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PATOGENIA EXPERIMENTAL DA INFECÇÃO PELO HERPESVÍRUS BOVINO TIPO 2 EM OVINOS E COBAIAS

DISSERTAÇÃO DE MESTRADO

Fabrício Dias Torres

Santa Maria, RS, Brasil
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PATOGENIA EXPERIMENTAL DA INFECÇÃO PELO HERPESVÍRUS BOVINO TIPO 2 EM OVINOS E COBAIAS

por

Fabrício Dias Torres

Dissertação apresentada ao Curso de Mestrado do 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.**

Orientador: Prof. Rudi Weiblen

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PATOGENIA EXPERIMENTAL DA INFECÇÃO PELO HERPESVÍRUS BOVINO TIPO 2 EM OVINOS E COBAIAS

elaborada por
Fabrício Dias Torres

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

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

PATOGENIA EXPERIMENTAL DA INFECÇÃO PELO HERPESVÍRUS BOVINO TIPO 2 EM OVINOS E COBAIAS

Autor: Fabrício Dias Torres
Orientador: Rudi Weiblen
Data e Local da Defesa: Santa Maria, 03 de março de 2009.

A biologia e a epidemiologia da infecção pelo herpesvírus bovino tipo 2 (BoHV-2), agente da mamilite herpérica bovina, são pouco conhecidas. Com isso, o presente estudo teve como objetivos elucidar alguns aspectos da epidemiologia e estudar a patogenia da infecção pelo BoHV-2 em ovinos e cobaias. Um estudo sorológico pesquisando anticorpos para o BoHV-2 em 2.213 amostras de bovinos com mais de 2 anos, provenientes de 136 municípios distribuídos em 7 mesoregiões do Rio Grande do Sul (RS), determinou uma soroprevalência de 24,5% (543/2.213). Os resultados demonstram que o BoHV-2 está disseminado no rebanho bovino no RS, com potencial envolvimento em casos de mamilite freqüentemente relatados em rebanhos leiteiros. Um segundo experimento visou caracterizar a infecção latente em ovinos, propostos como modelo experimental. Ovelhas lactantes inoculadas com o BoHV-2 na pele do úbere excretaram o vírus por até 5 dias, desenvolveram mamilite e soroconverteram. Entretanto, a tentativa de reativar a infecção latente pela administração de dexametasona (Dx) no dia 40 pós-infecção (pi) não resultou em reativação. Não obstante, o DNA viral latente foi detectado por PCR em gânglios neurais e/ou em diversos linfonodos (LNs) de todos os animais no dia 40 pós reativação (pr). Cordeiros que haviam sido inoculados com o BoHV-2 pela via nasal igualmente albergavam o DNA viral latente nos gânglios trigêmeos, tonsilas e LNs regionais. Esses resultados demonstram que o BoHV-2 estabelece infecção latente em gânglios neurais e tecidos linfóides regionais, embora a reativação viral não seja facilmente obtida com os protocolos usados em outros alfaherpesvírus. Um terceiro experimento foi realizado para validar cobaias (*Cavia porcellus*) como modelo experimental para estudo da biologia da infecção com o BoHV-2. Cobaias lactentes (30-40 dias) inoculadas com o BoHV-2 nas regiões genital e intra-dérmico no tetos e úbere desenvolveram sinais clínicos moderados a severos, notadamente mais pronunciados na região genital. O vírus foi isolado de suabes coletados entre os dias 3 e 7 pi da região genital (8/12) e em menor freqüência da região dos tetos (1/12). Todos os animais soroconverteram para o BoHV-2, apresentando títulos de anticorpos neutralizantes entre 16 e 128 no dia 30 p.i. A análise histológica de biópsias de pele coletada de lesões revelou corpúsculos de inclusão e infiltrado linfocitário e plasmocitário perivascular. PCR realizada com DNA total extraído dos tecidos coletados no dia 35 pi revelou a presença de DNA viral latente em gânglios neurais lombo-sacrais e LNs regionais. A administração de Dx no dia 35 pi cursou com recrudescência clínica na região genital, porém não foram detectados excreção viral nem soroconversão, indicadores de reativação da infecção latente. Estes resultados demonstram que cobaias podem ser usados como modelo para a infecção aguda e latente pelo BoHV-2, e confirmam que a reativação viral não é facilmente obtida pelo uso de protocolos usuais para outros alfaherpesvírus. Em conjunto, os resultados demonstram a utilidade de ovinos e cobaias como modelo experimental para a infecção pelo BoHV-2.
Palavras-chave: herpesvírus bovino tipo 2, BoHV-2, epidemiologia, ovino, cobaias, patogenia viral.

ABSTRACT

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EXPERIMENTAL PATHOGENESIS OF BOVINE HERPESVIRUS TYPE 2 INFECTION IN SHEEP AND GUINEA PIGS.

Author: Fabrício Dias Torres
Adviser: Rudi Weiblen
Santa Maria, March 3rd 2009.

The biology and epidemiology of bovine herpesvirus 2 (BoHV-2), the agent of bovine herpetic mammillitis (BHM), remain largely unknown. Thus, the present study aimed at addressing selected aspects of BoHV-2 epidemiology and pathogenesis in sheep and guinea pigs. A serological survey for BoHV-2 antibodies in 2.213 samples from cattle > 24-months-old from 136 counties of seven different mesoregions of Rio Grande do Sul (RS) revealed an overall prevalence of 24.5% (543/2.213). These results demonstrate that BoHV-2 infection is widespread among cattle in RS, and potentially involved in cases of mammillitis frequently described in dairy cows. A second experiment was conducted to characterize the latent BoHV-2 infection in a sheep model. Lactating ewes inoculated with BoHV-2 in the skin of the udder shed virus for up to five days, developed mammillitis and seroconverted. However, attempts to reactivate latent infection by dexamethasone (Dx) administration at day 40 post infection (pi) failed. Nevertheless, viral DNA was detected by PCR in several nerve ganglia and/or regional lymph nodes (LNs) of all animals at day 40 post-reactivation (pr). Lambs previously inoculated with BoHV-2 in the nose also harbored latent viral DNA in trigeminal ganglia, tonsils and regional LNs. These results demonstrated that BoHV-2 establishes latent infection in nerve ganglia and in regional lymphoid tissues, yet virus reactivation is not easily achieved by standard protocols used for other alphaherpesvirus. A third experiment was conducted to evaluate guinea pigs (*Cavia porcellus*) as an experimental model to study the biology of BoHV-2 infection. Weanling (30-40 days-old) guinea pigs inoculated into the genital area and over the skin of the udder and teats with a viral suspension developed moderate to severe clinical signs, noticeably more pronounced in the genital area. Infectious virus was recovered from swabs collected between days 3 and 7 from genital area (8/12) and less frequently (1/12) from teat skin. All animals seroconverted to BoHV-2 developing neutralizing titers from 16 to 128 at day 30 pi. Histological examination of skin biopsies collected from genital lesions showed intranuclear inclusion bodies and perivascular infiltrates composed by lymphocytes and plasmocytes. PCR examination of tissues collected from animals euthanized at day 35 pi revealed the presence of latent viral DNA in nerve ganglia and LNs. Dx administration at day 35 pi was followed by mild recrudescence of genital disease in some animals, yet virus isolation and/or seroconversion – which are usually taken as indicators of virus reactivation – were not observed. These results show that guinea pigs may be used as models to study BoHV-2 acute and latent infection and confirm that BoHV-2 reactivation is not easily achieved by using the standard protocols for other alphaherpesviruses. Taken together, these results demonstrate that both sheep and guinea pigs are suitable animal models for BoHV-2 infection.

Key words: Bovine herpesvirus type 2, BoHV-2, epidemiology, sheep, guinea pig, viral pathogenesis.

LISTA DE FIGURAS

4. CAPITULO 3

LISTA DE TABELAS

SUMÁRIO

1. INTRODUÇÃO

O herpesvírus bovino tipo 2 (BoHV-2), agente etiológico da mamilite herpética bovina (BHM), é um vírus envelopado que possui uma molécula de DNA de fita dupla como genoma. O BoHV-2 pertence à família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Simplexvirus*, juntamente com os vírus do herpes simplex humano tipos 1 e 2 (HSV-1 e HSV-2), com os quais apresenta semelhanças antigênicas e moleculares (ROIZMAN et al., 1992). Essas semelhanças sugerem que estes vírus tenham se originado de um ancestral comum, juntamente com outros herpesvírus de primatas (EHLERS, 1999).

A infecção pelo BoHV-2 tem sido associada com duas formas clínicas distintas e bem definidas: lesões localizadas nos tetos ou ocasionalmente disseminadas pelo úbere (mamilite herpética, BHM), e lesões generalizadas na pele (*pseudolumpy skin disease* - PLSD) (GIBBS; RWEYEMAMU, 1977). Um surto atípico de mamilite, caracterizado por severa deposição de tecido granular difuso na região de tetos e úbere, além de lesões vesiculares na região de focinho, foi recentemente descrito nos Estados Unidos (EUA), associado com a infecção pelo BoHV-2 (KEMP et al., 2008). As lesões de mamilite podem ser confundidas com outras infecções de pele de bovinos, incluindo aquelas causadas pelo vírus da *pseudocowpox* e pelo vírus vaccinia (JACKSON, 1993; LOBATO et al., 2005).

O BoHV-2 foi isolado pela primeira vez em 1957 na África do Sul, de bovinos que apresentavam lesões generalizadas na pele (ALEXANDER et al., 1957) e posteriormente em Ruanda Urandi, a partir de lesões nas tetas de vacas leiteiras (HUYGELEN et al., 1960). No entanto, a importância do BoHV-2 como agente de mamilite só foi destacada em 1964, após o isolamento do vírus de uma doença ulcerativa de tetas e úbere de vacas leiteiras (MARTIN et al., 1966). A infecção pelo BoHV-2 tem sido detectada por isolamento/sorologia em vários países, incluindo o Kênia (MARTIN; GWYNNE, 1968), Austrália (TURNER et al., 1974), Reino Unido (GIBBS; RWEYEMAMU, 1977), EUA (LETCHOWORTH; LA DUE, 1982), Itália (CASTRUCCI et al., 1972), Brasil (ALICE, 1977) e Japão (IMAI et al., 2005).

Os maiores prejuízos ocasionados pela infecção com o BoHV-2 ocorrem em gado leiteiro, embora a infecção também tenha sido diagnosticada em bovinos de corte (JOHNSTON, 1971). As perdas estão relacionados com a redução da produção leiteira devido à mastites intercorrentes, custo do tratamento e descarte do leite (GIBBS; RWEYEMAMU, 1977). No Reino Unido, um surto de mamilite herpética resultou em queda de

aproximadamente 20% na produção de leite como conseqüência da dificuldade de ordenha dos animais afetados, descarte do leite e o desenvolvimento de mastites (GIBBS; RWEYEMAMU, 1977).

Estudos sorológicos indicam que o vírus está disseminado no rebanho bovino de vários países (DARDIRI; STONE, 1972). MARTIN; GWYNNE (1968) detectaram 33,33% de soropositividade num total de 106 amostras testadas. Em outro estudo realizado na Holanda, 37,7% das amostras testadas foram positivas para anticorpos contra o BoHV-2 (SCOTT; MARTIN, 1978). No Japão detectou-se 0,99% de prevalência de anticorpos contra o BoHV-2 (IMAI et al., 2001). No Brasil, existem poucos relatos sobre a ocorrência da infecção. O BoHV-2 foi isolado em duas ocasiões no país, nas décadas de 70 e 80 (ALICE, 1977; CASTRO et al., 1988). No entanto, casos de mamilite com características clínicas compatíveis com a infecção pelo BoHV-2 têm sido freqüentemente relatados por veterinários de campo (PITUCO, E.M., 2005. comunicação pessoal). Anticorpos contra o BoHV-2 tem sido ainda detectados em bubalinos e várias espécies de animais silvestres no leste da África, mesmo na ausência de sinais clínicos da infecção, sugerindo que as infecções são freqüentemente subclínicas (MARTIN; GWYNNE, 1968).

A infecção já foi reproduzida experimentalmente em camundongos, coelhos e cobaias (PEPPER et al., 1966; SMEE; LEONHARD, 1994) e ovinos e caprinos (WESTBURY, 1981). Ovinos inoculados com o vírus podem desenvolver lesões semelhantes às observadas em bovinos (ALMEIDA et al., 2008). A capacidade do BoHV-2 estabelecer infecções latentes em bovinos foi confirmada por MARTIN; SCOTT (1979). A biologia da infecção latente, no entanto, ainda não foi satisfatoriamente esclarecida, com resultados inconsistentes de reativação experimental da infecção (WESTBURY, 1981; ALMEIDA et al., 2008).

Os relatos de ocorrência de infecções subclínicas (MARTIN; GWYNNE, 1968) e latentes em bovinos (MARTIN; SCOTT, 1979); a identificação da susceptibilidade de caprinos e ovinos à infecção experimental (WESTBURY, 1981) e evidências de que insetos possam ser possíveis vetores (GIBBS et al., 1973) são informações epidemiológicas importantes. Porém, a ausência de estudos que confirmem os mecanismos de transmissão envolvidos, bem como um melhor entendimento da patogenia do ciclo infecção aguda-latência-reativação contribuem para o relativo desconhecimento destes mecanismos comparativamente com outros alfaherpesvírus humanos e animais. Assim, esta dissertação apresenta três estudos independentes que visam contribuir para um melhor conhecimento da epidemiologia e patogenia da infecção pelo BoHV-2. Estes estudos serão apresentados na forma de capítulos:

- Capítulo 1: Prevalência de anticorpos contra o vírus da mamilite herpética em bovinos do Rio Grande do Sul, Brasil.

- Capítulo 2: Distribuição de DNA latente do herpesvírus bovino tipo 2 (BoHV-2) em tecidos de ovinos infectados experimentalmente (*Distribution of latent bovine herpesvirus 2 DNA in tissues of experimentally infected sheep*).

- Capítulo 3: Infecção aguda e latente pelo herpesvírus bovino tipo 2 em cobaias (*Acute and latent infection by bovine herpesvirus type 2 in a guinea pig model*).

2. CAPÍTULO 1

PREVALÊNCIA DE ANTICORPOS CONTRA O VÍRUS DA MAMILITE HERPÉTICA EM BOVINOS DO RIO GRANDE DO SUL, BRASIL.

Fabrício Dias Torres, Lucas Machado Bernardes, Rudi Weiblen, Eduardo Furtado Flores.

(Artigo submetido para publicação na revista Ciência Rural - 2009)

Prevalência de anticorpos contra o vírus da mamilite herpética em bovinos do Rio Grande do Sul, Brasil

Prevalence of antibodies to bovine herpes mammillitis virus in cattle of Rio Grande do Sul state, Brazil.

- NOTA -

RESUMO

O herpesvírus bovino tipo 2 (BoHV-2), agente da mamilite herpética bovina (BHM), possui distribuição mundial e foi identificado no Brasil na década de 70. A partir de então, casos de mamilite clinicamente compatíveis com a BHM têm sido relatados por técnicos e produtores, o que sugere a presença e disseminação do agente no rebanho bovino brasileiro. Este trabalho relata um inquérito sorológico da infecção pelo BoHV-2 no Estado do Rio Grande do Sul (RS), em amostras coletadas como parte do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCBT). Um total de 2.213 amostras de soro coletadas de fêmeas bovinas com idade igual ou superior a 24 meses, predominantemente de rebanhos leiteiros, de 136 municípios pertencentes a sete macroregiões do RS foram testadas para anticorpos anti-BoHV-2 pela técnica de soro-neutralização (SN). Anticorpos anti-BoHV-2 (títulos > 2) foram detectados em 24,5% (543/2.213) das amostras. Os índices de positividade variaram entre 12,9% (22/170) na região metropolitana de Porto Alegre e 48,9% (69/137) na região centro oriental do RS. Em geral, os rebanhos leiteiros apresentaram prevalência maior (32,3%), seguidos dos rebanhos mistos (22%) e de corte (17,6%). Estes resultados demonstram uma ampla disseminação da infecção pelo BoHV-2 no rebanho bovino do Estado, e sugerem a participação do agente em parte dos casos de mamilite descritos em gado leiteiro.

Palavras-chave: herpesvírus bovino 2, BoHV-2, mamilite herpética, soroprevalência.

ABSTRACT

Bovine herpesvirus type 2 (BoHV-2), the agent of bovine herpetic mammillitis (BHM), has a worldwide distribution and was identified in Brazil in the 70's. Thereafter, cases of bovine mammillitis clinically compatible with BHM have been frequently reported by cattle owners and veterinarians, mainly in dairy herds, suggesting the dissemination of BoHV-2 among Brazilian cattle. Herein we performed a serologic survey for BoHV-2 antibodies in cattle from Rio Grande do Sul (RS), using serum samples obtained upon a statistically planned sampling as a part of the Program of Control/Eradication of Brucellosis and Tuberculosis (PNCBT). A total of 2.213 serum samples from cows with 24 months or older, predominantly from dairy herds, from 136 counties located in seven geographical regions of RS were tested by virus neutralization (VN). Serum samples with VN titers > 2 were detected in 24,5% (543/2.213) of the animals. Prevalence rates varied from 12,9% (22/170) in Porto Alegre metropolitan area to 48,9% (69/137) in the central-eastern region. In most regions, the highest prevalence rates were observed in dairy herds (32.3%), followed by double proposit (22%) and beef herds (17.6%). These results demonstrate that BoHV-2 infection is widespread among cattle in RS, and potentially involved in cases of mammillitis described in dairy cows.

Key words: bovine herpevirus 2, BoHV-2, herpetic mammillitis, seroprevalence.

O herpesvírus bovino tipo 2 (BoHV-2), também conhecido como vírus da mamilite herpética, é um vírus DNA, com envelope, pertencente ao gênero *Simplexvirus,* subfamília *Alphaherpesvirinae*, família *Herpesviridae* (ROIZMAN et al., 1992). Uma propriedade biológica importante dos alfaherpesvírus é a capacidade de estabelecer e reativar infecções latentes e, dessa forma, se manter na natureza (FLORES, 2007). A infecção pelo BoHV-2 tem sido associada com duas formas clínicas distintas e bem definidas: lesões vesiculares e erosivas localizadas nas tetas ou no úbere (mamilite herpética) e lesões nodulares disseminadas na pele (*pseudo lumpy skin disease)* (GIBBS & RWEYEMAMU, 1977). As lesões nos tetos e úbere podem ser confundidas com outras infecções víricas

de pele, principalmente as causadas pelo vírus *pseudocowpox* e pelo vírus da *vaccinia* (LOBATO et al., 2005). Essas lesões dificultam a ordenha, predispõem à mastites, resultam em redução da produção leiteira e, ocasionalmente, podem levar ao descarte prematuro das vacas afetadas (GIBBS & RWEYEMAMU, 1977). Não existem vacinas comerciais disponíveis contra o BoHV-2, e as medidas de controle baseiam-se no isolamento dos animais afetados e em cuidados para evitar a transmissão do agente (SMEE & LEONHARDT, 1994). Uma vez introduzida em um rebanho, a infecção pode se perpetuar por meio dos portadores da infecção latente, que ocasionalmente reativam a infecção e a transmitem a animais susceptíveis (GIBBS et al., 1973).

A mamilite herpética bovina (*bovine herpetic mammillitis*, BHM) já foi descrita em vários países e, em alguns deles, parece possuir importante repercussão sanitária e econômica, sobretudo em gado leiteiro (GIBBS & RWEYEMAMU, 1977; SMEE & LEONHARDT, 1994). No Brasil, dois relatos nas décadas de 70 e 80 demonstraram a presença da infecção (ALICE, 1977; CASTRO et al., 1988). Apesar da falta de publicações mais recentes sobre a ocorrência da infecção no país, casos de mamilite com características clínicas compatíveis com a infecção pelo BoHV-2 têm sido freqüentemente relatados por veterinários de campo (KREUTZ, L.C., 2006, informe verbal; PITUCO, E. M. 2004, informe verbal). Assim, o objetivo do presente trabalho foi investigar a prevalência de anticorpos neutralizantes para o BoHV-2 em bovinos do Estado do Rio Grande do Sul.

Foram utilizadas 2.213 de um total de aproximadamente 18.000 amostras de soro coletadas de fêmeas bovinas com idade igual ou superior a 24 meses, por meio de um desenho amostral executado como parte do Programa Nacional de Controle e Erradicação da brucelose e Tuberculose (PNCBT). As amostras foram disponibilizadas pelo Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF). As amostras eram oriundas de rebanhos localizados em 136 municípios, foram separadas proporcionalmente em micro- e meso-regiões dentro de sete macro-regiões, conforme levantamento efetuado pela Secretaria de Agricultura e Abastecimento (SAA) do RS. Do total do rebanho de cada região, foram alocadas de maneira aleatoriamente 0,016% das amostras do rebanho de cada região, sendo agrupadas em categorias

(rebanho de leite, dupla aptidão e corte). Assim, a amostragem é representativa do rebanho bovino do Rio Grande do Sul.

As amostras foram testadas em duplicata pela técnica de soro-neutralização (SN), na qual incubaram-se diluições 1:2 de cada amostra de soro com 100 doses infectantes para 50% dos cultivos celulares ($DICC_{50}$) do BoHV-2 durante 2h, seguido da adição de uma suspensão de células de linhagem de rim bovino (CRIB) e incubação a 33°C em atmosfera de 5% CO_2. A leitura do teste foi realizada após 96h de incubação pelo monitoramento do efeito citopático. A técnica de SN para o BoHV-2 foi descrita com detalhes por ALMEIDA (2008).

Os resultados apresentados na tabela 1 demonstram índices variáveis de soropositivos para o BoHV-2 nas regiões amostradas. Os maiores índices de positividade foram observados na região centro-oriental, que abrange a região de Santa Cruz, Lajeado e Estrela (48,9%) e na região noroeste (48,2%), igualmente caracterizada pela existência de minifúndios e atividade leiteira desenvolvida. Os menores índices foram observados em regiões tradicionalmente dedicadas à pecuária de corte extensiva, como as regiões sudeste (19,7%) e sudoeste (22,6%). Em praticamente todas as regiões, observou-se a tendência de os rebanhos leiteiros apresentarem soroprevalência superior aos rebanhos mistos e de corte (Tabela 1). Embora relatos na literatura indiquem que rebanhos de corte também são afetados pelo BoHV-2, nestes rebanhos a infecção provavelmente possui repercussão econômica inferior à observada em gado leiteiro (GIBBS et al., 1973; SCOTT & MARTIN, 1978).

Os níveis de soropositividade observados revestem-se de especial importância, sobretudo porque sorologia positiva aos herpesvírus, em ausência de vacinação, indica a condição de portador (FLORES, 2007). Assim como em outras infecções herpéticas, o vírus permanece de forma latente nos hospedeiros após a infecção aguda, podendo ser reativado e re-excretado ocasionalmente (GIBBS et al. 1973). Estudos recentes em ovelhas – propostas como modelo experimental – demonstraram a presença de DNA do BoHV-2 em gânglios lombares durante a infecção latente, além de linfonodos regionais. Embora não tenha sido possível provocar a reativação e excreção viral pela administração de corticosteróides,

acredita-se que eventos de reativação e excreção viral, que acompanham episódios de estresse por causas diversas, sejam importantes para a transmissão e manutenção do vírus no ambiente natural (TORRES et al., 2008). A inexistência de vacinas comerciais contra o BoHV-2, aliada à ausência de reatividade sorológica entre o BoHV-2 e outros herpesvírus de ruminantes, também corroboram a observação de que o índice de 24,5% de positividade detectado no presente estudo representa a prevalência de animais portadores da infecção latente.

Em um estudo prévio, detectaram-se níveis de soropositividade semelhantes em bacias leiteiras do RS (27%) e do Paraná (29,8%) (ALMEIDA, S.R. informe verbal). Índices altos de prevalência foram detectados também em outros países como o Kênia (33,3%; MARTIN & GWYNNE, 1968) e Holanda (37,7%; SCOTT & MARTIN, 1978). Os índices de soropositividade observados no presente estudo vêem ao encontro das informações de veterinários de campo, que relatam a ocorrência freqüente de mamilite em vacas leiteiras (KREUTZ, L.C., informe verbal; PITUCO, E.M., informe verbal). As características clínicas descritas geralmente são compatíveis com a enfermidade causada pelo BoHV-2, embora outras infecções virais também possam cursar com quadros clínicos semelhantes, incluindo as causadas pelo vírus da varíola bovina, vírus *vaccinia* e vírus da pseudovaríola, infecções também já identificadas no Brasil (JACKSON, 1993; LOBATO et al., 2005). Devido a ocorrência dessas infecções que produzem quadros clínicos semelhantes à BHM, estudos adicionais envolvendo rebanhos de outros estados são necessários para fornecer dados mais abrangentes sobre a situação real e a relevância da infecção pelo BoHV-2 no rebanho leiteiro do país.

A importância sanitária da mamilite herpética é reconhecida em diversos países, principalmente em rebanhos leiteiros. O manejo intensivo desses animais pode favorecer a disseminação do vírus e da enfermidade. Acredita-se que a aglomeração de animais aliada à determinadas práticas de manejo durante a ordenha sejam importantes para a disseminação do agente (GIBBS & RWEYEMAMU, 1977; SCOTT & MARTIN, 1978).

Durante a análise dos resultados do presente estudo, um padrão de distribuição das amostras positivas ficou bem evidente: a concentração de amostras positivas em determinadas propriedades – nas quais uma grande parte dos animais era reagente - contrastando com propriedades negativas, muitas vezes localizadas na mesma macro-região. Esse tipo de distribuição – a ocorrência de rebanhos positivos e negativos numa mesma região, muitas vezes com proximidade geográfica – é característico de infecções que são transmitidas por contato direto ou indireto (FLORES, 2007). Embora as formas de transmissão do BoHV-2 ainda não sejam bem conhecidas, acredita-se que a transmissão ocorra principalmente por contato indireto, ou de forma indireta, com a participação das mãos dos ordenhadores, copos das ordenhadeiras e, mesmo, com a participação de insetos (moscas, mosquitos), que atuariam como vetores mecânicos (GIBBS et al., 1973). Assim, a transmissão entre rebanhos, mesmo vizinhos, aparentemente não ocorre com freqüência, ao contrário de transmissão entre animais de um mesmo rebanho, que parece ser mais freqüente (GIBBS et al., 1973).

Em resumo, os resultados dos testes sorológicos indicam que a infecção pelo BoHV-2 está amplamente difundida no rebanho bovino do Estado do RS com prevalência de 24,5%, e que os rebanhos leiteiros apresentam uma prevalência maior do que os rebanhos mistos e de corte. Após os primeiros relatos nas décadas de 70 e 80, este trabalho confirma a presença e disseminação do vírus no país.

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TABELA 1. Anticorpos neutralizantes para o herpesvírus bovino tipo 2 (BoHV-2) em bovinos do Estado do Rio Grande do Sul, de acordo com macro-regiões e aptidão dos rebanhos.

Macro-região	Leite	Misto	Corte	Total
Noroeste (49)[a]	94/329 (28,6%)	12/57 (21,1%)	2/26 (7,7%)	108/412 (26,2%)
Nordeste (11)	48/97 (49,5%)	20/44 (45,5%)	—[b]	68/141 (48,2%)
Centro Ocidental (14)	11/47 (23,4%)	27/179 (15,0%)	0/20 (0%)	38/246 (15,4%)
Centro Oriental (13)	32/81 (39,5%)	35/51 (68,6%)	0/5 (0%)	67/137 (48,9%)
Metropolitana de Porto Alegre (18)	8/62 (12,9%)	8/88 (9,1%)	6/20 (30%)	22/170 (12,9%)
Sudoeste (14)	40/117 (34,2%)	20/149 (13,4%)	113/501 (22,6%)	173/767 (22,6%)
Sudeste (17)	40/111 (36%)	24/97 (24,7%)	3/132 (2,3%)	67/340 (19,7%)
(136 municípios)	273/844 (32,3%)	146/665 (22%)	124/704 (17,6%)	**543/2213 (24,5%)**

[a]Número de municípios amostrados

[b]Não Amostrado

3. CAPÍTULO 2

DISTRIBUTION OF LATENT BOVINE HERPESVIRUS 2 DNA IN TISSUES OF EXPERIMENTALLY INFECTED SHEEP.

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Distribution of latent bovine herpesvirus 2 DNA in tissues of experimentally infected sheep

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Abstract

The biology of latent infection by bovine herpesvirus 2 (BoHV-2), the agent of mammillitis in cows, remains largely unknown. We herein report attempts to reactivate the latent infection and investigated the sites of BoHV-2 latency in experimentally infected sheep. Ewes inoculated with BoHV-2 in the udder's skin shed virus for up to five days, developed mammillitis and seroconverted. However, attempts to reactivate latent infection by dexamethasone administration at day 40 pi failed. Nevertheless, viral DNA – and non infectious virus - was detected by PCR in several nerve ganglia and/or regional lymph nodes (LNs) of all animals at day 40 post-reactivation. Likewise, lambs previously inoculated with BoHV-2 in the nose harbored latent viral DNA in trigeminal ganglia, tonsils and regional LNs. These results demonstrate that BoHV-2 establishes latent infection in nerve ganglia and in regional lymphoid tissues, yet virus reactivation is not easily achieved by standard protocols used.

Keywords: bovine herpesvirus 2, BoHV-2, latent infection, sheep.

1. Introduction

Bovine herpesvirus type 2 (BoHV-2) is the etiologic agent of bovine herpetic mammillitis (BHM), a vesicular, erosive and necrotic disease of the udder and teats of cows. The virus belongs to the subfamily Alphaherpesvirinae, genus Simplexvirus and is closely related to human herpes simplex viruses (HSV-1 and HSV-2) and other primate herpesviruses (Roizman et al., 1992; Ehlers et al., 1999). Initially identified in Africa in 1957, BoHV-2 infection has been shown to be widespread among cattle worldwide. Serological and/or virological data have demonstrated the presence of the infection in several countries, including the United States (Dardiri & Stone, 1972), Britain (Johnston et al., 1971), Brazil (Alice, 1977), Kenya (Martin & Gwynne, 1968), Australia (Turner et al., 1974) and Japan (Imai et al., 2005), among others. Positive serology to BoHV-2 has

also been detected in wild ruminants yet their potential role in the epidemiology of the infection is unclear (Plowright & Jesset., 1972). BoHV-2 infection has also been associated with a generalized skin nodular condition called pseudo-lumpy skin disease (PLSD) (Gibbs & Rweyemamu, 1977).

In nature, BoHV-2 infection occurs mainly in heifers and young cows at first lactation and may be subclinical or accompanied by a relatively mild disease (Turner et al., 1976; Scott & Holliman, 1984). Nonetheless, the infection frequently results in painful skin lesions characterized by erythema, edema, vesicle formation and deep ulceration. These lesions may range from small papules to large vesicles and extensive ulceration of the skin of the udder and teats (Martin et al., 1966; 1969; Lechtworth & LaDue, 1982). Although BHM is frequently benign and self-limiting, it may result in a significant drop in milk production, increase in the susceptibility of the mammary gland for bacterial mastitis and even the culling of productive cows due to chronic mastitis (Lechtworth & LaDue, 1982; Wellenberg et al., 2002). Small vesicular lesions may also develop in the muzzle of calves suckling on affected cows. Sheep and goats are susceptible to experimental BoHV-2 infection (Westbury, 1981) yet their possible role in the epidemiology of the infection has not been investigated.

The early reports of BHM in Europe and United States described a marked seasonal occurrence with most cases occurring during the fall season (Martin et al., 1966; 1969; Lechtworth & LaDue, 1982). Epidemiological evidence supported by some laboratory data suggests that BoHV-2 may be mechanically transmitted by insect vector like the common stable fly *Stomoxys calcitrans* (Gibbs et al., 1973) yet a definitive proof of this mode of transmission is still lacking. It has been shown that the intact skin surface is an effective barrier against experimental infection by BoHV-2 and the involvement of the dermis is essential for the development of severe lesions (Martin et al., 1969). This argues against transmission by the milking machine or human hands during manual milking, except when many cows present teat lesions concomitantly (Gibbs et al., 1973). In any

case, the source of infection in many outbreaks is not readily apparent and probably involves reactivation of pre-existing latent infection (Gibbs et al., 1973; Martin & Scott, 1979).

In contrast with other animal and human alphaherpesviruses, the latent infection by BoHV-2 has been poorly studied and many aspects of its biology remain obscure. Experimental reactivation of the infection has not been consistently demonstrated (Probert & Povey, 1975; Turner et al., 1976; Castrucci et al., 1980) and attempts to recover the virus upon explant cultures of sensory nerve ganglia have failed (Lechtworth & Carmichael, 1980). In some of the studies demonstrating latency and reactivation, biases may have been introduced by inoculating the virus intravenously (Martin & Scott, 1979; Castrucci et al., 1982). Likewise, the sites in which the virus may remain latent remain unknown (Lechtworth & Carmichael, 1980).

Sheep are susceptible to experimental BoHV-2 infection, develop lesions similar to those developed by cattle, and thus have been used to study several aspects of the pathogenesis of BoHV-2 infection (Westbury, 1981; Almeida et al., 2008). In a recent study, our group described the reproduction and characterization of acute mammillitis in lactating ewes inoculated with BoHV-2 in the skin of the udder and teats. Nevertheless, attempts to demonstrate virus latency through reactivation by dexamethasone (Dx) treatment failed (Almeida et al., 2008). Thus, the objective of the present study was to demonstrate BoHV-2 latent infection in lactating ewes and lambs inoculated experimentally, and to investigate the distribution of latent viral DNA in neural and non-neural tissues.

2. Material and methods

2.1. Cells and virus

All procedures of virus amplification, isolation from swabs, quantitation and virus-neutralizing (VN) assays used a MDBK (Madin-Darby bovine kidney) derived cell line named CRIB (Flores & Donis, 1995). Cells were cultivated in MEM (minimum essential minimum, Gibco,

BRL), containing ampicillin (1.6mg/L), streptomycin (0.4mg/L) and nistatin (0.02mg/L) supplemented with 5% horse serum. The BoHV-2 strain used for animal inoculation was provided by Dr. Fernando Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska at Lincoln, Lincoln, NE, USA). For optimizing virus replication, all procedures of virus growth were performed in cells cultured at 33ºC (O'Connor, 1985).

2.2. Experiment # 1 – BoHV-2 infection in lactating ewes

Seven lactating ewes, previously tested negative for BoHV-2 by VN, were inoculated with BoHV-2 and two remained as uninfected controls. The ewes were inoculated into the skin of teats and udder. Previous to inoculation, the local skin was submitted to mild abrasion by friction with a rough cleaning sponge. Then, a 2 mL inoculum containing $10^{5.6}$ $TCID_{50}$/mL of the virus was uniformly distributed over the skin upon using a cotton swab, followed by a slight friction. The controls were inoculated with MEM upon the same treatment. The animals were clinically examined on a daily basis for local and systemic signs of infection. Swabs collected from the sites of virus inoculation and lesions during 14 days were submitted to virus isolation in CRIB cells, according to standard protocols. The specimens were considered negative for virus after three passages of five days each without the appearance of cytopathic effect (CPE).

Serum samples collected on the day of inoculation and at day 28pi were submitted to standard VN assays, testing two-fold dilutions of sera against a fixed dose of virus (100-200 $TCID_{50}$/well). CRIB cells were used as indicators of virus growth. Virus neutralizing titers were expressed as the reciprocal of the highest dilution of sera that prevented the production of CPE.

Forty days after virus inoculation, two inoculated ewes and one control were euthanized for tissue collection. The genito-femoral (L3-L4); ileo-inguinal (L3-L2) and ileo-hipogastric (L2-L1) ganglia plus the supramammary, deep inguinal, medial iliac and prefemoral lymph nodes (LNs) were individually and aseptically collected for virus isolation and DNA extraction for PCR. At the

same day, the remaining five inoculated ewes plus one control were submitted to five daily administrations of Dx (Decadronal, Prodone Laboratories, SP, Brazil. 0.5mg/kg/day). Animals were monitored for 15 days, as described during acute infection. Forty days after Dx treatment, these ewes were euthanized for tissue collection and examination for virus and viral DNA.

2.3. Experiment # 2 – BoHV-2 infection in lambs

This experiment used lambs inoculated with BoHV-2 into the nostrils, in which the acute infection was characterized in its virological and clinico-pathological aspects (Almeida et al., 2008). Forty days after virus inoculation, the inoculated lambs (n = 10) plus the controls (two lambs inoculated with MEM) were submitted to five daily administrations of dexamethasone (0.25mg/kg/day). The animals were monitored clinically and nasal swabs for virus isolation were collected up to day 14 post-Dx and processed as described above. Forty days after Dx treatment, lambs were euthanized for tissue collection. Trigeminal ganglia (TG) and regional LNs were submitted to virus isolation and DNA extraction for PCR.

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, 8th , 1979). The experiments were approved by an institutional committee on Ethics on Animal Welfare and Experimentation (UFSM – Comitê de Ética e Experimentação Animal: process #23081-013798/2006-04).

2.4. DNA extraction and PCR

Approximately 50 to 100 mg of each tissue section were minced with a sterile razor blade and submitted to DNA extraction using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After extraction, the DNA was solubilized in 60µl of Tris-EDTA buffer and stored at – 20°C until testing. The DNA concentration was measured by UV absorbance at 260 nm.

Amplification of viral DNA sequences was performed by using a semi-nested PCR for the glycoprotein B (gB) gene of BoHV-2. This strategy was used to improve the sensitivity of the assay and adequate measures to avoid contamination were adopted. All tissue collections were aseptically performed using individual needles and blades and carefully processed to avoid cross-contamination. Known positive and negative tissues controls were included in each round of DNA extraction. These DNA controls, plus a DNA from infected cell culture and water negative control were included during the PCR assays.

The primers were designed based on the GeneBank (access number M21628 sequence). The target region (624 bp) was initially amplified with the external primers (forward) 5'-CTCCAGCGACGATCCTAATTT-3' (position 6528) and (reverse) 5'-TATGCGTTGTGCTCTGAGTG- 3' (position 7151). The second reaction used 2 µl of the first reaction as template and the same forward primer with an internal reverse primer 5'–CGGTGGTCTCAAGGTTGTTC–3' (position 6874), resulting in an internal fragment of 374 bp. Both PCR reactions were performed in a 25 µl volume, using 2 µl of template DNA (total DNA extracted from 50 - 100 mg of tissue), 0.5µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 1 x reaction buffer and 0.75 units of Taq polymerase (Invitrogen). The PCR conditions were: initial denaturation (94ºC for 5 min), followed by 35 cycles of 94ºC - 50 sec; 64ºC - 40 sec for primer annealing and 72ºC - 45 sec for primer extension; and a final extension of 7 min at 72ºC. PCR products were visualized in a 1.5% agarose gel, stained with ethidium bromide under UV light. In all reactions, DNA extracted from CRIB cells infected with BoHV-2 and from trigeminal ganglia of one lamb inoculated intranasally with BoHV-2 (Almeida et al., 2008) were used as positive controls. DNA extracted from TGs and parotideal LN of one control lamb was used as negative control. The identity of PCR amplification was determined by submitting PCR products obtained from lumbar ganglia of one ewe (#134) and from a TG of one lamb (# 01) to nucleotide sequencing. Tissues that

were positive by PCR were subsequently submitted to virus isolation in CRIB cells, as described for specimens collected during acute infection and following Dx administration.

3. Results

3.1. Acute and latent BoHV-2 infection in lactating ewes

The main virological and serological findings during acute infection of inoculated ewes are summarized in Table 1. Infectious virus was detected in swabs collected from the lesions of four ewes and shedding was detected from day 4 up to day 8 p.i., with the highest frequency of virus detection at day 7. Five ewes developed detectable VN titers (2 to 8) when examined serologically at day 28 pi. One ewe (# 01) shed virus for two days yet did not seroconvert. The other ewe failing to seroconvert (# 144) did not shed detectable amounts of virus. Low VN titers (or even lack of seroconversion detectable by VN) has been observed in other BoHV-2 inoculation in sheep and might reflect the restriction of virus replication to local sites (Almeida et al. 2008)

The body temperature of all ewes remained unaltered during the course of acute infection. All seven inoculated ewes developed moderate to severe signs of mammillitis. The clinical signs were first observed at day 3 pi, and were initially characterized by focal hyperemia and edema of the teats and udder. On day 4 pi, the hyperemia and edema were more pronounced and small vesicles (approximately 1 – 2 mm in diameter) appeared. The vesicles progressively increased in size, turned into pustules and eventually became coalescent and covered with a fibrinopurulent secretion, with an ulcerative aspect. During this period, new vesicles were appearing. Large brownish scabs appeared late in infection and gradually subsided after day 8 p.i. The severity of lesions and signs increased up to day 5 to 8 pi and then declined progressively until day 10 pi, when only reminiscent lesions were present. In the ewes that shed virus, the period of virus shedding coincided with the clinical course.

No infectious virus was recovered from swabs collected from the sites of virus inoculation after day 9 p.i., nor in the day preceding Dx administration (day 40 p.i.). Two ewes (# 01 and 04) were euthanized prior to Dx treatment (day 40 p.i.) for tissue collection. No infectious virus could be recovered from the sites of virus inoculation in the days following Dx administration. Close examination of these sites did not reveal any evidence of clinical recrudescence. Furthermore, serological examination of ewes at day 28 p.r. failed to demonstrate significant increase in VN titers following Dx treatment (Table 1).

The distribution of BoHV-2 DNA in regional nerve ganglia and lymph nodes of inoculated ewes is presented in Table 2. Viral DNA was detected in at least one lumbar ganglia of all animals, with frequencies varying among animals. The ganglia of ileo-inguinal were more consistently positive for viral DNA (7/14), followed by genitofemoral (6/14) and ileo-hipogastric (3/11). Viral DNA was also detected in regional LNs of all animals, the suprammamary being the most frequent (7/14), followed by deep inguinal (4/14), prefemoral (2/14) and iliac (1/6) (Table 2). Nucleotide sequencing of the amplicon obtained from ewe # 134 and lamb # 01 showed a near 100% homology with a BoHV-2 sequence deposited in Genebank. We were unable to demonstrate infectivity in any of the DNA positive tissues upon inoculation of macerates into CRIB cells.

These results show that, following virus replication in the skin of the udder and teats, BoHV-2 DNA is present in several lumbar nerve ganglia and regional LNs during latent infection. Attempts to reactivate the virus upon DX treatment, however, were unsuccessful.

3.2. Acute and latent BoHV-2 infection in lambs

The characterization of acute infection and the attempts to reactivate the latent infection by BoHV-2 in lambs have been previously reported (Almeida et al., 2008). Briefly, inoculation of BoHV-2 ($10^{5.1}TCID_{50}$/ml) with help of a cotton swab in the nose of lambs resulted in viral replication, shedding and the development of local vesicular and erosive lesions in seven out of ten

animals. Virus shedding was detected in swabs collected from lesions and nose of seven lambs, between days 2 and 8 p.i., and lasted one to three days. Mild signs of respiratory infection (nasal discharge, vesicles) were observed in most animals between days 3 and 8 p.i. All inoculated animals seroconverted to BoHV-2, developing VN titers from 2 to 8 at day 28 p.i. In that study, attempts to reactivate the infection by Dx administration at day 40 p.i. were fruitless since no virus shedding or seroconversion were observed after Dx treatment (Almeida et al., 2008).

The distribution of BoHV-2 DNA in nerve ganglia and LNs of inoculated lambs, collected 40 days post-Dx (day 80 p.i.) is summarized in Table 3. Viral DNA was detected in the TG and/or lymphoid tissues of all animals, with varied frequencies among the lambs. The TGs were more consistently positive (7/10); tonsils were positive in three cases. Viral DNA was also detected in some regional LNs, the parotideal LN being the most frequently positive (6/10), followed by mandibular (5/10) and lateral retropharyngeal (4/10). All tissues positive for viral DNA were negative for infectious virus, as ascertained by failure to detect infectivity by virus isolation.

4. Discussion

The results presented herein demonstrate that BoHV-2 DNA is present in regional nerve ganglia and lymph nodes of sheep 40 days after virus inoculation and 40 days after Dx administration. The presence of viral DNA in the absence of infectivity fulfills the biological requirements to define latent infection. Nevertheless, in spite of the presence of latent DNA in nerve ganglia, Dx administration – in a similar protocol used to reactivate other animal herpesviruses (Silva et al., 1999; Vogel et al., 2003; 2004; Diel et al., 2007; Henzel et al., 2008) - failed to induce BoHV-2 reactivation. Thus, it is reasonable to speculate that reactivation of BoHV-2 latency in sheep may require stimuli either quantitatively and/or qualitatively different from those used to reactivate other alphaherpesviruses. To our knowledge, this is the first report on molecular detection

and distribution of latent BoHV-2 DNA. On the other hand, these findings clearly demonstrate that the biology of BoHV-2 latent infection is poorly understood compared to its counterparts.

The biology of latent infection by BoHV-2 has been difficult to characterize compared to other alphaherpesviruses. Experimental studies trying to demonstrate and characterize the latent infection by BoHV-2 virus have yielded conflicting results. Virus reactivation and shedding upon corticosteroid treatment was demonstrated in calves several months after intravenous or intradermal inoculation (Martin & Scott, 1979; Castrucci et al., 1982). Other studies have failed to induce virus reactivation by corticosteroid treatment (Probert & Povey, 1975; Castrucci et al., 1980) and to recover virus by explant cultures of nasal mucosa and sensory nerve ganglia (Lechtworth & Carmichael, 1984). In the present and also in a previous experiment (Almeida et al., 2008), none of the inoculated sheep shed virus or seroconverted following Dx administration, in spite of active virus replication and seroconversion observed during acute infection. The lack of reactivation was demonstrated by absence of virus shedding and/or seroconversion. At first glance, one would argue whether the virus was capable of establishing latent infection in these animals after acute infection. The detection of viral DNA in nerve ganglia clearly demonstrated the colonization of these sites with latent viral genomes. All inoculated animals (ewes and lambs) presented at least one site positive for viral DNA and, in most cases, more than one ganglion and/or LN were positive. Examination of tissues from two ewes (# 01 and 04) euthanized prior to Dx treatment also revealed several DNA positive sites. No major differences in the frequency of detection and distribution of viral DNA were observed between these and the group of ewes examined at day 40 post-Dx.

The reasons for the failure to reactivate the infection upon corticosteroid treatment are merely speculative. The drug and protocol of Dx treatment were essentially the same used to reactivate BoHV-5 in sheep (Silva et al., 1999) and goats (Diel et al., 2007); and to reactivate BoHV-1 and BoHV-5 in calves (Vogel et al., 2003). In the present experiment, lambs received a

2.5-fold and ewes received a 5-fold Dx dose compared to that routinely used to reactivate BoHV-1 in cattle (Winckler et al., 1999, Vogel et al., 2003). In our previous experiment (Almeida et al., 2008) the same dose used in lambs failed to reactivate the infection in inoculated ewes. The failure to induce virus reactivation by Dx may also reflect particular virus-host interactions. Sheep are not the natural hosts of the virus and differences in the biology of the infection - reflecting peculiar interactions of the virus with different species - might occur. It is possible that virus/host interactions leading to the establishment and reactivation of latency are not identical to those observed during infection of natural hosts. Reactivation of BoHV-5 infection in rabbits (Caron et al., 2002) and goats (Diel et al., 2007), for example, and PRV reactivation in pigs (Thawley et al., 1984) require unusually high Dx doses. The use of alternative protocols and drugs to induce virus reactivation, as well as more sensitive techniques (e.g. virus detection in latency tissues during the expected course of virus reactivation) may be able to induce and/or to demonstrate BoHV-2 reactivation. In any case, BoHV-2 reactivation in nature likely occurs as a mean to assure virus perpetuation and transmission, as for other alphaherpesviruses (Rock, 1993).

The major sites of alphaherpesvirus latency are sensory nerve ganglia innervating the sites of primary infection. In our study, latent viral DNA was readily detected in several lumbar ganglia of inoculated ewes and in the TGs of inoculated lambs (Tables 2 and 3). These lumbar ganglia receive sensory and autonomic nerves distributed in the mammary gland and skin (Dyce et al., 1997). Even in the animals that apparently did not seroconvert to BoHV-2 after virus inoculation (ewes # 01 and 144), latent viral DNA was demonstrated in nerve ganglia. In three lambs (# 106, 110 and 128), we were unable to detect viral DNA in TGs, yet these animals harbored at least one PCR positive LN or tonsil. It is possible that the amount of DNA in these nerve ganglia were below the detection limit of our PCR.

In addition to sensory and autonomic nerve ganglia, persistent/latent alphaherpesvirus DNA has been detected in other neural and non-neural sites as well. Latent human herpes simplex virus DNA has been detected in human nodose ganglia, vagus nerve, bone marrow and occasionally in the central nervous system (Lohr et al., 1990; Cantin et al., 1994; Croen, 1991). Besides the TG, pseudorabies virus (PRV) latent DNA has been detected in tonsils and in some brain regions (Wheeler & Osorio, 1991; Cheung, 1995). Latent BoHV-1 DNA has been detected in neural (TG, CNS) and also in lymphoid tissues (tonsils, regional lymph nodes) and cells (PBMCs) following nasal and genital infection (Winckler et al., 2000; Lovato et al., 2000; Vogel et al., 2004; Henzel et al., 2008). Thus, our findings that BoHV-2 DNA is present in regional lymphoid tissue (LNs, tonsils) both after nasal or udder skin inoculation are not surprisingly. The biological significance of latent infection in non-neural sites is unclear since virus reactivation from these sites has not been demonstrated (Steiner & Kennedy, 1995; Lovato et al., 2000). Nonetheless, recovery of infectious virus from blood of calves upon corticosteroid treatment several months after IV inoculation (Martin & Scott, 1979) suggests that BoHV-2 might reactivate from lymphoid tissues.

In summary, we demonstrated that acute BoHV-2 infection of ewes (udder's skin) and lambs (nose and muzzle) is followed by establishment of latent infection in nerve ganglia. Latent viral DNA was also detected in some regional lymphoid tissues. Latent infection could not be readily reactivated by routine drug and protocols used to reactivate other alphaherpesviruses, suggesting that BoHV-2 reactivation in sheep might require qualitatively and/or quantitatively different stimuli. The cycle latency-reactivation likely represents a major means of BoHV-2 perpetuation in nature and its understanding is critical towards the adoption of measures to control the infection in the field.

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Table 1 – Virological and serological findings during acute infection and after dexamethasone (Dx) treatment in ewes inoculated with bovine herpesvirus type 2 in the udder and teats.

	Acute infection			Post Dx treatment[a]		
Animal #	Viral shedding[c]	VN antibodies[b]		Viral shedding	VN antibodies	
		Day 0	Day 28 pi		Day 0	Day 28 pDx
01	6 -7	<2	<2	-	<2	Eut[d]
02	4 -7	<2	2	-	4	2
03	5 -7	<2	8	-	4	8
04	-	<2	4	-	8	Eut
134	-	<2	2	-	2	2
142	4 -8	<2	2	-	2	2
144	-	<2	<2	-	<2	<2
C1	-	<2	<2	-	<2	<2
C2	-	<2	<2	-	<2	<2

[a] Dexamethasone treatment began at day 40 pi;

[b] Virus neutralizing antibodies expressed as the reciprocal of the highest serum dilution capable of preventing CPE;

[c] First day pi of virus shedding/duration of shedding (days);

[d] Euthanized before Dx treatment.

Table 2 - Detection of bovine herpesvirus type 2 DNA by semi-nested PCR in neural and non neural tissues collected from experimentally infected ewes.

Tissue	Animal #								
	01	02	03	04	134	142	144	C1	C2
Dorsal root (lumbar) ganglion of nerve:									
Ileo-hipogastric	(+[a] / -[b])	(- / -)	(- / +)	(- / -)	(+ / -)	(nt[c] / -)	(nt / nt)	(- / -)	(- / -)
Ileo-inguinal	(+ / -)	(- / -)	(- / -)	(+ / +)	(+ / +)	(+ / -)	(+ / -)	(- / -)	(- / -)
Genitofemoral	(+ / -)	(+ / -)	(+ / -)	(- / -)	(+ / +)	(+ / -)	(- / -)	(- / -)	(- / -)
Lymph node									
Supramammary	(+ / +)	(+ / +)	(- / -)	(+ / -)	(+ / +)	(- / -)	(- / -)	(- / -)	(- / -)
Deep inguinal	(+ / +)	(- / -)	(+ / -)	(- / -)	(+ / -)	(- / -)	(- / -)	(- / -)	(- / -)
Iliac[d]	+	-	-	nt	-	-	-	-	-
Prefemoral	(- / -)	(- / -)	(- / -)	(+ / -)	(- / -)	(+ / -)	(- / -)	(- / -)	(- / -)

[a] Positive; [b] Negative; [c] not tested; [d] only one tissue.

Table 3 - Detection of bovine herpesvirus type 2 DNA by semi-nested PCR in neural and non-neural tissues collected from lambs inoculated into the nostril and muzzle[a].

Tissue	Animal #											
	101	102	104	106	110	128	132	134	135	137	C1	C2
Trigeminal ganglia	+[a]	+	+	-[b]	-	-	+	+	+	+	-	-
Lymph nodes												
Parotideal	+	+	-	+	-	+	+	+	-	-	-	-
Retropharyngeal	+	-	-	-	+	-	+	+	-	-	-	-
Mandibular	+	+	-	-	-	-	+	+	+	-	-	-

[a] The acute infection and attempts to reactivate latent infection in these animals have been described previously (Almeida et al., 2008). [b] Positive, [c] Negative

4. CAPÍTULO 3

ACUTE AND LATENT INFECTION BY BOVINE HERPESVIRUS TYPE 2 IN A GUINEA PIG MODEL.

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8 **Acute and latent infection by bovine herpesvirus type 2 in a guinea pig model.**

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Abstract

Bovine herpetic mammillits (BHM) is a self-limiting disease of cows associated with bovine herpesvirus type 2 (BoHV-2) infection. The disease has a worldwide distribution, yet its pathogenesis is poorly understood. Herein we evaluate guinea pigs (*Cavia porcellus*) as an experimental model to study the biology of acute and latent infection by BoHV-2. Twelve weanling female guinea pigs (30-40 days-old) inoculated subcutaneously with BoHV-2 in the genital area and on the teats developed clinical signs (hyperemia, edema, vesicles, scabs) and shed virus between days 3 and 7 post-infection (pi). Virus was more consistently recovered from swabs collected from the genital area (9/12) than teat lesions (1/12). All animals seroconverted to BoHV-2, developing virus neutralizing (VN) titers from 16 to 128 at day 30 post-infection (pi). Histological examination of skin biopsies collected from lesions at different intervals showed intranuclear inclusion bodies and perivascular infiltrates composed mainly by lymphocytes and plasmocytes. PCR examination of tissues collected from all animals euthanized at day 35 pi revealed the presence of latent viral DNA in lumbosacral nerve ganglia and in regional lymph nodes. In a paralel experiment, eight females (60 days-old) previously inoculated in the genital area were treated with dexamethasone (Dx) at day 35pi, trying to reactivate latent infection. Dx administration was followed by apparent recrudescence of genital disease in some animals, yet no viral shedding or seroconversion could be demonstrated. Taken together these results demonstrate that guinea pigs are susceptible to BoHV-2, and that experimental infection reproduces many clinical and virological aspects of acute infection and disease. Moreover, acute infection is followed by the establishment of latent infection in regional nerve ganglia. Thus, this species may be potentially used to study selected aspects of BoHV-2 biology.

Keywords: bovine herpesvirus 2, BoHV-2, latent infection, guinea pig.

1. Introduction

Bovine herpesvirus type 2 (BoHV-2) is the etiologic agent of herpetic mammillitis (BHM), a vesicular, erosive and necrotic disease of the udder and teats of cows (Martin & Scott, 1979). BoHV-2 is an alphaherpesvirus, genus *Simplexvirus*, closely related to human herpes simplex viruses (HSV-1 and HSV-2) and other primate herpesviruses (Roizman et al., 1992; Ehlers et al., 1999). Initially identified in Africa in 1957, BoHV-2 infection has been shown to be widespread among cattle in several countries (Martin & Gwynne, 1968; Johnston et al., 1971; Dardiri & Stone, 1972; Turner et al., 1974; Imai et al., 2005; Torres et al., 2009b).

In nature, BoHV-2 infections occur mainly in cows at first lactation and may be subclinical or accompanied by a relatively mild disease (Turner et al., 1976; Scott & Holliman, 1984). In some cases, the infection results in painful skin lesions characterized by erythema, edema, vesicle/pustule formation and deep ulceration of the skin of the udder and teats (Martin et al., 1966; 1969; Lechtworth & LaDue, 1982). Although BHM is frequently benign and self-limiting, it may result in a significant drop in milk production, increase in the susceptibility of the mammary gland for bacterial mastitis and even the culling of productive cows due to chronic mastitis (Lechtworth & LaDue, 1982; Wellenberg et al., 2002).

The early reports of BHM in Europe and United States described a marked seasonal occurrence with most cases occurring during the fall (Martin et al., 1966; 1969; Lechtworth & LaDue, 1982). In some outbreaks in Ireland, meteorological condition (with over rainfall) seemed to be determinant for the occurrence of the disease (O'Connor, 1995). Epidemiological evidence supported by some laboratory data suggests that BoHV-2 may be mechanically transmitted by insect vector like the common stable fly *Stomoxys calcitrans* (Gibbs et al., 1973), yet a definitive proof of this mode of transmission is still lacking. The source of infection in many outbreaks is not readily

apparent and probably involves reactivation of pre-existing latent infection (Gibbs et al., 1973; Martin & Scott, 1979).

Although latent infection probably plays an important role in the perpetuation of BoHV-2 in nature, the biological and molecular basis of latency are poorly understood. Experimental reactivation of the infection has not been consistently demonstrated (Probert & Povey, 1975; Turner et al., 1976; Castrucci et al., 1980) and attempts to recover the virus upon explant cultures of sensory nerve ganglia have failed (Lechtworth & Carmichael, 1982). Likewise, the sites in which the virus may remain latent in the natural host remain unknown (Lechtworth & Carmichael, 1982).

Sheep are susceptible to experimental BoHV-2 infection and have been used to study the pathogenesis of acute and latent infection (Westbury, 1981; Almeida et al., 2008; Torres et al., 2009a). Our group described the reproduction and characterization of acute mammillitis in lactating ewes (Almeida et al., 2008) and the characterization of latent infection in lambs and ewes, including a fine mapping of the distribution of latent viral DNA in tissues (Torres et al., 2009a). Nonetheless, BoHV-2 latency in sheep remained unsolved, including the unability of current protocols to reactivate the virus (Torres et al., 2009a). Guinea pigs have been used to study some aspects of BoHV-2 biology and the efficacy of anti-viral vaccines (Smeed & Leonhard, 1994). The use of animal models may help in understanding BoHV-2 pathogenesis, including the latency-reactivation cycle. Animal models may be also useful for antiviral drug and vaccine testing. As BoHV-2 is closely related to HSV-1 and HSV-2, it has been proposed as a model to *simplexviruses* (Ehlers et al., 1999). Thus, in the present study we characterized the acute and latent infection by BoHV-2 in guinea pigs, thereby proposing this species as an animal model to study the biology of BoHV-2.

2. Material and methods

Two experiments were conducted independently. The first experiment comprised the characterization (clinico-pathological, virological and serological) of the acute infection following inoculation of 12 females into the genital area and in the skin of the teats. After acute infection (day 35 post-infection), the presence of latent viral DNA was investigated by PCR in neural and non-neural regional tissues. The second experiment investigated the reactivation of latency by dexamethasone (Dx) administration in eight animals previously inoculated with BoHV-2 in the genital area.

2.1 Cells and virus

All procedures of virus amplification, isolation from swabs, quantitation and virus-neutralizing (VN) assays used a MDBK derived cell line named CRIB (Flores & Donis, 1995). Cells were cultivated in MEM, containing ampicillin (1.6mg/L), streptomycin (0.4mg/L) and nistatin (0.02mg/L) supplemented with 5% horse serum. The BoHV-2 strain used for animal inoculation was provided by Dr. Fernando Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska at Lincoln, Lincoln, NE, USA). For optimizing virus replication, all procedures of virus growth were performed in cells cultured at 33ºC (O'Connor, 1985).

2.2 Experiment #1 – Characterization of acute infection and mapping of latent viral DNA

Twelve weanling female guinea pigs, 30 days-old, previously tested negative for BoHV-2 antibodies by VN, were inoculated with the virus and three remained as controls. The animals were inoculated subcutaneously in the teats or genital area by using an insulin needle (13 x 4.5 mm), with 0.1mL of a viral suspension containing $10^{5.1}$ $TCID_{50}$ (median tissue culture infectious dose). The controls were inoculated with the same volume of MEM. The animals were clinically examined on a

daily basis for local signs of disease. Swabs collected from the sites of virus inoculation were submitted to virus isolation in CRIB cells, according to standard protocols. The specimens were considered negative for virus after three passages of five days each without producing cytopathic effect (CPE). Skin biopsy from inoculated sites collected at days 3pi (one animal), 4pi (two animals), 5pi (three animals) and 8pi (two animals) were submitted to histological examination according to standard techniques.

Serum samples collected on the day of inoculation and at day 28pi were submitted to standard VN assays, testing two-fold dilutions of sera against a fixed dose of virus (100-200$TCID_{50}$/well). CRIB cells were used as indicators of virus replication. Virus neutralizing (VN) titers were expressed as the reciprocal of the highest dilution of sera that prevented the production of CPE.

Thirty five days after virus inoculation, all animals were euthanized for tissue collection. The segment of lumbo-sacral spinal cord between L1 and L3, and between L3 and the end of sacral segment, comprehending ileo-inguinal nerve ganglia and pudendal nerve ganglia, respectively, plus the iliac lymph nodes, were individually and aseptically collected for DNA extraction for PCR.

2.3 DNA extraction and PCR

The collected tissues were initially minced with a sterile razor blade and submitted to DNA extraction using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After extraction, the DNA was solubilized in 60µl of Tris-EDTA buffer and stored at –20°C until testing. The DNA concentration was measured by UV absorbance at 260 nm.

Amplification of viral DNA sequences was performed by using a semi-nested PCR for the glycoprotein B (gB) gene of BoHV-2 (Torres et al., 2009a). All tissue collections were aseptically performed using individual needles and blades and carefully processed to avoid cross-

contamination. Known positive and negative tissues were included in each round of DNA extraction. These DNA controls, plus a DNA from infected cell culture and water negative control were included during the PCR assays.

The primers were designed based on the GeneBank (access number M21628 sequence). The target region (624 bp) was initially amplified with the external primers (forward) 5'-CTCCAGCGACGATCCTAATTT-3' (position 6528) and (reverse) 5'-TATGCGTTGTGCTCTGAGTG- 3' (position 7151). The second reaction used 2 µl of the first reaction as template and the same forward primer with an internal reverse primer 5'–CGGTGGTCTCAAGGTTGTTC–3' (position 6874), resulting in an internal fragment of 374 bp. Both PCR reactions were performed in a 25 µl volume, using 2 µl of template DNA (total DNA extracted from 50 - 100 mg of tissue), 0.5µM of each primer, 2.5 mM MgCl2, 0.2 mM of dNTPs, 1 x reaction buffer and 0.75 units of Taq polymerase (Invitrogen). The PCR conditions were: initial denaturation (94ºC for 5 min), followed by 35 cycles of 94ºC - 50 sec; 64ºC - 40 sec for primer annealing and 72ºC - 45 sec for primer extension; and a final extension of 7 min at 72ºC. PCR products were visualized in a 1.5% agarose gel, stained with ethidium bromide under UV light. In all reactions, DNA extracted from CRIB cells infected with BoHV-2 and from trigeminal ganglia of one lamb inoculated intranasally with BoHV-2 (Almeida et al., 2008) were used as positive controls. DNA extracted from TGs and parotideal LN of one control lamb was used as negative control.

The identity of PCR amplification was determined by submitting PCR products obtained from L1-L3 of one animal (#1) of the first experiment to nucleotide sequencing. Tissues that were positive by PCR were subsequently submitted to virus isolation in CRIB cells, as described for specimens collected during acute infection and following Dx administration.

2.4 Experiment #2 – Attempts to reactivate latent infetion

In the second experiment, eight 60-days-old female guinea pigs were inoculated with a viral suspension containing $10^{5.1}$ $TCID_{50}$ in the genital area, using the same protocol described in the first experiment; three additional animals were inoculated with MEM (control group). The animals were monitored as described above. At day 35 pi, all animals were treated with Dx (Decadronal, Prodone Laboratories, SP, Brazil. 2.0 $mg.kg^{-1}.day^{-1}$) via intramuscular for five consecutive days. These animals were monitored thereafter during 14 days, as described in experiment 1. Serum was collected at day the first day of Dx administration and at day 28 post-reactivation (pr) to assay for VN antibodies. These animals were euthanized at day 30 pr for tissue collection for PCR.

3. Results

3.1 Experiment #1

Table 1 summarizes the main findings during acute infection in twelve weanling female guinea pigs experimentally inoculated with BoHV-2. All inoculated animals developed clinical signs in both sites, yet the signs were more severe in the genital area (Figure 1B and C). The observed signs were hyperemia and edema, progressing to the development of vesicles that subsequently erupted and gave place to scabs. In general, the lesions first appeared between days 2 to 3 pi, increasing in severity for one or two days, lasting for 3 to 4 days and regressing by days 5 to 7-8 pi.

Histological examination of biopsies collected from teat lesions in alternate days from different animals revealed pustular ulcerative dermatitis, with perivascular lymphoplasmocytic inflammatory infiltrate, ulceration and amphiphylic viral intranuclear inclusion bodies (Figure 2).

Virus isolation was more consistently achieved from swabs collected from genital lesions (8/12) than from inoculated teats (1/12). Virus shedding was first detected at days 3-4pi and lasted from one to 4 days (Table 1).

Serological testing at day 28 pi revealed seroconversion in moderate to high titers in all inoculated animals (VN titers ranging from 16 to 128). Control animals (C1, C2 and C3) remained healthy, did not show any clinical signs nor seroconverted during the experiment (Table 1).

As expected, BoHV-2 established latent infection in neural tissues after acute infection. Table 2 presents the distribution of viral DNA – detected by a semi-nested-PCR – in neural and non-neural tissues of inoculated animals at day 35 pi. Viral DNA, in the absence of infectious virus (as demonstrated by failure to isolate virus), was detected in both neural sites consistently examined (lumbar 11/12, sacral 10/12). Among the examined LNs, only two specimens were positive (Table 2).

Taken together, these results demonstrate that weanling female guinea pigs are susceptible to experimental BoHV-2 infection (as demonstrated by virus replication and seroconversion), develop clinical signs of infection (mainly in the genital area) and harbor viral DNA in lumbar and sacral nerve segments during latent infection.

3.2 Experiment #2

The clinical monitoring of animals from Experiment 2 during acute infection revealed mild to moderate edema, hyperemia and some small vesicles and ulcers. Lesions appeared between days 3 and 4, lasting for up to 4 days, with the same aspect observed in the first experiment. However, no infectious virus could be recovered from lesions in the days following virus inoculation. Serological examination of sera at day 30 pi revealed seroconversion (VN titers from 16 to 256) in all inoculated animals, demonstrating that virus replication indeed took place.

After Dx treatment at day 35 pi, animals developed mild to moderate clinical signs, including edema, hyperemia, small vesicles and ulceration. Three animals (#5, 6 and 8) presented moderate edema, scabs formation, hyperemia and noticeable pain (Figure 1D). The lesions began at day 4 pr., increasing in severity after 1 to 2 days, regressing after 3 to 4 days. However, as observed during acute infection, no virus shedding could be detected in swabs collected from the inoculated areas. Likewise, no increase in VN titers was observed after Dx treatment. Controls animals remained healthy and did not shed virus or seroconverted during the experiment.

4. Discussion

Our experiments demonstrate that weanling female guinea pigs are susceptible to BoHV-2 infection upon experimental inoculation. The inoculated animals shed virus in secretions, developed clinical signs - mainly in the genital area – during acute infection and harbored viral DNA in neural tissues during latent infection. Previous articles described the susceptibility of guinea pigs to BoHV-2, testing a mutant thymidine kinase (TK) deleted virus as a vaccine candidate (Smeed & Leonhard, 1994) and for antiviral drug testing (Smee et al., 1994). However, to our knowledge the present article is the first detailed description of acute infection and disease and latent infection by BoHV-2 in guinea pigs. Moreover, these results suggest that this animal species may represent a suitable experimental model to study selected aspects of BoHV-2 biology, including pathogenesis, immunology, vaccine and antiviral drug testing, among others. On the other hand, the lack of virus reactivation upon Dx treatment used in other animal herpesviruses – fact already demonstrated for BoHV-2 in sheep (Almeida et al., 2008) – represent an interesting finding worthwhile of further investigation.

The first experiment demonstrated that BoHV-2 is capable of replicating efficiently in the genital submucosa – and to a lesser extent in the subcutaneous skin of teats - of weanling female

guinea pigs (30 days-old). Virus replication - monitored by virus isolation -, clinical signs (hyperemia, edema, vesicles, scabs) were somewhat similar to those described in cows naturally (Kemp et al., 2008) and experimentally infected (Castrucci et al., 1982); and following experimental infection of lactating ewes (Almeida et al., 2008). The overall clinical evolution with the appearance of hyperemia and edema in early stages, followed by the development of vesicles that erupted giving rise to necrotic areas was already described in cows (Kemp et al., 2008) and in a sheep model (Almeida et al., 2008). The major difference was the duration of clinical course, noticeably shorter in this animal model. In sheep, lesions develop after 4 to 5 days after virus inoculation, lasting for 5 to 6 days (Almeida et al., 2008). In contrast, guinea pigs developed genital hyperemia and edema as early as at day 2 pi, increasing in severity for approximately 24 h and rapidly subsiding thereafter. Only mild, reminiscent lesions were still observed at days 3 to 4 pi. The preference of BoHV-2 for replication in mucosal surfaces (genital, in this case) rather than in intact skin has been previously reported and likely reflects the nature of each epithelial surface (dry ceratinized skin surface versus mucosal epithelium) (Smeed & Leonhard, 1994; Dyce, 1997; Almeida et al., 2008). This difference could be readily observed by comparing the virus isolation from genital area versus skin (teats) swabs (Table 1). A more efficient virus replication in mucosal surfaces than in intact skin has also been observed upon inoculation of lambs with BoHV-2 in the nose (Almeida et al., 2008).

Another noticeable difference in BoHV-2 experimental infection in sheep and guinea pigs was the magnitude of the VN response. While sheep usually develop low to moderate titers (up to 16), guinea pigs developed higher (16 to 256). The reasons for this discrepancy may be interesting to investigate as might reflect differences in host susceptibility and/or peculiarities in the immunological response to the virus.

The microscopic examination of skin biopsies demonstrated that BoHV-2 replication in guinea pig tissues resulted in lesions histologically similar to those develop in cows (Letchworth &

Carmichel, 1984) and sheep (Almeida et al., 2008). In particular, intranuclear inclusion bodies in ceratinocytes (Figure 2D) and lymphoplasmocytic inflammatory cellular infiltrate (Figure 2A) were the most prominent histological findings in all phases of acute infection.

In the first experiment, clinical signs and virus shedding during acute infection were more pronounced than in the second group, despite de same virus dose and inoculation protocol. Although purely hypothetical and based solely in one observation, it is tempting to speculate that the age (weanling, 30-days-old versus 60-days-old) of the animals might somehow influence their susceptibility and the outcome of virus infection. Thus, in any case weanling guinea pigs rather than older animals should be preferred to study the biology of BoHV-2.

The failure to reactivate the latent infection by standard Dx protocols – used successfully for a number of other alphaherpesviruses – might be related to specific host-virus interactions (BoHV-2-guinea pigs) and/or to specific mechanisms underlying the interactions of BoHV-2 with non-natural hosts, since a similar finding has been observed in sheep (Almeida et al., 2008; Torres et al., 2009a). Since no virus could be detected in swabs collected after Dx treatment (and no seroconversion was detected), the nature of the clinical signs developed in this phase is unknown. These signs are likely not related to the activity of the drug on the animal since the controls remained healthy. Even though virus reactivation was not achieved (or demonstrated), latent infection was definitively and conclusively shown by detection of virus DNA – in the absence of concomitant virus replication – in neural and non-neural tissues. In other words, latent infection was demonstrated – and the latency sites mapped – yet virus reactivation was not achieved by the methods used. In the future, alternative drugs and/or protocols should be tested to demonstrate virus reactivation.

In summary, we describe acute clinical infection and demonstrate latent infection in guinea pigs experimentally inoculated with BoHV-2. These findings support the use of this species to study

selected aspects of BoHV-2 biology. As BoHV-2 shares many biological and genetic similarities with HSV-1 and HSV-2, this animal model may be used for early drug and/or vaccine testing. The issue of virus reactivation – which is not readily achieved in animal models upon routine protocols – remains unsolved and, as such and due to its relevance for BoHV-2 epidemiology, deserves further investigation.

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Table 1. Clinical signs, viral shedding and virus neutralizing (VN) antibodies in guinea pigs experimentally infected with bovine herpesvirus type 2 in the perigenital area and teats.

Animal #	Clinical signs		Viral shedding day (duration)		VN titer	
	Genital	Teats	Genital	Teats	Day 0	Day35pi
1	e, h, x,d, u, v	e, h, c	4 (1)	-	<2	64
2	e, h, x	h, c	-	5 (1)	<2	32
3	e, h, d, c, v, u	e, h, d	4 (1)	-	<2	32
4	e, h, d	e, h	4 (1)	-	<2	64
5	e, h, d, v, c	e, h	3 (2)	-	<2	64
6	e, h, d, v, u, x	e, h	4 (4)	-	<2	64
7	e, h, v, u, n	e, h	4 (1)	-	<2	128
8	e, h, c, u	e, h, c	-	-	<2	32
9	e, h, d	e, h	-	-	<2	16
10	e, h, v, n	h	4 (1)	-	<2	64
11	h, e, v, x	h	3 (2)	-	<2	128
12	e, h, v, x, u	e, h	-	-	<2	128
C1	-	-	-	-	<2	<2
C2	-	-	-	-	<2	<2
C3	-	-	-	-	<2	<2

e: edema; h: hyperemia, d: hyperesthesia; v: vesicles; u: ulcers; x: exudates c: crusts; n: necrosis.

Table 2 – Distribution of bovine herpesvirus type 2 DNA detected by semi-nested PCR in neural and non-neural tissues collected from guinea pigs inoculated into the genital area and teats.

Tissue	Animal #														
	1	2	3	4	5	6	7	8	9	10	11	12	C1	C2	C3
Ganglia															
Lumbar dorsal root	+ [a]	+	+	+	+	+	+	+	- [b]	+	+	+	-	-	-
Sacral dorsal root	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-
Lymph node															
Iliac	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-

[a] Positive, [b] Negative

Figure 1

A) Genital area of animal control (C1); B) Inoculated animal #6 at day 5 pi.: genital area presenting edema, hyperemia, vesicles (arrows), ulcer and exsudate. C) Inoculated animal #6 at day 7 pi.: genital area presenting edema, hyperemia, vesicles (arrows), ulcer and exsudate. D) Inoculated animal #8 at day 6 pr.: genital area presenting severe edema, hyperemia, necrosis and ulcers (arrows).

Figure 2

Histological findings of pustular ulcerative dermatitis by BoHV-2 in teat skin biopsies of guinea pigs. A) Inoculated animal #7: severe neutrophilic exocytosis in the epidermis; B) Focal ulceration of epidermis, with scab formation and severe inflammatory neutrophilic and lymphoplasmacytic infiltrate; C) Ulcerative pustular dermatitis, with severe endothelial swelling, congestion, multifocal hemorrhagic, inflammatory infiltrate lymphoplasmacytic and fibrosis. D) Inoculated animal #8: amphophylic viral intranuclear inclusion bodies in keratinocytes (arrows).