times the usual concentration used for cell culture) and 2% calf serum free from antibodies against IBR virus. With a disposable syringe the contents were squeezed into a tube for immediate inoculation of cell cultures, or storage at –80°C until use. Serum samples were collected with intervals of 4–6 days and heated at 56°C for 1/2 h to inactivate complement. On day 36 the 2 contact heifers were challenged by intranasal instillation of $10^{7.5}$ $TCID_{50}$ of the strain isolated from one of the hinds. The cattle were surveilled clinically and serologically for the next 3 weeks according to the procedure described above. Virus isolation was attempted daily during the first 3 days after challenge and then every second day until Day 11.

Cell cultures, isolation and propagation of virus

Samples (1 ml) were adsorbed for 3 h at 37°C to monolayers of primary or secondary calf kidney cells grown in roller tubes. The cell cultures were washed once and supplied with maintenance medium consisting of Eagles MEM, 2 % calf serum and antibiotics (100 i.u. of penicillin, 100 ug of streptomycin sulphate, 50 ug of neomycin and 50 i.u. of mycostatin). Monolayers were observed the following 4 days. If no cytopathogenic effect occurred, the cell culture was harvested by a single freeze/thaw procedure, and 0.2 ml of the material served as inoculum for a new passage in cell culture. Cultures infected with isolates were prepared on coverslips in Leighton tubes for verification of infection by specific immunofluorescence staining technique.

Virus for restriction fragment pattern (RFP) analysis was propagated in 1 l roller bottles with confluent monolayers of primary bovine kidney cells (deer virus & BHV-1) and porcine kidney cells (Suid herpesvirus type 1, SHV-1) established with Hanks medium, 10 % bovine fetal serum and antibiotics. Before inoculation of cultures with seed virus (0.1–1.0 m.o.i.) the medium was replaced by 40 ml medium without serum. Cultures were harvested after 48 h at 37°C.

Virus strains

One virus isolate from deer II (W70/24) was purified by limiting dilution and passaged 3 times to produce a virus stock. Strains of BHV-1 (Brørup 82/SVSL) and SHV-1

(3275/81-/SVIVR) both isolated from severe acute outbreaks of disease served as reference strains in the RFP analysis.

Indirect immunofluorescence
Cultures infected with herpesvirus from deer were fixed in acetone followed by incubation (1/2 h 37°C) with rabbit hyperimmune serum against the BHV-1 strain (dil. 1:200 in PBS) and stained with fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (Dakopatts). In addition, cell cultures infected with the deer isolate or the BHV-1 strain respectively were used for a 2-fold titration of the rabbit hyperimmune serum in an immunofluorescence antibody test (IFAT) in order to estimate the level of serological cross reaction between the 2 strains.

Serum neutralisation (SN) test
The test was performed by means of the micro-neutralization system using 4 wells per 2-fold serum dilution (*Bitsch* 1978). Briefly serum dilutions and 100 $TCID_{50}$ of deer herpes virus or BHV-1 virus in equal amounts were incubated for 24 h at 37°C before a suspension of calf kidney cells was added. Microplates were incubated at 37°C for at least 5 days before reading. The neutralizing titre was recorded as the reciprocal of the highest serum dilution that inhibited cytopathogenicity.

Blocking ELISA
This test was carried out as the routine test during the present Danish IBR eradication campaign. Briefly the test included coating of microplates (Nunc immuno II) overnight at 22°C with BHV-1 virus diluted in carbonate buffer. Incubation with undiluted serum sample overnight at 22°C. Demonstration of unblocked antigen (Ag) was done by reaction with peroxydase-conjugated cattle anti-BHV-1 IgG diluted in neutral phosphate buffer with 0.05 % Tween 20 for 1 h at 22°C. Substrate consisted of OPD in citrate buffer with hydrogen peroxide. Each step was followed by a washing procedure with PBS and Tween 20. The results were read photometrically. Positive samples included sera which inhibited the reaction ≥ 50 %, while a blocking effect of 40–49 % was recognized as a weak or suspicious reaction.

Purification of virus DNA for RFP analysis
Harvests from cell cultures each infected with 1 of the 3 herpesvirus strains were clarified by low-speed centrifugation, and virions were pelleted by centrifugation for 2 h at 14000 rpm and 5°C (Beckman, rotor JA 20). The pellet was resuspended in 5 ml 0.5 % N-lauryl sarcosine, 10 mmol/l EDTA, 100 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 8.0 and kept on ice-bath for 10 min. The lysed virions were treated thrice with proteinase K (Boehringer, Mannheim, FRG) each time at a final concentration of 200 ug/ml for 1/2 h at 37°C. The resulting solution was subsequently extracted twice with equal volumes of phenol and chloroform. The supernatant was finally mixed with 2 volumes of 96 % ethanol containing 0.4 % LiCl w/v. High molecular weight virus DNA was collected on a bended glassrod and rinsed in ice-cold 80 % ethanol, air dried and dissolved in 0.5 ml TE buffer (10 mmol/l Tris-HCl, 1 mmol EDTA, pH 8.0).

RFP analysis
Restriction enzymes (RE: BamHI, BclII, BglII, KpnI, XhoI) were obtained from Anglian Biotechnology, Ltd. and applied according to the recommendations of the manufacturer. Purified virus DNA (1.5 ug) was digested with the different RE and electrophoresed overnight in a 0.6 % agarose gel with TBE buffer (89 mmol/l Tris, 89 mmol/l boric acid, 2 mmol/l EDTA) at

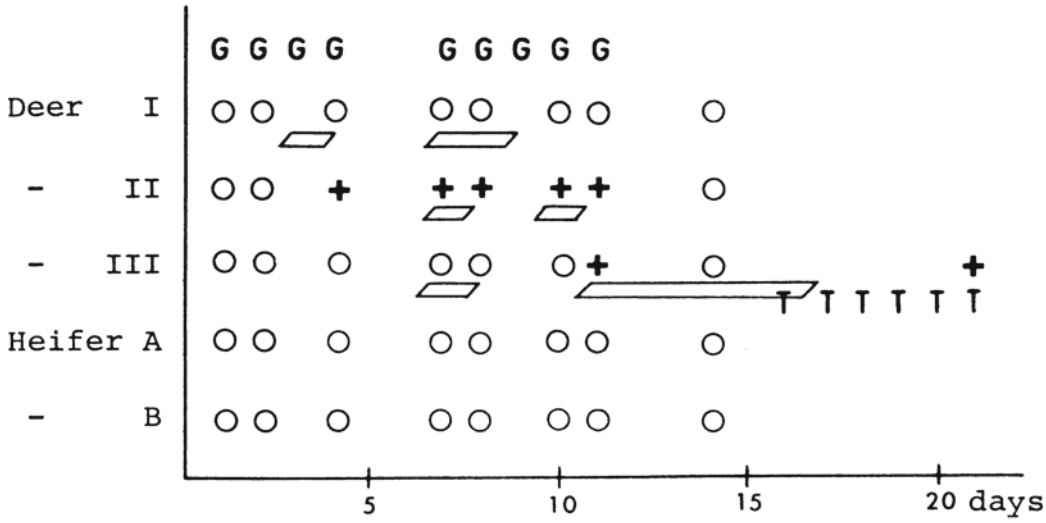

Figure 1. Clinical scores and results of virus isolation attempts from red deer (I, II & III) and cattle (A & B) related to days after being housed together. (▱): Rectal temperatures above 40°C (red deer) and above 39°C (cattle); (T): Tachnypnoea; (+/0): Positive/negative virus isolation results; (G) Glucocorticoid treatment of the red deer.

3V/cm. The DNA bands were stained with ethidium bromide, and the gel was UV-illuminated (302 nm) and photographed with a Polaroid MP 4 camera.

Results

Activation of infection in deer

Following the initiation of glucocorticoid treatment the body temperatures of the two BHV-1 seropositive red deer (I & II, Fig. 1) temporarily raised above 40°C on Day 3 and between Day 7 and 10 (Fig. 1). The seronegative hind III had a similar fever attack on Day 7 followed by a constant pyrexic period from Day 11 culminating on Day 16 with a temperature of 41.4°C. Probably due to antibiotic treatment (Streptocillin, DAK) the temperature declined to 39.3°C the following day. From Day 16 the animal was obviously weakened and had besides little serous nasal discharge, tachypnoea with a respiratory rate of 120 per min. The animal died on Day 21.

Except for deer III, no clinical symptoms could be registered neither in the other deer slaughtered at Day 36, nor in the cattle which were observed for a further 3-week-period during the direct challenge experiment.

On necropsy deer III macroscopically showed diffuse, moderate erythematous areas of the tracheal mucosa, while the lungs had disseminated small (1–3 mm) consolidations and petecchial haemorrhages besides a few larger (1–2 cm) greenish abscesses. The pleural cavity contained blood and there was a fibrinous to fibrous pleuritis and pericarditis. Apart from splenomegaly all other organs were macroscopically normal. Deer I & II and the cattle slaughtered later in the experiment showed no post mortem abnormalities.

From pools of nasal and vaginal secretions

of 1 of the glucocorticoid treated, herpesvirus seropositive hinds (Deer II, Fig. 1) it was possible to isolate a cytopathogenic virus during a period of at least 1 week (Day 4–11). From the secretions of the seronegative hind (Deer III) virus was only isolated on Day 11 and 21, however only 1 attempt was made during the period in between. On necropsy virus was also isolated from the tracheal mucosa and from lung material. No virus infection could be demonstrated in deer I or in the 2 contact heifers.

Serological identification of virus isolate

When virus was isolated the cytopathogenic changes were generally seen during the first 4 days of the primary passage. On subpassage the typical cytopathogenic effect exhibited by other type 1 suid-, equid- and bovid herpervirus, i. e. fast progressive cell rounding and detachment of individual cells in plaques, was already observed within 24 h. Immunofluorescent staining of infected cultures at that stage with hyperimmune serum against BHV-1 virus demonstrated bright fluorescence mainly of membrane and cytoplasm with masked intranuclear staining. Comparative titration of hyperimmune BHV-1 serum against cultures infected with homologous virus or the red deer virus isolate showed a 4-fold decrease of titre against the latter.

RFP analysis of virus isolate

Purified DNA from the red deer herpesvirus, SHV-1 and BHV-1 was digested with 5 different restriction enzymes for comparisons of the RFPs generated. From Fig. 2 it appears that the RFPs of all 3 virus strains were different, although similarities in band migration did appear. The similarities are indicated by arrows and illustrated schematically below the photographic representation of the gels. These similarities are obvious for the digest obtained with BamHI (Fig. 2a) where 2 fragments of the red deer herpesvirus genome comigrate with 2 BHV-1 fragments and the largest one of them comigrates with 1 SHV-1 fragment. The similarities are especially distinct for the XhoI RFPs (Fig. 2, e1), where the red deer virus share a triplet of bands with the BHV-1 fragment pattern. The nethermost band of the triplet is however obscured by a double band. The 2 uppermost bands of the red deer triplet are also shared with the RFP of SHV-1.

Furthermore 2 smaller bands of the red deer herpesvirus comigrate with SHV-1 bands. The XhoI RFPs of BHV-1 and SHV-1 are aligned in Fig. 2, e2 to show the numerous similar bands in the RFPs of these 2 genomes expressed especially with this restriction enzyme. It appears from the digest with XhoI that the RFPs of the red deer herpesvirus differ more from those of SHV-1 and BHV-1, than the 2 latter differ from each other.

Challenge of cattle with virus isolate

Following direct intranasal inoculation of the cattle with the red deer herpesvirus isolate, virus could only be reisolated from 1 heifer 1 day after challenge and not during the following 11 days.

Serological response of animals

Among the red deer the seronegative animal (III, Table 1) during the observation period never managed to produce an immune response against the herpesvirus to which it had been exposed. On the other hand deer I and II, which were suspected to be latently infected, demonstrated a significant titre increase in the SN test 25 days and 8–11 days, respectively, after initiation of glucocorticoid treatment. There was serological cross

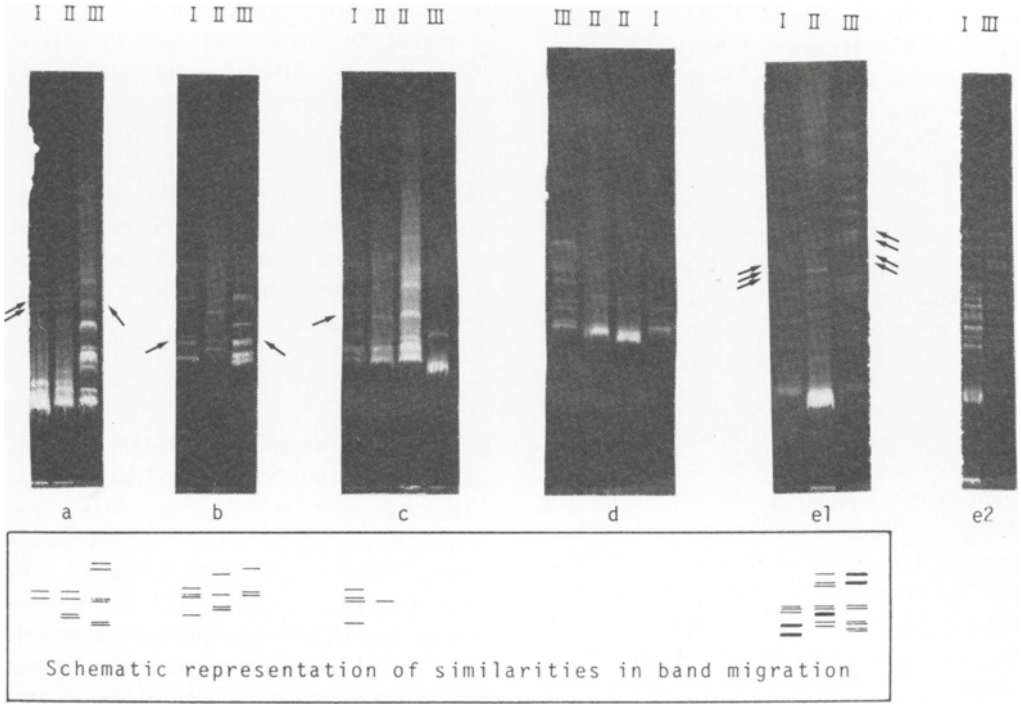

Figure 2. Restriction fragment patterns of genomic DNA from BHV-1 (I), CHV-1 (II) and SHV-1 (III), digested with the restriction enzymes BamHI (a), BclI (b), BglII (c), KpnI (d) and XhoI(e). Digest of duplicate preparations of DNA drom CHV-1 have been included in the BglII and KpnI digest. Arrows indicate bands of BHV-1 and SHV-1 comigrating with bands of the red deer virus.

reaction but no identity between the 2 viruses employed in the SN test. The SN titres were 5–38 fold higher with the homologous virus than with BHV-1. The difference was especially pronounced at high levels of antibodies.

Among the cattle no serological response was detected during the 5-week-period of contact exposure. However, following nasal instillation of the virus isolate low serological titres to the homologous virus were noted during the next 2 weeks.

The sera from deer (Day 25) and cattle (Day 58), containing the highest level of antibodies according to the SN test, were also tested in the routine blocking ELISA against the BHV-1 virus. All sera reacted in the test, a response which was significant for the sera from deer (80 % inhibition), while the cattle sera only caused 40 % inhibition, which fell within the range of weak or suspicious reactions.

Discussion

As previously demonstrated with herpesvirus infection in cattle (*Bitsch* 1973), the present study confirmed, that BHV-1 seropositive red deer may carry a latent herpesvirus infection, which can be reactivated in a stress situation. Despite the failure to isolate virus from 1 of the hinds, there was serologi-

Table 1. Development of virus neutralizing antibodies in red deer (I, II & III) and cattle (A & B) against the red deer herpesvirus strain (CHV) and BHV-1 virus – days after initiation of glucocorticoid treatment of the deer.

	Day	1	4	8	11**	14	17	21	25	36***	42	46	49	58
Deer I	CHV	76*	76	76	91	76	108	256	304					
	BHV	8	11	14	11	19	11	23	45					
Deer II	CHV	91	91	181	1447	4096	4096	>4096	4096					
	BHV	14	11	16	64	108	108	108	108					
Deer III	CHV	<1	<1	<1	<1	<1	<1	<1						
	BHV	<1	<1	<1	<1	<1	<1	<1						
Heifer A	CHV	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	2	5	6
	BHV	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Heifer B	CHV	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	2	4
	BHV	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

* : Reciprocal value of serum dilution inhibiting cytopathogenicity of 100 $TCID_{50}$.
** : Last day of glucocorticoid treatment of the deer.
***: Day for direct virus challenge of cattle.

cal evidence for virus propagation in both seropositive animals.

The close serological relationship between the isolate and BHV-1 in IFAT excluded the possibility that the red deer virus belonged to any other known bovine herpesvirus type (*Osorio et al.* 1985). In addition its biological behaviour in vitro i.e. nonrestrictive cell culture conditions rejected the isolate as a BHV-2 candidate (*O'Connor* 1985). Also the fast replication cycle and cytopathogenic pattern, characteristic for the bovine type 1 herpesvirus, and the diffuse immunofluorescence picture without stained intranuclear inclusions excluded the red deer virus from the remaining known bovine herpesvirus types (*Osorio et al.* 1985, *Storz et al.* 1984). On the other hand the serological difference observed between the BHV-1 and the red deer isolate in IFAT and the SN test pointed at a specific Alpha herpesvirus strain of red deer.

This was also supported by the RFPs of the isolate. However, the well-documented genomic variation within the species of the Alpha herpesvirus group and the genetic instability, which has been documented for several species of this group, necessitated that interpretation from RFP analysis should be done with great care and on the basis of proper reference material. For this reason we compared the RFP of the red deer herpesvirus isolate with the RFPs of both BHV-1 and SHV-1. The latter virus represents a genus, which is morphologically, biochemically and partly serologically related to BHV-1 and yet their nucleotide sequences are only approximately 8 % homologous (*Bush & Pritchett* 1985). Attempts to differentiate between genital and respiratory isolates of BHV-1 by comparisons of RFPs have so far been contradictory (*Gregersen et al.* 1985, *Seal et al.* 1985), and IBR and IPV virus were often shown to be more than 95 % homologous (*Seal et al.* 1985). We therefore only included 1 isolate of BHV-1 in our comparisons.

The RFPs of different bovine herpesvirus types differ extremely (*Osorio et al.* 1985). Likewise, from the experience with more

than fifty Danish isolates of SHV-1 we have observed a great RFP variation within this type (*Siig Christensen,* unpublished results). In spite of this, significant similarities in band migration appeared between all three viruses employed in the present investigation. This clearly supports the relationship of the red deer herpesvirus to the Alpha herpesvirus group.

By aligning the RFPs of SHV-1, BHV-1 and the red deer virus, from digestion with Xho 1 it seems that the 2 former viruses may be more related to each other than the red deer virus is related to any of them. However, in accordance with the great variation in the RFPs of the different SHV-1 isolates, the apparently more pronounced similarity between SHV-1 and BHV-1 may be a coincidence and not significant. Apart from the serological results, the predominant overall picture in the RFPs of the three viruses lead to the conclusion, that the isolate from red deer is a unique herpesvirus, as it was also recently proposed by *Nettleton et al.* (1986) and *Reid et al.* (1986). It is therefore suggested that the virus be designated Cervid Herpesvirus type 1 (CHV-1) in accordance with the systematic classification and nomenclature of viruses (*Matthews* 1982).

Reactivation of the latent CHV-1 infection caused a temperature rise, and the virus spread to the seronegative hind, where it could be demonstrated in the upper respiratory tract and lungs leading to fatal pneumonia.

Although infection with an IBR virus strain in deer has previously been established (*Chow & Davis* 1964), other IBR virus strains do not seem to infect the red deer even folloving experimental inoculation (*Reid et al.* 1986). On the other hand, this study indicate, that the cattle are likewise not susceptible to the topical CHV-1 isolate, except in the extreme experimental situation, where intranasal instillation of the deer virus induced a weak immune response. The host specificity thus also classified the CHV-1 strain as a distinct strain of deer.

The provoked immune response in cattle cross reacted only weakly with IBR virus in the blocking ELISA, and this could indicate that the CHV infection of red deer may only cause minor inconvenience for IBR virus serological surveys in cattle.

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Sammendrag

Latent herpesvirus infektion hos kronhjort: Karakterisering af et specifikt hjorte herpesvirus underbygget ved sammenligning af genom restriktionsfragmentmønstre.

Glycocorticoid behandling af importerede krondyr (Cervus elaphus), der var seropositive overfor Infektiøs Bovin Rhinotracheitis (IBR) virus inducerede en opblussen af en latent herpesvirus infektion med efterfølgende smittespredning til et seronegativt krondyr. Imidlertid spredtes infektionen ikke til kvæg opstaldet i tæt kontakt med disse dyr, og kun ved direkte podning kunne virus til en vis grad etablere sig. Den isolerede virusstamme havde egenskaber tilfælles med andre Alpha herpesvirus og specielt Bovin Herpesvirus type 1 (BHV-1), men adskilte sig fra sidstnævnte ved værtsspecificitet, serologisk reaktion og ved restriktionsfragment mønsteret af virusgenomet. Dette specifikke type 1 herpesvirus foreslås betegnet Cervid Herpesvirus type 1 (CHV-1). Det kan konkluderes, at selvom CHV-1 seropositive krondyr kan give anledning til problemer i dyrefarme, vil infektionen sandsynligvis kun skabe mindre problemer hos kvæg i forbindelse med en aktuel serologisk undersøgelse for IBR.

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