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Dissertação de Mestrado

DISTRIBUIÇÃO DO DNA DOS HERPESVÍRUS BOVINO TIPOS 1 (BHV-1) E 5 (BHV-5) NO ENCÉFALO DE COELHOS DURANTE A INFECÇÃO LATENTE

Sandra Vanderli Mayer

PPGMV

Santa Maria, RS, Brasil

2005

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por

Sandra Vanderli Mayer

Dissertação apresentada ao Programa de Pós-graduação em Medicina Veterinária, Área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM/RS), como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-graduação em Medicina Veterinária

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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Elaborada por
Sandra Vanderli Mayer

como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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Luizinho Caron

Santa Maria, 21 de março de 2005.

Mayer, Sandra Vanderli, 1974-
M468d

Distribuição do DNA dos herpesvírus bovino tipo i (BHV-1) e 5 (BHV-5) no encéfalo de coelhos durante a infecção latente / por Sandra Vanderli Mayer ; orientador Eduardo Furtado Flores. – Santa Maria, 2005.
39 f . ; il.

Dissertação (mestrado) – Universidade Federal de Santa Maria, 2005.

1. Medicina veterinária 2. Herpesvírus bovino 3. Infecção latente 4. Coelhos I. Flores, Eduardo Furtado, orient. II. Título

CDU: 619:636.92

Ficha catalográfica elaborada por
Luiz Marchiotti Fernandes – CRB 10/1160
Biblioteca Setorial do Centro de Ciências Rurais/UFSM

AGRADECIMENTOS

Ao Professor Eduardo Furtado Flores, pela orientação, ensinamentos, disponibilidade e apoio demonstrados na execução desse trabalho;

Ao Professor Rudi Weiblen, pela colaboração e apoio demonstrados durante o convívio;

À Fernanda Silveira Flores Vogel pela ajuda na elaboração desse trabalho e também pela amizade de todos esses anos.

Ao meu esposo Evandro Reinoldo Winkelmann, pelo incentivo, ajuda em todos os momentos, amor e compreensão sempre demonstrados;

A minha irmã Vânia Mayer Steckel e ao meu cunhado Edegar Steckel pelo incentivo e apoio prestados;

A minha irmã Shawana Stephanie Mayer pela colaboração e constante presença;

Aos colegas e amigos do laboratório, em especial Valter Leonardo de Quadros pela ajuda na execução dos experimentos e Fernando Groff pelo companheirismo prestados;

A todos os mestrandos do Setor de Virologia não citados aqui e bolsistas de iniciação científica que de alguma forma contribuíram para a execução dos experimentos;

À professora Ione Denardin e bolsistas do setor de cuniculura do CASM pelo cuidado aos animais durante o experimento.

Ao CNPq pelo fornecimento de bolsas e ao MCT/CNPq/CAPES/Finep pelo apoio financeiro que viabilizaram esse trabalho.

A Universidade Federal de Santa Maria e ao Programa de Pós-graduação em Medicina Veterinária pela formação acadêmica e científica.

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DISTRIBUIÇÃO DO DNA DOS HERPESVÍRUS BOVINO TIPOS 1 (BHV-1) E 5 (BHV-5) NO ENCÉFALO DE COELHOS DURANTE A INFECÇÃO LATENTE

AUTORA: SANDRA VANDERLI MAYER
ORIENTADOR: EDUARDO FURTADO FLORES
Santa Maria, 21 de março de 2005.

O herpesvírus bovino tipo 5 (BHV-5) é um importante agente etiológico de meningoencefalite em bovinos e estabelece infecção latente em seus hospedeiros, principalmente nos gânglios dos nervos sensoriais. No entanto, a colonização de áreas profundas do cérebro com DNA viral pode ter implicações importantes na patogenia da infecção pelo BHV-5 após a reativação da infecção latente. Neste estudo, foi investigada a distribuição do DNA do BHV-5 no cérebro de coelhos infectados experimentalmente, antes e após a reativação da infecção latente, utilizando um nested-PCR para uma seqüência do gene da glicoproteína B. Dezoito coelhos infectados pela via intranasal com um isolado brasileiro de BHV-5 foram divididos em dois grupos: coelhos do grupo A (n=8) foram submetidos a eutanásia 60 dias pós inoculação (pi) para a coleta de tecidos; animais do grupo B (n=7) foram submetidos a administração de dexametasona no dia 60 pi. Para comparação foram utilizados dois grupos de coelhos inoculados com o BHV-1 (C, n=3 e D, n=3), cada grupo sendo submetido a um dos tratamentos acima, respectivamente. Nos animais do grupo A, o DNA viral foi consistentemente detectado no gânglio trigêmeo (8/8), freqüentemente no cerebelo (6/8), com menor freqüência na ponte e córtex anterior (3/8) e ocasionalmente no tálamo (2/8) e córtices ventro-lateral, dorso-lateral e posterior, pedúnculo cerebral e tálamo (1/8). Nos animais previamente submetidos à reativação, a distribuição do DNA viral foi mais ampla, sendo detectado mais consistentemente, além do TG (7/7), nos córtices ventro-lateral (6/7), e posterior (5/7), ponte e tálamo (4/7) e menos freqüentemente no pedúnculo cerebral (3/7). Em contrapartida, os animais inoculados com o BHV-1 apresentaram o DNA viral latente em poucos tecidos além do TG, e não apresentaram alterações importantes na distribuição do DNA viral após a reativação. Esses resultados demonstraram que o DNA latente do BHV-5 - e não o do BHV-1 - pode estar presente em várias áreas do cérebro de

coelhos infectados experimentalmente. A reativação induzida por dexametasona resulta na reativação viral e provavelmente na colonização de áreas adicionais no cérebro. Com base nesses resultados pode-se especular que a reativação do DNA viral latente nestas regiões pode contribuir para a recrudescência de doença neurológica observada após a reativação da infecção latente pelo BHV-5.

Palavras-chave: herpesvírus bovino tipos 1 e 5, BHV-5, BHV-1, infecção latente, coelhos.

ABSTRACT

Master's Dissertation
Programa de Pós-graduação em Medicina Veterinária
Universidade Federal de Santa Maria

DISTRIBUTION OF BOVINE HERPESVIRUS TYPES 1 (BHV-1) AND 5 (BHV-5) DNA IN THE BRAIN OF RABBITS DURING LATENT INFECTION

AUTHOR: SANDRA VANDERLI MAYER
ADVISER: EDUARDO FURTADO FLORES
Santa Maria, March, 21st , 2005.

Bovine herpesvirus type 5 (BHV-5) is a major etiologic agent of meningo-encephalitis in cattle and establishes lifelong latent infection in trigeminal ganglia and also in other areas of the brain. Colonization of deep areas of the brain with latent viral DNA may have important implications on the pathogenesis of BHV-5 neurological infection upon reactivation. In this study, we investigated the distribution of BHV-5 DNA in the brain of experimentally infected rabbits – a laboratory model for BHV-5 infection - prior and subsequently to virus reactivation, using a nested PCR for the glycoprotein B gene. Eighteen rabbits inoculated intranasally with a Brazilian BHV-5 isolate were divided in two groups: group A rabbits (n=8) were euthanized 60 days post-inoculation (pi) for tissue collection; group B (n=7) were submitted to dexamethasone administration at day 60pi for reactivation of latent infection and euthanized for tissue collection 60 days later. To compare, we used two groups of BHV-1-infected rabbits (C, n=3 and D, n=3), each group being submitted to one of the above treatments, respectively. In group A rabbits, viral DNA was consistently detected in trigeminal ganglia (8/8), frequently in cerebellum (6/8), anterior cortex, pons medulla (3/8) and only occasionally in thalamus (2/8), ventro-lateral, dorsal and posterior cortices, midbrain (1/8). In rabbits previously submitted to virus reactivation, viral DNA showed a broader distribution, being detected more frequently – besides the TG (7/7) - in ventro-lateral (6/7) and posterior cortices (5/7), pons-medulla and thalamus (4/7) and midbrain (3/7). In contrast, rabbits inoculated with BHV-1 harbored latent viral DNA in a few tissues in addition to TG and did not show significant changes in distribution of viral DNA post-reactivation. These results demonstrate that latency by BHV-5 DNA – and not BHV-1 DNA – may be established in several areas of the brain of experimentally infected rabbits. Further, dexamethasone-induced virus reactivation is followed by a wider distribution of latent viral DNA, probably due to virus dissemination from the original sites. Thus, it is reasonable to

speculate that reactivation of latent infection from deep areas of the brain may contribute to the recrudescence of neurological disease frequently observed upon reactivation of latent BHV-5 infection.

Key words**:** *bovine herpesvirus type 5, BHV-5, BHV-1, latent infection, rabbits.*

1. INTRODUÇÃO

Os herpesvírus bovino tipos 1 (BHV-1) e 5 (BHV-5) são *Alphaherpesvirus* estreitamente relacionados entre si em vários aspectos biológicos, estruturais, antigênicos e moleculares, porém apresentam uma patogenia distinta. O BHV-1 está associado à doença respiratória (rinotraqueíte infecciosa bovina, IBR) e/ou genital (vulvovaginite/balanopostite pustular infecciosa, IPV/IBP), enquanto o BHV-5 é o agente etiológico de meningoencefalite de curso geralmente fatal (ROIZMAN et al., 1992). Após a infecção primária, tanto o BHV-1 quanto o BHV-5 estabelecem infecções latentes, principalmente em neurônios de gânglios sensoriais e autonômicos (ROCK, 1994; VOGEL et al., 2003). A infecção latente pode ser reativada experimentalmente em virtualmente todos os animais infectados pelo BHV-1 através da administração de dexametasona (Dx) (ROCK, 1994). A capacidade de estabelecer e reativar infecções latentes constitui-se no ponto chave da epidemiologia e patogenia dessas infecções e contribui para a perpetuação dos *Alphaherpesvirus* na natureza (PASTORET & THIRY, 1985; ROCK, 1994).

Embora já tenha sido demonstrado que tanto o BHV-1 quanto o BHV-5 estabelecem infecção latente nos seus hospedeiros, diferenças importantes na região do gene relacionado à latência (LAT/LTR) entre o BHV-1 e BHV-5 sugerem que esses vírus possam diferir na capacidade de estabelecer e reativar a infecção latente (DELHON et al., 2003; VOGEL et al., 2003; 2004).

Após infecção ocular ou oronasal, o BHV-1 estabelece latência nos neurônios sensoriais do gânglio trigêmeo (TG). Entretanto, quando a infecção é genital, a latência é estabelecida nos neurônios dos gânglios sacrais (ACKERMANN et al., 1982; VOGEL et al., 2004). O DNA latente do BHV-1 já foi encontrado em outros sítios além do TG, como em tonsilas (WINKLER et al., 2000) e em linfócitos T CD4+ circulantes (WINKLER et al., 1999) e em células mononucleares sangüíneas (LOVATO et al., 2000) após infecção intranasal. Além disso, também já foi demonstrada a presença do DNA latente nos gânglios dos nervos gênito-femural, obturador, pudendo, ciático e gânglio do nervo retal-caudal em touros latentemente infectados com o BHV-1 através da via intraprepucial (VOGEL et al., 2004). O DNA latente do vírus do herpes simplex (HSV) pesquisado no gânglio ciliar de cadáveres foi detectado em 66% das amostras, demonstrando que o gânglio ciliar constitui-se em um sítio adicional para a latência (BUSTOS & ATHERTON, 2002). A presença do DNA latente do HSV também foi detectada no gânglio sacral dorsal em camundongos (CROEN et al., 1991). Este local como sítio periférico de latência já havia sido descrito por GORDON et

al. (1991) em coelhos e camundongos. CANTIN et al. (1994) detectaram o DNA latente do HSV na medula óssea de humanos. OBARA et al. (1997) detectaram o DNA latente do HSV no gânglio espinhal em 207 dos 262 gânglios espinhais analisados. MITCHELL et al. (2003) também detectaram o DNA latente do HSV no gânglio torácico de humanos. SCHULZ et al. (1998) analisaram a presença de DNA latente do HSV, encontrando 56% de amostras positivas no glânglio facial, 61% no gânglio vestibular e em 50% no gânglio espiral dos indivíduos analisados. TAKASU et al. (1992) demonstraram o DNA latente do HSV em 88% das amostras de gânglio geniculado de indivíduos infectados.

A latência do vírus da pseudoraiva (PRV) também não é exclusiva dos tecidos neurais. Assim como no BHV-1 e BHV-5, o principal sítio de latência do PRV após a infecção aguda é o gânglio trigêmeo. Porém, o DNA latente do PRV já foi detectado no bulbo olfatório e medula oblonga, e com menor freqüência no tronco cerebral e medula espinhal (RZIHA et al., 1986). O DNA latente do PRV também já foi detectado nas tonsilas (WHEELER & OSORIO, 1991; CHEUNG, 1995; WHITE et al., 1996). ROMERO et al. (2003) detectaram DNA latente do PRV com maior freqüência no gânglio sacral (90%) de suínos selvagens quando comparado ao gânglio trigêmeo (60%), tanto em infecção natural como experimental.

Assim como o BHV-1, o BHV-5 estabelece infecção latente no gânglio trigêmeo, após infecção intranasal (ASHBAUGH et al., 1997; VOGEL et al., 2003). Sítios adicionais de latência já foram identificados em tecidos do sistema nervoso central (SNC) de bezerros latentemente infectados (MEYER et al., 2001; VOGEL et al., 2003). VOGEL et al. (2003) detectaram o DNA latente do BHV-5 no gânglio TG e em diferentes secções do SNC de bezerros após infecção intranasal. Estes achados demonstram que tanto o BHV-1 como o BHV-5 podem estabelecer latência em sítios neurais e não-neurais, além dos gânglios sensoriais.

Tem sido demonstrado que os coelhos constituem-se em modelos adequados para o estudo da infecção aguda e latente pelo BHV-1 e BHV-5 (ROCK et al., 1992; MEYER et al., 1996; SILVA et al., 1999; CARON et al., 2002). No entanto, vários aspectos da infecção aguda e latente pelo BHV-5 ainda são desconhecidos e podem ser potencialmente investigados nesse modelo animal. O presente estudo teve como objetivo investigar a distribuição do DNA viral latente, antes e depois da reativação viral induzida por dexametasona, no encéfalo de coelhos inoculados pela via nasal com uma amostra de BHV-1 (SV-265) e de BHV-5 (SV- 507).

2. CAPÍTULO 1

Dexamethasone-induced reactivation of bovine herpesvirus type 5 latent infection in experimentally infected rabbits results in a wider distribution of latent viral DNA in the brain[1].

Running title: Distribution of latent bovine herpesvirus type 5 DNA

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(Artigo a ser submetido ao *Brazilian Journal of Medical and Biological Research*)

[1] Work supported by a MCT/CNPq/CAPES/Finep grant (PRONEX em Virologia Veterinária. 215/96).

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Abstract

Bovine herpesvirus type 5 (BHV-5) is a major etiologic agent of meningo-encephalitis in cattle and establishes lifelong latent infection in trigeminal ganglia and also in other areas of the brain. Colonization of deep areas of the brain with latent viral DNA may have important implications on the pathogenesis of BHV-5 neurological infection upon reactivation. In this study, we investigated the distribution of BHV-5 DNA in the brain of experimentally infected rabbits – a laboratory model for BHV-5 infection - prior and subsequently to virus reactivation, using a nested PCR for the glycoprotein B gene. Fifteen rabbits inoculated intranasally with a Brazilian BHV-5 isolate were divided in two groups: group A rabbits (n=8) were euthanized 60 days post-inoculation (pi) for tissue collection; group B (n=7) were submitted to dexamethasone administration at day 60pi for reactivation of latent infection and euthanized for tissue collection 60 days later. To compare, we used two groups of BHV-1-infected rabbits (C, n=3 and D, n=3), each group being submitted to one of the above treatments, respectively. In group A rabbits, viral DNA was consistently detected in trigeminal ganglia (8/8), frequently in cerebellum (6/8), anterior cortex and pons (3/8) and occasionally in medulla oblongata and dorso-lateral cortex (2/8), ventro-lateral and posterior cortices (1/8), midbrain and thalamus (1/8). In rabbits previously submitted to virus reactivation, viral DNA showed a broader distribution, being detected more frequently – besides the TG (7/7) - in ventro-lateral (6/7) and posterior cortices (5/7), pons, medulla oblongata and thalamus (4/7) and midbrain (3/7). In contrast, rabbits inoculated with BHV-1 harbored latent viral DNA in a few sections in addition to TG and did not show significant changes in distribution of viral DNA post-reactivation. These results demonstrate that latency by BHV-5 DNA – and not BHV-1 DNA – may be established in several areas of the brain of experimentally infected rabbits. Further, dexamethasone-induced virus reactivation is followed by a wider distribution of latent viral DNA, probably due to virus dissemination

from the original sites. Thus, it is reasonable to speculate that reactivation of latent infection from deep areas of the brain may contribute to the recrudescence of neurological disease frequently observed upon reactivation of latent BHV-5 infection.

Key words: bovine herpesvirus type 5, BHV-5, BHV-1, latent infection, rabbits.

Introduction

The establishment of latent infection in neurons of sensory and autonomic nerve ganglia is the hallmark of the infection by human and animal alphaherpesviruses and has profound implications in their epidemiology and pathogenesis (1). Viral reactivation and shedding may occur under natural or induced stimuli and provide adequate means for viral transmission and spread (2). Recrudescence of clinical disease (orolabial, nasal and genital lesions, encephalitis) are well documented consequences of reactivation of human and some animal herpesviruses (3-5).

Bovine herpesvirus type 5 (BHV-5) is an alphaherpesvirus associated with severe, usually fatal meningo-encephalitis in cattle (1, 6). The disease is characterized by tremors, nystagmus, teeth grinding, circling, ataxia, recumbency, paddling and death (5, 7). Severe outbreaks of neurological disease by BHV-5 have been frequently reported mainly in Brazil and Argentina (7, 8). In animals surviving acute infection, BHV-5 establishes a lifelong latent infection that can be reactivated under certain natural or induced stimuli (4, 5). In contrast with many other animal herpesviral infections, BHV-5 reactivation is frequently accompanied by recrudescence of clinical disease, both in the natural host and in a rabbit model (4, 5).

The major sites of latent infection by human (i.e. herpes simplex virus [HSV-1]) and animal alphaherpesviruses (BHV-5, bovine herpesvirus type 1[BHV-1], pseudorabies virus [PRV]) are the sensory nerve ganglia innervating the site of primary viral replication (2, 9-11). Therefore, oronasal infection results in the establishment of latency mainly in trigeminal ganglia (TG) and genital infection ensues colonization of sacral ganglia with latent viral DNA (2, 9-12). However, other possible neural and non-neural sites of latent infection or virus persistence by these viruses have also been described (13-15). The major site of latent infection by BHV-1 is also the sensory nerve ganglia, where the virus replicates lytically during acute infection (2, 14). In addition, BHV-1 DNA has been detected in tonsils; in CD_4+

T lymphocytes and in peripheral blood mononuclear cells of latently infected animals (16, 17). After experimental genital infection, latent BHV-1 DNA was consistently found in sacral ganglia of heifers (18) and in sacral, other nerve ganglia and in regional lymph nodes of bulls (12). The biological significance of latent infection in non-neural sites and neural sites other than sensory nerve ganglia remains obscure since reactivation from these sites has not been unequivocally demonstrated.

BHV-5 is very neuroinvasive and neurovirulent in both the natural host (19-21) and in animal models (4, 21, 22). The ensuing neurological disease is often fatal, yet cases of mild infection followed by clinical recovery or even subclinical neurological infection seem not to be rare (4, 5, 19, 20). Natural and/or dexamethasone-induced BHV-5 reactivation in calves and rabbits is followed by virus shedding and frequently by recrudescence of neurological disease (4, 5, 21). Recently, we demonstrated that – in addition to TG - BHV-5 does establish latent infection in several areas of the brain of experimentally inoculated calves (23). Further, reactivation of latent infection was followed by a wider distribution of the virus in the brain and establishment of latency in additional sites (23). The dissemination of virus to other sites in the brain upon reactivation is believed to play a role in human encephalitis by HSV-1 (3, 15) and may be determinant in the recrudescence of neurological disease frequently observed in BHV-5 latently infected calves and rabbits (4, 5, 21).

Rabbits have been successfully used to study several aspects of BHV-5 acute and latent infections (4, 21, 24). In this study we investigated the distribution of latent BHV-5 DNA in the brain of experimentally infected rabbits, prior and subsequently to dexamethasone-induced reactivation, to determine whether reactivation of latent viral DNA may lead to the establishment of latency in other brain regions. Our results are consistent with those observed in cattle (23) and point out for a biological importance of latent infection in

neural sites other than the TG in the pathogenesis of herpesvirus encephalitis upon reactivation.

Material and methods

Experimental design

Twenty four rabbits were divided in four groups (A, B, C, D) and inoculated intranasally with bovine herpesvirus type 5 (BHV-5, groups A and B) or BHV-1 (groups C and D). Animals were monitored clinically during the acute infection; virus replication was monitored by testing nasal swabs for infectivity. Sixty days after inoculation, inoculated rabbits from groups A e C were euthanized for collection of brain tissue. The other inoculated rabbits (groups B and D) were treated with dexamethasone (Dx) to reactivate the infection. Sixty days after Dx administration, these rabbits were euthanized for tissue collection. The distribution of latent viral DNA in different sections of the brain of rabbits of all groups was investigated by nested-PCR, using a set of primers to the glycoprotein B (gB) gene.

Cells and viruses

A bovine cell line named CRIB (25), derived from Madin-Darby bovine kidney cells (MDBK; American Type Culture Collection, CCL-22) was used for virus multiplication, quantitation and isolation from nasal swabs and tissues. Cells were routinely maintained in Eagle's minimal essential medium (MEM, Cultilab, Campinas, SP. Brazil) containing penicillin (1.6mg/L), streptomycin (0.4mg/L) and 5% fetal calf serum (Cultilab). The BHV-1 SV-265 strain was isolated from a calf with respiratory disease and the BHV-5 SV-507 strain was isolated from an outbreak of meningo-encephalitis in southern Brazil and has been submitted to nucleotide sequencing of the entire DNA genome (26).

Animals, virus inoculation and dexamethasone (Dx) treatment

Twenty four weanling rabbits (30 to 35 days old), seronegative to BHV-5 and BHV-1, were used for virus inoculation. The inoculated groups were kept in separated cages. The rabbits were inoculated by the intranasal route with 0.5 ml of viral suspension in each nostril (total viral dose: $10^7 TCID_{50}$/animal), with the respective virus as follows: group A: BHV-5, n=11; group B: BHV-5, n=7; group C: BHV-1, n=3; group D: BHV-1, n=3. Sixty days post inoculation (pi), animals of groups A and C were euthanized for tissue collection. Rabbits of groups B and D were submitted to daily administrations of dexamethasone (2.6 mg/kg/day during five days) starting at day 60pi. The DX-treated rabbits were euthanized at day 60 post-dexamethasone (pDx) for tissue collection. All procedures of animal handling and experimentation were performed under veterinary supervision and according to recommendations by the Brazilian Committee on Animal Experimentation (COBEA; law # 6.638 of May, 8^{th} , 1979).

Animal monitoring, sample collection and processing

After virus inoculation and Dx treatment, rabbits were monitored clinically on a daily basis. Nasal swabs for viral isolation were collected daily up to the day of Dx administration from animals of groups A and C and then up to day of euthanasia in groups B and D rabbits. The swabs were immersed in 1ml of MEM containing 5 x penicillin and stored at -70ºC. After vortexing and centrifugation (3.000 x g, 10 min), the supernatant of nasal swabs were inoculated onto monolayers of CRIB cells grown in 24-well plates and submitted to three passages of five days each, with the cells being monitored for cytopathic effect (CPE). At necropsy, different sections of the brain and peripheral nervous system (TG) were aseptically and individually collected for virus isolation and PCR.

The following sections were collected individually: cerebral cortex (anterior, posterior, ventro-lateral and dorso-lateral), olfactory bulb, thalamus, midbrain, pons, medulla oblongata, cervical medulla, cerebellum and trigeminal ganglia (TG). The location of the sections examined for the presence of viral DNA is depicted in Figure 1. Tissue collection was performed bilaterally, when applicable. Virus isolation was only attempted in sections that were positive for viral DNA by PCR. For virus isolation, the tissue samples were processed by preparing a 10% (wt/vol.) homogenized suspension, which was inoculated onto CRIB monolayers. Monitoring of virus replication was performed as described above.

DNA extraction

Strict precautions were taken to avoid DNA carryover, including: isolation of pretarget, posttarget and amplification into separated facilities; use of plastic disposable labware, positive-displacement pipettes in all steps of DNA manipulation; and use of coverings for operator's hair, clothing's and face. Total DNA for PCR was extracted from approximately 0.2 g of each section. Template DNA was prepared by using the Easy-DNA Isolation Kit (Invitrogen), according to the manufacturer's protocol. The DNA concentration was measured by UV absorbance at 260nm. For large areas (dorso-lateral, ventro-lateral and cerebellum cortices, for example), representative fragments were collected bilaterally, pooled and submitted to DNA extraction.

Polymerase chain reaction

The PCR reaction was performed using two sets of primers corresponding to positions 57.338 and 57.782 (primers 1 and 2) and 57.143 and 57.416 (primers 3 and 4) of the glycoprotein B (gB) gene coding region of the BHV-5 strain SV-507 (26). The target region (273 bp) was initially amplified with the external primers: (forward) 5' -

CCAGTCCAGGCAACCGTCAC- 3' (57.338) and (reverse) 5' -CTCGAAAGCCGAGTACCTGCG- 3' (57.782) and then with the internal primers: (forward) 5'-GTGGTGGCCTTTGACCGCGAC-3' (position 57.143) and (reverse) 5'-GCTCCGGCGAGTAGCTGGTGTG-3' (position 57.416). These two sets of primers recognize the homologous sequence of glicoprotein B of BHV-1 (Flores, E.F. personal communication). The PCR reaction was performed in a 25µl volume, using 1µl of template DNA (containing approximately 1µg of total DNA in TE buffer), 100ng of each primer, 1mM $MgCl_2$, 10mM of dNTPs, 10% DMSO, 1x reaction buffer and 0.5 units of Taq Polymerase (Gibco, BRL). The PCR conditions were: initial denaturation (94ºC for 10 min), followed by 35 cycles of 94ºC-1 min; 56ºC- 40 sec for primer annealing and 72ºC - 40 sec for primer extension; and a final extension of 7 min at 72ºC. The second PCR reaction was performed by used 2µl of PCR products for first PCR reaction. The product obtained next two PCR reaction was an amplicon of 273 base pairs (bp). PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. To increase the sensitivity of the detection, the PCR solutions (2µl) of negative tissues in the first PCR, were used as template in a second PCR reaction, using the primers and conditions described above. DNA extracted from the brain of the rabbits mock-inoculated was used as negative control. DNA extracted from the brain of a BHV-5 acutely infected calf, was used as positive controls. The specificity of the PCR amplification was confirmed in a previous report (23).

Results

Acute infection

All rabbits inoculated with BHV-5 (groups A and B) excreted virus in nasal secretions up to day 7 post-inoculation (pi); four were still shedding virus at day 9 pi and three at day 10 pi. Virus shedding was not discontinued after day 10 pi. Thereafter, a few rabbits shed virus

for one day sporadically within the interval between days 19 and 60 pi. No virus shedding was detected in the days preceding dexamethasone treatment, demonstrating that rabbits were latently infected. Three rabbits (# 09, 03 and 05) developed neurological disease and died or were euthanized in extremis at days 15, 20 and 21 pi, respectively. Infectious virus was detected in several areas of the brain of these animals. The rabbits dying of neurological disease during acute infection were not included in our study. Rabbits inoculated with BHV-1 shed virus in nasal secretions up to day 9 pi; one was still shedding at day 11 pi. No virus shedding was detected thereafter and up to dexamethasone treatment (day 60 pi). Control rabbits did not shed virus in any swab collection.

Latent infection

At day 60 pi (and in days preceding it), no infectious virus was detected in nasal swabs collected from all groups. Thus, these animals fulfilled the classical definition of latent infection. At day 60 pi, group A (BHV-5) and C (BHV-1) rabbits were euthanized and several brain sections were collected to investigate the distribution of latent viral DNA by nested-PCR. The results of nested-PCR performed in total DNA extracted from these brain sections are presented in Tables 1 and 3; representative nested-PCR results are shown and Figures 2 and 3. BHV-5 DNA was consistently detected in trigeminal ganglia (8/8), frequently in cerebellum (6/8), occasionally in pons and anterior cortex (3/8), dorso-lateral cortex (2/8), thalamus (2/8), ventro-lateral and posterior cortices (1/8), midbrain and of group A rabbits (Table 1, Figure 2). In contrast, in group C rabbits BHV-1 DNA was detected consistently in TG, and only occasionally found in other sections (Table 3, Figure 3). All DNA positive tissues were negative in virus isolation, confirming that the rabbits were latently infected.

To investigate whether the distribution of latent viral DNA in the brain would change after virus reactivation, rabbits from groups B (BHV-5) and D (BHV-1) were submitted to

five daily administrations of Dx beginning at day 60 pi. Following Dx treatment, all group B rabbits shed virus in nasal secretions, starting at day 3 post-Dx (pDx) and lasting from four to eleven days. All group D rabbits also excreted virus after Dx treatment, with shedding starting at day 4 pDx and lasting from 7 to 9 days. At day 60 pDx, rabbits from both groups were euthanized for tissue collection. At the time of tissue collection, no animal was shedding virus. The distribution of latent viral DNA in the brain of BHV-5 infected rabbits, after Dx treatment, is shown on Table 2 and Figure 2. Briefly, viral DNA was detected in roughly the same areas of group A rabbits, yet with higher frequency in several sections, mainly in ventro-lateral (6/7) and posterior cortices (5/7), pons-medulla and thalamus (4/7) and midbrain (3/7). The sections which showed the highest increased positivity were the ventro-lateral cortex (1/8 to 6/7), posterior cortex (1/8 to 5/7), thalamus (2/8 to 4/7), medulla oblongata (2/8 to 4/7) and midbrain (1/8 to 3/7). The other sections showed a roughly similar frequency or of latent viral DNA in both groups. Comparing with group A, group B rabbits showed an almost two-fold increase in the number of total DNA positive sections (43/77 or 55.8% positive sections against 29/88 or 32.9%). Likewise, rabbits from group B showed a higher mean number of PCR positive sections (mean: 6.1 versus 3.6). Again, in group D rabbits (BHV-1), latent viral DNA was almost exclusively restricted to TG, with only a few other sections being positive. All sections PCR-positive were negative in virus isolation, fulfilling the classical requirements for defining latent infection.

Altogether, these results demonstrated that BHV-5 DNA is present in several areas of the brain of experimentally infected rabbits during latent infection, both prior and after Dx-induced virus reactivation. After Dx treatment, however, the number of positive sections per animal and the frequency of positive areas were higher than prior to reactivation. These findings suggest that virus reactivation ultimately results in colonization of additional areas of

the brain with latent viral DNA. In contrast, latent BHV-1 DNA was found almost exclusively in TG and seemed not to expand its distribution upon virus reactivation.

Discussion

The results presented herein corroborate and extend previous findings from our lab in which BHV-5 DNA was detected in several areas of the brain of experimentally infected calves (23). In the present study, BHV-5 DNA was detected in several areas of the brain of latently infected rabbits. At the time of tissue collection, rabbits from all groups met the biological criteria traditionally used to define latent infection: presence of viral DNA in the absence of productive viral replication (2, 14, 15). The failure to demonstrate infectious virus in DNA-positive tissues is compatible with latent infection rather than viral persistence. Furthermore, examination of brain sections of rabbits previously submitted to Dx-induced virus reactivation demonstrated a wider distribution of viral DNA in the brain. In particular, the ventro-lateral, posterior cortices and pons-medulla showed an increased frequency of colonization with latent viral DNA. Taken together, these results demonstrate that further viral dissemination within the brain may occur upon reactivation and point out for a potential role of latency in CNS sites – not excluding TG - in the pathogenesis of recrudescent BHV-5 neurological disease that frequently follows virus reactivation.

In contrast, BHV-1 latent DNA was almost exclusively restricted to TG, regardless whether the examination was performed in rabbits submitted or not to prior virus reactivation. These findings are also consistent with previous studies which showed the TG as a major site of latency by BHV-1 in the natural hosts (2, 9, 14, 18) and in rabbits used as a model (4, 27). Nevertheless, a few other brain areas also harbored latent DNA, indicating that BHV-1 is capable of invading some brain regions during acute infection. Indeed, in a parallel experiment, we were able to detect infectious virus in some areas of the brain of rabbits

inoculated with a BHV-1 isolate (data not shown). These and previous data (28) indicates that BHV-1, even not being as neuroinvasive as BHV-5, may be able to reach the brain after intranasal inoculation. The replication and spread within the brain, however, would not suffice to produce clinical meningo-encephalitis.

Sensory nerve ganglia are the major sites of latent infection by human and animal alphaherpesviruses, yet additional sites of latency have been identified for these viruses. Latent HSV-1 DNA has been detected in human nodose ganglia, vagus nerve, bone marrow and in some areas of the CNS; in mouse cornea and in non-neural sites in other animal models (3, 13-15). PRV DNA has also been detected in olfactory bulbs, brain stem, pons/medulla and cervical medulla and tonsils (10, 11). The major site of latent infection by BHV-1 is also the sensory nerve ganglia, yet other sites including autonomic nerve ganglia, lymph nodes, tonsils and peripheral blood mononuclear cells have been shown to harbor latent DNA (9, 12, 16, 17). The biological significance of alphaherpesvirus latency in non-neural sites and other neural sites than the TG is controversial since attempts to reactivate virus from some of these tissues by explant cultures have failed (13-17).

Previous studies have detected latent BHV-5 DNA in some areas of the brain of experimentally infected calves (9, 20). Further, we recently demonstrated that BHV-5 does establish latency in several areas of the brain of experimentally infected calves and that the distribution of latent DNA changes after virus reactivation, resulting in colonization of additional sites (23). The biological significance of the presence of viral DNA in several areas of the brain during latent infection, and the possible implications for the pathogenesis of BHV-5 infection are unclear at this point. However, colonization of deep areas of the brain with latent viral DNA may have important implications on the pathogenesis of BHV-5 neurological disease that frequently occurs after reactivation (4, 5, 21, 23, 28). Encephalitis due to reactivating virus is one of the possible mechanisms explaining HSV encephalitis in

humans (3, 15) and thus may also contribute to the neurological disease that frequently accompanies reactivation of BHV-5 in rabbits and calves (4, 5, 21, 23, 28). It is conceivable that virus reactivating from deep areas of the brain - in addition to virus reactivating from the TG - may serve as a source of virus for neurological infection during reactivation. Favoring this hypothesis, it was recently demonstrated that the timing, kinetics and distribution of histological changes in the CNS after BHV-5 reactivation differed from that observed during acute infection (4, 5, 21). In the present study, the ventro-lateral, posterior cortices and pons-medulla showed the most prominent increase in the frequency of viral DNA detection from pre- to post-reactivation. Virus reactivating from TG (first order sensory neurons), pons-medulla (second order neurons) and thalamus (third order sensory neurons) – sites shown to harbor viral DNA prior to reactivation – might have been transported by anterograde axonal transport and contributed to the colonization of these areas after Dx-treatment. However, the demonstration that BHV-5 DNA in CNS sites is biologically active, i.e. it can be reactivated as the DNA in the TG, for example, is necessary to support this hypothesis. Sequential studies after dexamethasone administration, analyzing the kinetics of viral messenger RNAs, protein and/or progenie virus production in these sites are underway in our lab and may answer this question.

In summary, the results presented herein corroborates our previous findings in cattle and indicate that: 1. BHV-5 does establish latent infection in several areas of the brain and 2. The distribution of DNA changes after Dx-induced reactivation such that additional areas became positive for viral DNA. In contrast, BHV-1 latent infection in rabbits is almost exclusively restrited to TG and virus reactivation appears not to change significantly the distribution of latent viral DNA. The biological significance of these findings and the possible role of these additional latency sites in the pathogenesis of BHV-5 are currently under investigation.

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Table 1. Detection of bovine herpesvirus 5 (BHV-5) DNA by PCR in brain sections of rabbits 60 days after intranasal inoculation (group A).

Animal	Section[a]										
	ol	ac	vlc	dlc	pc	cb	tg	po	mo	mb	th
07	-[b]	-	-	+[c]	-	+	+	-	-	-	-
11	-	-	-	-	-	+	+	-	+	-	-
12	-	+	-	-	-	-	+	-	-	-	-
16	-	-	-	-	-	+	+	-	-	-	-
18	-	+	-	+	+	+	+	+	+	+	+
20	-	-	-	-	-	-	+	+	-	-	+
22	-	+	-	-	-	+	+	-	-	-	-
24	-	-	+	-	-	+	+	+	-	-	-

[a] Ol: olfactory lobe; ac: anterior cortex; vlc: ventro-lateral cortex; dlc: dorso-lateral cortex; pc: posterior cortex; cb:cerebellum; tg: trigeminal ganglia; po: pons; mo: medulla oblongata; mb: midbrain; th: thalamus.

[b] Negative result.

[c] Positive result.

Table 2. Detection of bovine herpesvirus 5 (BHV-5) DNA by PCR in brain sections of rabbits 60 days after dexamethasone-induced virus reactivation[a] (group B).

Animal	Section[b]										
	ol	ac	vlc	dlc	pc	cb	tg	po	mo	mb	th
01	-	-	-	-	+	-	+	+	-	-	-
02	-	-	+	-	-	+	+	+	-	-	-
04	-	+	+	-	+	+	+	-	+	-	-
06	-	+	+	+	+	-	+	-	-	+	+
08	-	-	+	-	-	-	+	+	+	-	+
10	-	+	+	+	+	-	+	-	+	+	+
14	-	-	+	+	+	-	+	+	+	+	+

[a] Virus reactivation with dexamethasone was induced 60 days after virus inoculation.

[b] Ol: olfactory lobe; ac: anterior cortex; vlc: ventro-lateral cortex; dlc: dorso-lateral cortex; pc: posterior cortex; cb:cerebellum; tg: trigeminal ganglia; po: pons; mo: medulla oblongata; mb: midbrain; th: thalamus.

Table 3. Detection of bovine herpesvirus 1 (BHV-1) DNA by PCR in brain sections of rabbits 60 days after intranasal (group C) and 60 days after dexamethasone-induced virus reactivation[a] (group D).

Animal	Section[b]										
	ol	ac	vlc	dlc	pc	cb	tg	po	mo	mb	th
Group C											
15	-	-	-	-	-	-	+	-	+	-	-
17	-	-	-	+	-	+	+	-	+	-	-
19	-	-	-	-	-	-	+	-	-	-	-
Group D											
13	-	-	-	-	+	-	+	-	+	-	-
26	-	-	-	-	+	-	+	-	-	+	-
28	-	-	-	-	-	+	+	-	-	-	-

[a] Virus reactivation with dexamethasone was induced 60 days after virus inoculation.

[b] Ol: olfactory lobe; ac: anterior cortex; vlc: ventro-lateral cortex; dlc: dorso-lateral cortex; pc: posterior cortex; cb:cerebellum; tg: trigeminal ganglia; po: pons; mo: medulla oblongata; mb: midbrain; th: thalamus.

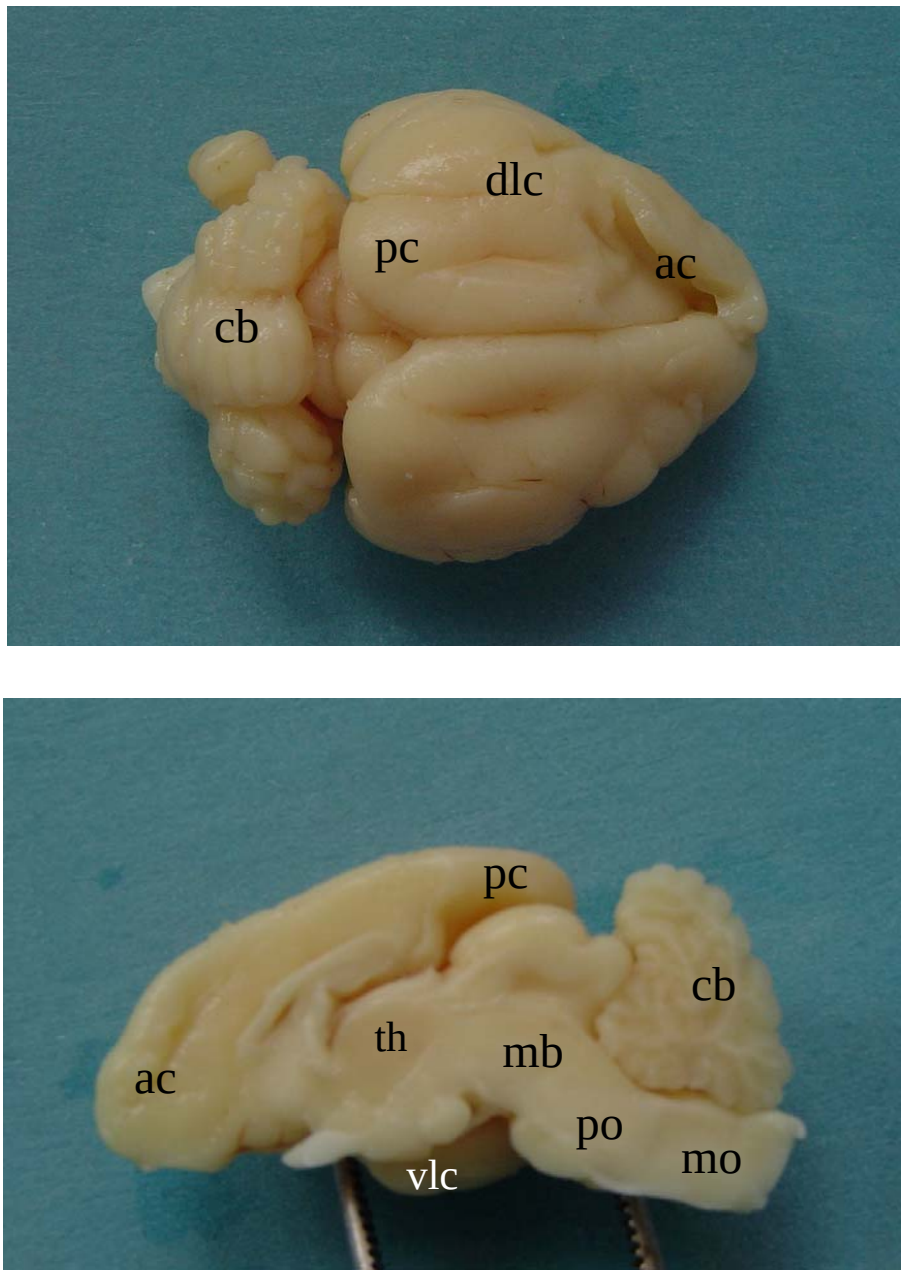

Figure 1. Rabbit brain showing the sections examined by PCR for the presence of latent bovine herpesvirus type 1 (BHV-1) or 5 (BHV-5) DNA. A. Dorsal view; B. Sagital view.

ac: anterior cortex; vlc: ventro-lateral cortex; dlc: dorso-lateral cortex; pc: posterior cortex; cb: cerebellum; po: pons; mo: medulla oblongata; mb: midbrain; th: thalamus.

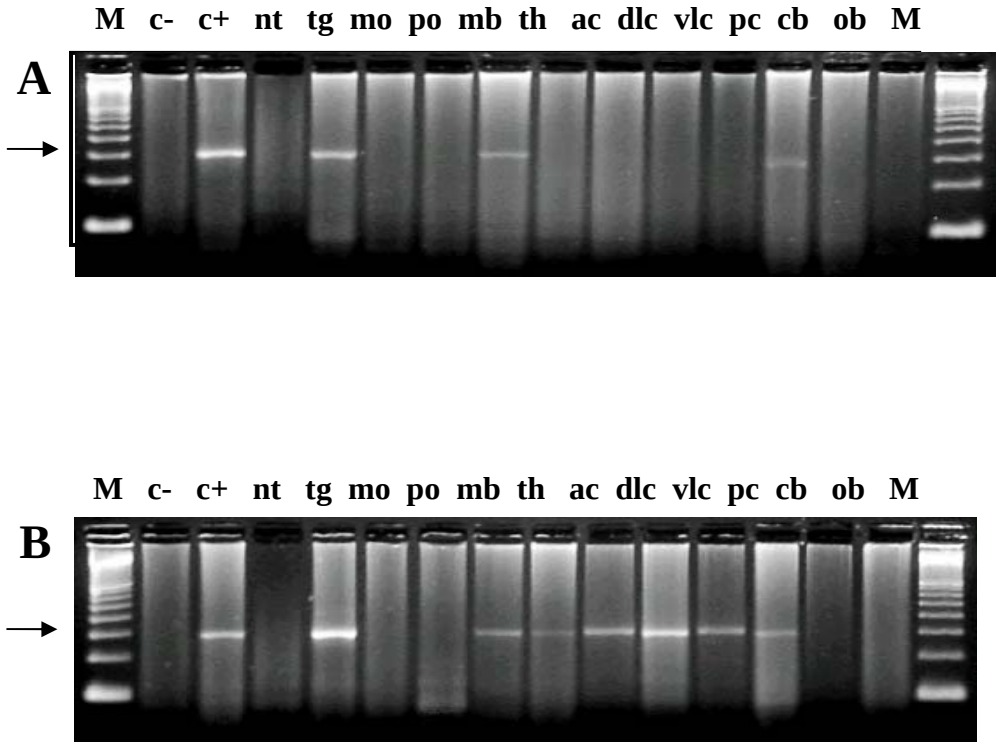

Figure 2. Detection of bovine herpesvirus type 5 DNA in the brain of experimentally infected rabbits prior (A) and subsequently (B) to dexamethasone-induced viral reactivation. A. Brain sections of rabbit # 07 (group A); B. Brain sections of rabbit # 06 (group B). The lanes of both panels show: M, molecular weight marker (100bp ladder); c-, negative control; c+, positive control; nt, no template; tg, trigeminal ganglia; mo, edulla oblongata; po, pons; mb, midbrain; th, thalamus; ac, anterior cortex; dlc, dorso-lateral cortex; vlc, ventro-lateral cortex; pc, posterior cortex; cb, cerebellum; ob, olfactory bulb; M, molecular weight marker. PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The size of amplified products is indicated by arrows.

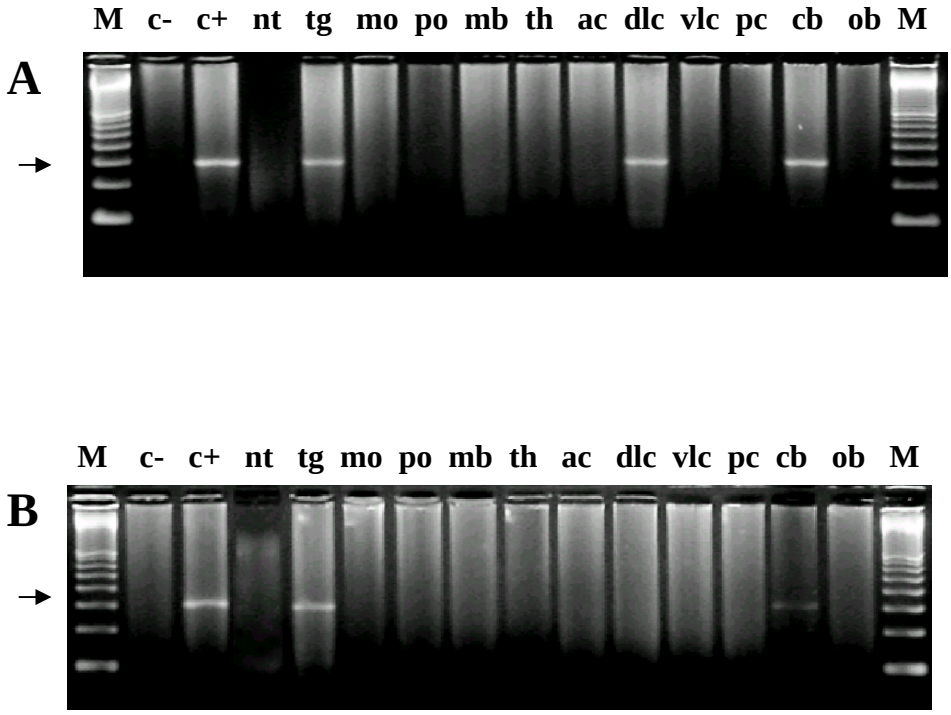

Figure 3. Detection of bovine herpesvirus type 1 DNA in the brain of experimentally infected rabbits prior (C) and subsequently (D) to dexamethasone-induced viral reactivation. A. Brain sections of rabbit # 28 (group C); B. Brain sections of rabbit # 15 (group B). The lanes of both panels show: M, molecular weight marker (100bp ladder); c-, negative control; c+, positive control; nt, no template; tg, trigeminal ganglia; mo, edulla oblongata; po, pons; mb, midbrain; th, thalamus; ac, anterior cortex; dlc, dorso-lateral cortex; vlc, ventro-lateral cortex; pc, posterior cortex; cb, cerebellum; ob, olfactory bulb; M, molecular weight marker. PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The size of amplified products is indicated by arrows.

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