VANESSA LOPES DIAS QUEIROZ DE CASTRO

DETECÇÃO DO *bovine herpesvirus* 1 EM ÓRGÃOS GENITAIS DE VACAS NATURALMENTE INFECTADAS E NO INTERIOR DE OVÓCITOS INFECTADOS *in vitro*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para a obtenção do título de *Doctor Scientiae*.

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APROVADA: 23 de julho de 2018.

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Eduardo Paulino da Costa
(Orientador)

“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original”

Albert Einsten

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BIOGRAFIA

Vanessa Lopes Dias Queiroz de Castro, natural de Jacareí – São Paulo, filha de José Ramos de Queiroz e Leni Maria Lopes Dias Queiroz, casada com Nero Lemos Martins de Castro. Graduada em Medicina Veterinária em janeiro de 2012 pela Universidade Federal de Viçosa. Atuou dois anos como bolsista de Iniciação Científica, sob orientação do Prof. Eduardo Paulino da Costa, na área de Reprodução Animal. Mestre em Ciência Animal pela Universidade Federal de Mato Grosso do Sul, sob orientação da Professora Eliane Vianna Costa e Silva. Obteve o título em fevereiro de 2014, com o título: Hormônio anti-Mülleriano sérico como preditor da puberdade de touros jovens Nelore. Nos dias atuais, defende a tese para obter o título de *Doctor Scientiae*.

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RESUMO GERAL

QUEIROZ-CASTRO, Vanessa Lopes Dias, D.Sc., Universidade Federal de Viçosa, julho de 2018. **Detecção do *bovine herpesvirus* 1 em órgãos genitais de vacas naturalmente infectadas e no interior de ovócitos infectados *in vitro*.** Orientador: Eduardo Paulino da Costa. Coorientadores: Abelardo Silva Junior, José Domingos Guimarães e Mariana Machado Neves.

Bovine herpesvirus 1 (BoHV-1) pertence a ordem *Herpesvirales*, família *Herpesviridae*, subfamília *Alphaherpesvirinae* e gênero *Varicellovirus*. É o agente causador da Rinotraqueíte Infecciosa Bovina (IBR). Apresenta dominância cosmopolita e alta disseminação em rebanhos bovinos de todo o mundo, acarretando prejuízos econômicos associados aos problemas produtivos e reprodutivos. O objetivo desta pesquisa foi estudar a interação do BoHV-1 com o útero, ovários e tubas uterinas, além de investigar a presença e a capacidade de penetração do BoHV-1 em complexos *cumulus*-ovócitos de vacas não vacinadas contra o BoHV-1, por meio da imunomarcação viral utilizando a microscopia confocal de varredura a laser. Coletou-se amostras de sangue e os órgãos genitais de 75 vacas abatidas em frigorífico e avaliou-se a imunomarcação viral nos tecidos uterino, tubárico e ovariano. Total de 719 COCs foram divididos em dois grupos: COCs derivados de vacas soropositivas e de vacas soronegativas e processados para a microscopia confocal de varredura a laser. Além disso, este estudo investigou a capacidade de penetração do BoHV-1 nos ovócitos de vacas soronegativas. Para tal, os ovócitos foram divididos em dois grupos: Grupo I: COCs (n=312) e ovócitos desnudados (n=296) infectados experimentalmente com BoHV-1 e incubados durante uma hora a 38,5°C e 5% de CO_2. O controle negativo formado por COCs (273) e ovócitos desnudados (n=310) foram submetidos ao mesmo protocolo, exceto a exposição ao BoHV-1 que não foi realizada. Grupo II: COCs (n=425) e ovócitos desnudados (n=405) foram submetidos ao mesmo protocolo de co-incubação com o BoHV-1, porém durante 24 horas. O controle negativo deste grupo foi formado por 398 COCs e 425 ovócitos desnudados submetidos ao mesmo protocolo exceto a exposição ao BoHV-1. Os resultados gerados nesta tese apresentam um grande significado para a área de reprodução animal. Os achados sugerem novos esclarecimentos sobre a infecção do BoHV-1 nos tecidos reprodutivos. As partículas virais do BoHV-1 foram detectadas no útero de 100% das vacas soropositivas ($P<0,01$). As tubas uterinas apresentaram o BoHV-1 em 73,2% e os ovários em 58,5% ($P>0,05$). Não foi observada a presença do vírus em nenhum órgão genital dos 34 animais soronegativos. Nenhum COC (n=276) proveniente de vacas soronegativas apresentou o

vírus. No entanto, foi evidenciada a presença do BoHV-1 no citoplasma das células do *cumulus* em alta porcentagem de COCs (n=158) dos 443 COCs aspirados de vacas soropositivas. Em relação aos ovócitos com e sem a presença das células do *cumulus*, co-incubados com o BoHV-1, o vírus foi evidenciado no seu interior após 24 horas. Conclui-se que, aparentemente, este estudo é o primeiro a relatar a detecção de BoHV-1 no útero em 100% das vacas soropositivas, o que sugere que este órgão atua como uma fonte de infecção fetal e esteja implicado em desempenhar um papel no abortamento. A detecção do BoHV-1 nas células do *cumulus* de vacas soropositivas, sugere que vacas infectadas naturalmente e assintomáticas podem apresentar os COCs infectados pelo vírus. Ademais, este estudo é o primeiro a detectar o BoHV-1 no interior de ovócitos bovinos apresentando a zona pelúcida intacta.

ABSTRACT

QUEIROZ-CASTRO, Vanessa Lopes Dias, D.Sc., Universidade Federal de Viçosa, July, 2018. **Detection of bovine herpesvirus 1 in genital organs of naturally infected cows and into in vitro infected oocytes.** Adviser: Eduardo Paulino da Costa. Co-Advisers: Abelardo Silva Junior, José Domingos Guimarães and Mariana Machado Neves.

Bovine herpesvirus 1 (BoHV-1) belongs to the *Herpesvirales* order, *Herpesviridae* family, *Alphaherpesvirinae* subfamily and *Varicellovirus* genus. It is the causative agent of Infectious Bovine Rhinotracheitis (IBR). Its cosmopolitan distribution and high dissemination in cattle herds around the world lead to economic losses related to productive and reproductive disorders. This research aimed to study the interaction of BoHV-1 in the uterus, oviducts, and ovarian tissues, also to investigate the ability of virus penetration in cumulus-oocyte complexes (COCs) from cows unvaccinated against BoHV-1 by immunofluorescence using confocal laser scanning microscopy. Blood samples and genital organs were collected from 75 cows to assess viral immunolabeling in the uterus, oviducts, and ovarian tissues. A total of 719 COCs were divided into two groups: COCs derivate from seropositive cows and those from seronegative cows. Then, the samples were processed for confocal microscopy analysis. Moreover, this study investigated the penetration capacity of BoHV-1 in oocytes from seronegative cows. Subsequently, oocytes were divided into two groups: Group I: COCs and denuded oocytes experimentally infected with BoHV-1 and incubated for 1 h at 38.5 °C and 5% CO_2. The negative control group were formed by COCs (n = 273) and denuded oocytes (n = 310), which were subjected to the same protocol except for exposure to BoHV-1. Group II: COCs (n = 425) and denuded oocytes (n = 405) were subjected to the same co-incubation protocol with BoHV-1, but for 24 h. The negative control of this group consisted of 398 COCs and 425 denuded oocytes that were subjected to the same protocol except exposure to BoHV-1. The results generated in this thesis have a great significance for the field of animal reproduction. The findings suggest new insights regarding the BoHV-1 infection in reproductive tissue. The virus was detected in the uterus in 100% of seropositive cows ($P<0.01$), 73.2% of oviduct samples and 58.5% of ovarian tissue samples ($P>0.05$). No genital organ samples from the 34 seronegative animals had virus presence. None of 276 COCs from the seronegative cows presented BoHV-1. However, BoHV-1 was present in the cytoplasm of cumulus cells from 158 out of 443 COCs recovered from seropositive cows. The virus was detected after 24h co-incubation with the BoHV-1within oocytes (with or without the presence of *cumulus* cells). In conclusion,

the virus detection of 100% in the uterine samples suggests this organ as a fetal infection source to be implicated in playing a role in abortion. The detection of BoHV-1 in the COCs of seropositive cows suggests that COCs from naturally infected and asymptomatic cows may be infected with BoHV-1. Moreover, this study appears to be the first to detect BoHV-1 within COCs and denuded oocytes exhibiting intact ZP, co-incubated with the virus for 24 h.

1. INTRODUÇÃO GERAL

O Brasil possui, hodiernamente, o maior rebanho comercial de bovinos do mundo, com efetivo animal de 218,2 milhões de cabeças no ano de 2016 (IBGE, 2017). Paralelamente, o país assumiu papel de destaque quanto às biotecnologias da reprodução animal, sendo considerado, no ano de 2015, o maior produtor mundial de embriões *in vitro*, com a marca de 275.918 unidades, representando 61,57 % de todos os embriões bovinos produzidos *in vitro* no mundo (IETS, 2016).

Apesar de milhões de embriões bovinos produzidos *in vitro* serem transferidos comercialmente, sem reportar a transmissão de doenças, estes riscos devem ser considerados. O entendimento do potencial de influência das doenças infecciosas nas biotécnicas reprodutivas, principalmente em embriões e gametas, acarreta preocupações, já que estes podem atuar como potenciais disseminadores de agentes infecciosos no rebanho (Perry, 2005).

Neste contexto, ressalta-se o *bovine herpesvirus* 1 (BoHV-1), agente etiológico da Rinotraqueíte Infecciosa Bovina (IBR). Este patógeno é endêmico em rebanhos bovinos de todo o mundo (Ackermann e Engels, 2005; Nandi et al., 2011; Ravishankar et al., 2013). Tem sido descrita elevada prevalência de rebanhos soropositivos em países europeus: 40 a 50 % na Grã-Bretanha, 62 % na Bélgica e 74,9 % na Irlanda, conforme relatos de Raaperi et al. (2010) e Cowley et al. (2011). Nos Estados Unidos da América, o BoHV-1 foi relatado como o diagnóstico mais frequente de abortamento oriundo de causa viral (Kirkbride, 1992).

Dados nacionais, obtidos a partir de levantamentos sorológicos, revelaram prevalência de 42,2 % em São Paulo (Mueller et al., 1981), 52,9, 71,3 e 81,7 % no Rio Grande do Sul (Ravazzolo et al., 1989; Vidor et al., 1995 e Piovesan et al., 2013, respectivamente). No estado de Minas Gerais, estudo envolvendo 335 municípios, demonstrou que em 93,4 % houve a presença de animais soropositivos (Rocha et al., 2001). No Paraná, 71,3 % dos 2.018 rebanhos não vacinados, foram positivos para o BoHV-1 (Dias et al., 2013). No Estado de Goiás, estudo com 6.932 animais apresentou soroprevalência de 51,9 % e do total das propriedades amostradas, 98,5 % apresentaram, pelo menos, um animal soropositivo, enquanto que 100 % dos municípios pesquisados apresentaram pelo menos uma propriedade positiva, evidenciando a disseminação deste agente (Barbosa et al., 2005).

Tendo em vista a elevada frequência da infecção, este agente causa perdas econômicas significativas à pecuária, interferindo no desempenho produtivo e reprodutivo. O prejuízo econômico gerado pela infecção é enorme e diverso. Estima-se um gasto financeiro médio de US$ 379,00 por vaca infectada (Can et al., 2016).

O difícil controle desta doença infecciosa relaciona-se a capacidade dos *Alphaherpesvirus* estabelecerem latência. Esta principal característica biológica induz, nos animais, após a primoinfecção, o estado de portadores vitalícios e potenciais transmissores do vírus, devido aos episódios de re-excreção viral. Os animais portadores abrigam o genoma viral na forma episssomal, no gânglio trigêmeo e sacral e, sob condições de estresse ou de terapia com corticoide, o BoHV-1 pode ser reativado, com consequente liberação de progênies virais infectantes (Kramps et al., 1996; Jones et al., 2011; El-Mayet et al. 2017). Desta forma, o número de animais infectados tende a ser significativamente maior que o daqueles que apresentam sintomatologia clínica (Pellet e Roizman, 2013).

A vacinação é recomendada em locais onde a infecção por *bovine herpesvirus* 1 é endêmica (Patel, 2005). Entretanto, a resposta sorológica é indistinguível daquela induzida pela infecção natural, exceto quando é utilizada a vacina com marcador antigênico para o BoHV-1, a qual permite esta distinção. Nesta vacina marcada o gene que codifica a glicoproteína gE é deletado e este vírus mutante é o utilizado na vacina. Assim, com o uso de um teste imunoenzimático específico, é possível distinguir os animais vacinados dos infectados com o vírus de campo por meio da observação da presença ou ausência da glicoproteína (Franco e Roehe, 2007). No entanto, esta vacina não é comercializada no Brasil (Flores e Cargnelutti, 2012; Pritchard et al., 2003). Ademais, as vacinas são capazes apenas de diminuir os sinais clínicos da doença e não impedem o desenvolvimento da fase latente da infecção (Galeota et al., 1997).

No contexto reprodutivo, o BoHV-1 pode determinar repetição de estros a intervalos regulares ou irregulares, natimortalidade, mortalidade perinatal, redução da fertilidade, infertilidade temporária, morte embrionária ou abortamento (Miller e Van der Maaten, 1986; Muylkens et al., 2007).

Adicionalmente, durante os últimos anos, foi demonstrado que o BoHV-1 pode estar presente no material utilizado no sistema de produção *in vitro* de embriões (Vanroose et al., 1999), em tecido ovariano (Pereira et al., 2015), associado às células epiteliais da tuba uterina (Bielanski et al., 1993), no fluido folicular (Weber et al., 2013) e nos espermatozoides de touros infectados (Rocha et al., 1998).

Estudos demonstraram que a presença do BoHV-1 em um sistema de produção *in vitro* de embriões afetou a fertilização e o desenvolvimento embrionário (Bielanski et al.,

1997; Vanroose et al., 1999; Makarevich et al., 2007). Ademais, ovócitos oriundos de vacas soropositivas sem sintomatologia clínica apresentaram comprometimento na taxa de maturação nuclear ovocitária, evidenciando a influência da presença do BoHV-1 (Mendes, 2012).

Tendo em vista a importância da infecção pelo BoHV-1 na reprodução de fêmeas bovinas e a ausência de estudos da presença do vírus nos órgãos genitais de vacas infectadas naturalmente, este trabalho teve como objetivo avaliar a presença ou ausência do BoHV-1 nos órgãos genitais de vacas infectadas naturalmente, assim como investigar a eficiência da zona pelúcida como barreira à infecção viral em ovócitos bovinos infectados *in vitro* por meio de ensaios de imunofluorescência utilizando microscopia confocal de varredura a laser.

2. REVISÃO DE LITERATURA

2.1 *Bovine herpesvirus* 1 (BoHV-1)

O BoHV-1 pertence à ordem *Herpesvirales*, família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus.* É o agente causador da Rinotraqueíte Infecciosa Bovina (IBR). A partícula viral apresenta o diâmetro entre 70 e 110 nm e é constituída por um capsídeo icosaédrico, um envelope glicoproteíco e DNA linear de fita dupla. O DNA de cadeia dupla codifica cerca de 70 proteínas, entre enzimas virais, proteínas estruturais, não estruturais e reguladoras (Fenner, 1987; Davison et al., 2009).

O envelope viral é composto por dez glicoproteínas, denominadas gB, gC, gD, gE, gG, gH, gI, gK, gL e gM, as quais apresentam propriedades antigênicas, moleculares e atuam nos processos de interação com a célula hospedeira (Schwyzer e Ackermann, 1996). A Figura 1 ilustra a estrutura geral de uma partícula viral dos herpesvírus.

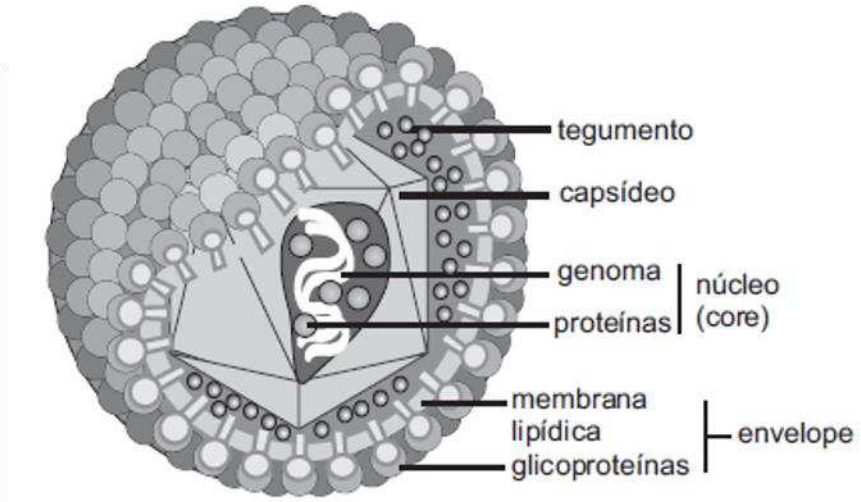

Figura 1: Ilustração da partícula viral da família *Herpesviridae* e seus componentes. Fonte: Franco e Roehe (2007).

A IBR foi descrita pela primeira vez na Alemanha, durante o século XIX (Graham, 2013), sendo que, no Brasil, ela foi registrada, inicialmente, no estado da Bahia, em 1962, e o agente etiológico foi isolado pela primeira vez em 1978, a partir de pústulas vaginais de vacas (Alice, 1978).

Estudos utilizando enzimas de restrição possibilitaram a divisão do BoHV-1 em dois subtipos, BoHV-1.1 e BoHV-1.2, este último em 2a e 2b. A maioria das cepas de

BoHV1.1 estão relacionadas com distúrbios respiratórios, reprodutivos e casos de conjuntivite. O subtipo BoHV-1 2a tem sido associado a abortos e infecções fetais e o subtipo 2b é menos virulento e incomum no Brasil (Muylkens et al., 2007).

As mucosas do sistema respiratório superior, do epitélio conjuntival e dos órgãos genitais são conhecidos sítios de entrada do BoHV-1. O vírus apresenta glicoproteínas em seu envelope responsáveis pela adsorção e fusão com a membrana da célula hospedeira. Posteriormente, como é comum para todos os vírus, adentra no ambiente intracelular e inicia um ciclo replicativo lítico de modo a permitir sua expressão gênica para a produção de novas progênies virais. A célula hospedeira morre e há liberação de grande quantidade dessas progênies por meio das secreções corpóreas (Muylkens et al., 2007).

A transmissão pode ocorrer por inalação de aerossóis contaminados ou por contato direto com secreções nasais de animais infectados. Ambas as formas de transmissão são consideradas importantes na disseminação do vírus (Van Donkersgoed e Babiuk, 1991). A transmissão indireta ocorre, principalmente, pela ingestão de água e alimentos contaminados e pelo uso nas coletas de sêmen de vaginas artificiais contaminadas (Engels e Ackermann, 1996).

Outra forma de transmissão é a venérea, que pode ocorrer pela monta natural ou por meio da inseminação artificial. Nesta última, o sêmen desempenha papel fundamental na cadeia epidemiológica (Philpott, 1993). O sêmen pode se contaminar durante a ejaculação, quando o líquido seminal entra em contato com a mucosa prepucial contaminada ou ainda pela técnica de transferência de embriões, em que o patógeno pode estar associado à zona pelúcida do ovócito ou estar presente nos meios de manipulação de embriões (D'Angelo, 1998).

A infecção caracteriza-se por diversas formas clínicas, que comprometem os órgãos respiratório e reprodutivo. Nas fêmeas, o BoHV-1 pode determinar repetição de estros a intervalos regulares/irregulares, abortamentos, natimortalidade, mortalidade perinatal, redução da fertilidade e infertilidade temporária (Miller e Van Der Maaten, 1986). Os abortamentos causados por este agente viral são, frequentemente, observados do quarto ao oitavo mês de gestação e sugere-se que estes sejam causados a partir do carreamento do agente infeccioso por meio de leucócitos sanguíneos até as veias placentárias. A morte fetal ocorre dentro de 24 horas após a entrada do vírus nas veias (Givens, 2006).

Mais especificamente, a forma genital da infecção manifesta-se clinicamente pelo aparecimento de pequenas vesículas, de um a dois milímetros de diâmetro, que evoluem para pústulas e erosões na vulva e vagina. O epitélio vulvar apresenta-se edemaciado,

hiperêmico e com secreção, que pode vir a tornar-se mucopurulenta devido à contaminação bacteriana secundária. Esta condição clínica é chamada vulvovaginite pustular infecciosa. Em touros, lesões similares são encontradas no prepúcio e pênis, condição patológica conhecida como balonopostite pustular infecciosa (Miller e Van Der Maaten, 1986).

Além disso, o BoHV-1 pode, também, causar lesões necrosantes nos ovários, principalmente se a infecção ocorrer no período da ovulação, afetando o corpo lúteo em formação, com consequente redução da concentração de progesterona, resultando em falha na prenhez (Gibbs e Rweyemann, 1977; Miller e Van Der Maaten, 1986).

O BoHV-1, assim como todos os vírus pertencentes a subfamília *Alphaherpesvirinae,* é capaz de estabelecer infecções latentes nos gânglios neuronais após a infecção primária, tornando os animais portadores vitalícios e potenciais disseminadores (Kramps et al., 1996; Winkler et al., 2000; Jones et al., 2000). O número de animais na fase de infecção latente tende a ser significativamente maior que aqueles que apresentam sintomatologia clínica (Pellet e Roizman, 2013). A imunossupressão do hospedeiro acarreta a recrudescência do BoHV-1, seguida de episódios de re-excreção viral, tornando-se de fácil transmissão e difícil controle (Jones et al., 2011; El-Mayet et al., 2017).

2.2 Epidemiologia da infecção pelo BoHV-1

O BoHV-1 é um agente cosmopolita e apresenta alta disseminação em rebanhos bovinos de todo o mundo (Ackermann e Engels, 2005; Nandi et al., 2011; Ravishankar et al., 2013).

Na maioria dos países europeus, a situação é endêmica e as taxas de infecção descritas são muito variáveis. Na Grã-Bretanha, o percentual de rebanhos soropositivos atinge 40 a 50 % e, na Bélgica, 62 %. Na Estônia, grande parte dos rebanhos leiteiros é endemicamente infectada com o BoHV-1 (Raaperi et al., 2010). No Reino Unido, rebanhos de corte e leite irlandeses atingiram 74,9 % de soropositividade (Cowley et al., 2011).

Na Índia, o governo tomou medidas para rastrear todas as amostras de sêmen de touros, a fim de detectar a presença de organismos causadores de doenças sexualmente transmissíveis, incluindo o BoHV-1, antes de permitir a utilização da dose na inseminação artificial. Estudos ressaltaram 38,6 % de soropositividade (Nandi et al., 2011). Ao norte

do país, 96,92 % dos reprodutores avaliados apresentaram anticorpos neutralizantes e, destes, 61,54 % apresentaram o DNA viral no sêmen, indicando que nem todos os touros soropositivos estavam na fase de excreção viral (Ravishankar et al., 2013).

Na América do Norte, a infecção apresenta caráter endêmico, sendo que os sinais clínicos são controlados por meio de programas de vacinação. No Canadá, foram descritas taxas de 37,8 % para animais e 59,5 % para rebanhos infectados pelo BoHV-1 (Durham e Hassard, 1990). Nos Estados Unidos da América, este patógeno foi relatado como o diagnóstico mais frequente de abortamento oriundo de causa viral (Kirkbride, 1992).

No Oriente Médio, na região central do Irã (província Esfahan), a soroprevalência atingiu 72 % em áreas de produção leiteira mais importante do país (Shirvani et al., 2012).

Na América do Sul, a soroprevalência foi de 82,1 % no Equador (Carbonero et al., 2011). No Brasil, dados regionais, obtidos a partir de levantamentos sorológicos, revelaram 42,2 % de animais reagentes no Estado de São Paulo (Mueller et al., 1981). No Rio Grande do Sul, foram descritas taxas de soropositividade de 81,7 % (Ravazzolo et. al., 1989), 71,3 % (Vidor et al., 1995) e 52,9 % (Piovesan et al., 2013). Ainda neste estado, 91,9 % dos municípios estudados apresentam soropositividade para o BoHV-1 (Lovato et al., 1995).

No estado de Minas Gerais, 93,4 % (313/345) dos municípios avaliados apresentaram pelo menos um bovino com sorologia positiva e 58,2 % dos soros apresentaram anticorpos neutralizantes para o BoHV-1 (Rocha et al., 2001).

No Estado de Goiás, a soroprevalência foi de 51,9 % e, do total das propriedades amostradas, 98,5 % apresentaram, pelo menos, um animal soropositivo. Adicionalmente, 100 % dos municípios pesquisados apresentaram pelo menos uma propriedade positiva, evidenciando a disseminação do BoHV-1 (Barbosa et al., 2005). Ainda neste estado, foi detectado o DNA viral em 19,4 % das amostras de sêmen, 23,6 % em secreções vaginais e 12 % em secreções nasais (Silva, 2013).

No Paraná, amostras de sangue foram coletadas de 14.803 fêmeas com idade ≥ 24 meses, provenientes de 2.018 rebanhos não vacinados. O total de 2.018 rebanhos (71,3 %) mostraram-se positivos para BoHV-1, enquanto 7.125 animais foram soropositivos, com prevalência de 59,0 % (Dias et al., 2013). Esses autores ressaltaram que, no Brasil, não há um programa de controle oficial de IBR e que a elevada soropositividade, relatada em diversos trabalhos, acarreta grande perda econômica para o agronegócio brasileiro.

Em rebanhos com problemas de fertilidade, o BoHV-1 deve ser considerado como um dos fatores de risco. A erradicação deste vírus pode acarretar melhorias ao desempenho reprodutivo do rebanho (Raaperi et al., 2012). A vacinação é recomendada

em locais onde a infecção por herpesvírus é endêmica (Patel, 2005). Contudo, as vacinas disponíveis comercialmente são capazes de reduzir os sintomas clínicos, mas não os eliminar e, ainda, não impedem o desenvolvimento da latência (Galeota et al., 1997).

Destaca-se que, no Brasil, inexiste programa de controle e erradicação do BoHV-1 e nem há disponibilidade comercial de vacinas marcadas (capazes de distinguir animais vacinados dos infectados naturalmente), estratégias essas que conduziram países como a Áustria, Dinamarca, Finlândia, Suíça, Suécia e Noruega para a condição de livres do vírus (Can et al., 2016).

De acordo com Aono et al. (2013), propriedades brasileiras que realizam a vacinação contra doenças reprodutivas, como IBR, Diarreia Viral Bovina (BVD) e Leptospirose, apresentam incidência significativamente reduzida de perdas gestacionais, quando comparadas às outras que não a praticam. Estes autores ressaltam que vacas vacinadas contra IBR e BVD apresentaram maior taxa de prenhez em programas de inseminação artificial em tempo fixo.

2.3 Abortamento e risco de contaminação dos órgãos genitais

É de conhecimento de longa data que o BoHV-1 é capaz de causar abortamento em vacas, após a exposição inicial ao vírus, a reativação da latência ou vacinação utilizando vírus vivo durante a prenhez (Muylkens et al., 2007). As lesões apresentadas pelos fetos abortados não diferem entre abortamentos causados por infecção natural, experimental ou vacinal (Kennedy e Richards, 1964).

A patologia visceral associada à infecção fetal tem sido descrita por vários autores e a lesão mais consistente relatada é uma necrose multifocal em vários órgãos do feto, sendo o fígado o mais afetado. Ademais, uma placentite necrótica é observada, caracterizada por uma necrose das vilosidades placentárias com o epitélio coriônico (Kennedy e Richards, 1964; Molello et al., 1966; Kirkbride, 1992; Anderson, 2007; Rodger et al., 2007; Crook et al., 2012; Mahajan et al., 2013). Extenso grau de autólise do feto também foi mencionado, sendo que é sugerido que a morte fetal ocorra de 24 a 48 horas antes da expulsão (Kennedy e Richards, 1964; Kirkbride, 1992; Givens, 2006).

Estudos mais antigos ressaltavam que as lesões placentárias eram secundárias à infecção fetal (Molello et al., 1966; Kendrick el al., 1971). Com a evolução das técnicas histopatológicas e moleculares, a detecção do antígeno viral foi associada a extensas lesões no fígado fetal e também com os vasos sanguíneos da placenta, dando origem à hipótese de uma disseminação hematogênica da mãe para o feto (Smith, 1997).

Por meio da PCR em tempo real, o BoHV-1 foi evidenciado em células endoteliais da placenta e cotilédones, provenientes de vacas infectadas naturalmente que sofreram abortamento (Crook et al., 2012; Mahajan et al., 2013). Vacas que foram inoculadas com o BoHV-1, no início do terceiro mês de prenhez, também apresentaram o vírus na placenta (Rodger et al., 2007). Estes achados reforçam a hipótese da disseminação hematogênica do vírus da placenta para o fígado, por meio da veia umbilical e, em seguida, para o resto dos órgãos. Todavia, ainda não há suporte molecular para esta teoria, em casos naturais de abortamentos causados pelo BoHV-1. É sugerido que o carreamento do agente infeccioso se dá por meio de leucócitos sanguíneos até as veias placentárias (Givens, 2006).

Uma das possíveis vias de contaminação dos órgãos genitais seria por meio da monta natural ou inseminação artificial, com o sêmen desempenhando papel fundamental na cadeia epidemiológica. O BoHV-1 pode estar presente no plasma seminal de touros infectados e também associado aos espermatozoides (Philpott, 1993; Van Oirschot et al., 1995; Wratall et al., 2006; Tanghe et al., 2005). Uma vez infectados, touros podem excretar o vírus no sêmen ao longo da vida, sendo que a presença do BoHV-1 não afeta a motilidade espermática (Van Oirschot, 1995; Tanghe et al., 2005).

O sêmen geralmente é contaminado durante a ejaculação, por contato com o vírus presente na mucosa prepucial. Touros soropositivos e sem sintomatologia clínica podem eliminar o BoHV-1 durante episódios de reativação viral, o que ressalta o risco de se utilizar touros soropositivos (Van Der Engelenburg et al., 1995). Dias et al. (2013) identificaram a monta natural como fator de risco para a infecção por BoHV-1, em bovinos no estado do Paraná. Um ponto que também deve ser destacado é que estudos comprovaram a resistência do BoHV-1 à criopreservação (Chapman et. al., 1979; Van Der Engelenburg et al., 1993).

Apesar do BoHV-1 ser um dos agentes infecciosos em que os reprodutores devem ser avaliados, antes de ingressarem como doadores de sêmen (segundo a Organização Mundial de Saúde Animal), a legislação no Brasil ainda carece de requisitos a respeito. Assim, a Instrução Normativa N° 48 de 17 de junho de 2003 do MAPA não estabelece que o BoHV-1 esteja dentre os requisitos sanitários mínimos para a produção e comercialização de sêmen bovino e bubalino no país.

Estudos envolvendo lesões nos órgãos genitais devido à infecção do BoHV-1 são escassos. Este patógeno viral foi isolado do conteúdo uterino de vacas cobertas por touro sabidamente soropositivo (Elazhary et al., 1980). Em outro estudo, Parsonson e Snowdon

(1975) observaram endometrite crônica e lesões nas tubas uterinas de vacas inseminadas com sêmen positivo para o BoHV-1.

Biópsias endometriais indicaram o desenvolvimento de endometrite necrótica crônica em vacas inseminadas com sêmen infectado com o BoHV-1 e alterações histopatológicas também foram observadas nas tubas uterinas (Graham et al., 2013).

Nos ovários, é sabido que a infecção ocorre por via hematogênica. Van Der Maaten e Miller (1985) observaram lesões ovarianas em novilhas inoculadas por via intravenosa com o BoHV-1. O vírus foi recuperado dos ovários de todas as fêmeas, mas não dos outros órgãos genitais. Os autores concluíram que o BoHV-1 adquire facilmente o acesso ao tecido ovariano através do sangue.

2.4 Risco de contaminação dos embriões e gametas

A transmissão de um patógeno pelo embrião pode ocorrer desde que o agente etiológico esteja presente nas células embrionárias (infecção embrionária verdadeira), em associação ou mesmo aderido à zona pelúcida (ZP), ou que esteja presente nos fluidos onde os embriões são recolhidos, manipulados, criopreservados ou transferidos (Wrathall, 1995; Wrathall e Sutmöller, 1998).

Durante os últimos anos, o BoHV-1 já foi detectado no fluido folicular (Bielanski et al., 1993; Weber et al., 2013), tecido ovariano (Pereira et al., 2015), células epiteliais da tuba uterina (Bielanski et al., 1993) e associado aos espermatozoides (Rocha et al.,1998; Tanghe et al., 2005). Estudos demonstraram que a presença do BoHV-1 em sistema de produção *in vitro* de embriões afetou a fertilização e o desenvolvimento embrionário (Bielanski et al., 1997; Vanroose et al., 1999; Makarevich et al., 2007).

A infecção viral de células embrionárias é uma possibilidade muito importante a ser considerada, já que estas células irão comprometer posteriormente o embrião, o feto ou, subsequentemente, o animal recém-nascido. Nesse tipo de infecção, o patógeno pode estar associado ao ovócito, ou ser carreado para o seu interior durante o processo de fecundação, por meio da fusão com o espermatozoide contaminado (Wrathall e Sutmöller, 1998).

Ademais, são consideradas fontes comuns de contaminação os materiais de origem animal utilizados para suplementação dos meios de cultura, as células somáticas utilizadas na maturação e/ou manutenção dos embriões, os meios de lavagem, além do próprio profissional, instrumentos ou equipamentos (Galuppo, 2005).

Vale destacar que existem diferenças na relação de associação de patógenos com embriões produzidos *in vivo* ou *in vitro*, tendo em vista que embriões desenvolvidos pela fertilização *in vitro* (FIV) diferem morfológica e fisiologicamente daqueles fertilizados *in vivo* (Wright e Ellington, 1995). Segundo Bielanski (2006) as técnicas de FIV possuem riscos específicos quanto à transmissão de patógenos, devido às condições artificiais de cultivo, que são substancialmente diferentes daquelas encontradas *in vivo*.

Stringfellow e Givens (2000a) ressaltaram que, na produção *in vitro* de embriões, por exemplo, são utilizados sistemas de cultivo específicos, que, por conter células animais, se tornam uma possível fonte de contaminação embrionária. Os fluidos utilizados *in vitro* também podem ser fonte de contaminação viral, já que este permanece em estreita proximidade com o embrião até o momento da transferência.

Adicionalmente, Vanroose et al. (2000) observaram que a ZP possui peculiaridades diferentes, dependendo do tipo de embrião. Neste contexto, alguns patógenos são capazes de aderir mais prontamente em embriões produzidos *in vitro* do que *in vivo* (Thibier, 2011). Também Pollard e Leibo (1993) salientaram que a dissolução da ZP pela enzima pronase é mais lenta em embriões produzidos *in vivo* (387 segundos) do que nos produzidos *in vitro* (132 segundos), evidenciando diferenças na estrutura da mesma.

2.5 Zona pelúcida como barreira mecânica e veículo passivo para o BoHV-1

A zona pelúcida (ZP) é uma matriz extracelular única que envolve o ovócito e também o embrião em sua fase inicial. Ela é secretada durante o desenvolvimento inicial dos ovócitos, nos folículos secundários, e pelas células foliculares circundantes, como remendos extracelulares que se unem em uma camada uniforme. Possui a função de conferir especificidade na fertilização, bloqueio à polispermia e proteção durante os estádios iniciais do desenvolvimento embrionário. Ela demonstra fulcral importância na proteção do embrião. Em bovinos, apresenta entre 10 e 15 μm de espessura (Epifano e Dean, 1994; Betteridge, 1995).

Pesquisadores sugerem que a maioria dos patógenos é incapaz de penetrá-la quando intacta e infectar as células dos embriões coletados (Stringfellow e Givens, 2000a). Os canais da ZP deixados pelas expansões das células da *corona radiata* ou pela passagem do espermatozoide se fecham muito rapidamente (Wrathall e Sutmöller, 1998).

Por meio da microscopia eletrônica de varredura, foi observado que a ZP de mamíferos é composta por vários poros e que as superfícies interna e externa diferem

morfologicamente, sendo que a interna demonstra uma aparência áspera, enquanto a externa exibe forma de camadas (Dudkiewicz e Williams, 1977; Phillips e Shalgi, 1980).

Outro estudo ressaltou que, em ovócitos imaturos, a superfície externa da ZP é irregular e apresenta diversidade na distribuição de poros, fendas e projeções (Riddell et al., 1993). Além disso, a parte interna da ZP apresenta numerosas malhas largas e poros profundos que, após a maturação, se tornam menos profundos (Suzuki et al., 1994; 1996). Os menores poros da ZP são observados nos bovinos (Dudkiewicz e Williams, 1977). No entanto, os poros da ZP são grandes o suficiente para permitir a entrada do BoHV-1 (Vanroose et al., 1999b).

Vanroose et al. (2000) constataram dois padrões na superfície externa da ZP de embriões: uma rede com aparência esponjosa, contendo vários poros, e uma estrutura mais compacta, apresentando menor quantidade de poros, porém maiores em diâmetro. Avaliando ovócitos maduros, zigotos, embriões no estágio de oito células e mórulas, estes autores observaram que o diâmetro dos poros diminuía quanto mais próximo da superfície interna da ZP.

Apesar de trabalhos ressaltarem que a maioria dos microrganismos patogênicos não atravessam a ZP intacta, o risco de infecção viral não deve ser excluído, uma vez que, ainda que não transpassem, as partículas virais podem ser retidas nas camadas mais externas, podendo penetrar no ovócito no momento da fertilização (Bowen, 1979). Além disso, os vírus podem atravessar a membrana plasmática e infectar o embrião, quando o mesmo já não está sob a proteção da ZP, e também infectar a receptora, quando o embrião for transferido juntamente com o agente aderido (Bielanski, 2006; Ponsart e Pozzi, 2013).

No entanto, quando aderidos à superfície do embrião, os vírus são vulneráveis a tratamentos realizados com o objetivo de removê-los ou inativá-los. Experimentos demonstraram que, em embriões bovinos, os tratamentos realizados com o objetivo profilático podem ser realizados com sucesso (Wrathall e Sutmöller, 1998).

Stringfellow e Givens (2000b) consideram que os agentes virais podem ser, frequentemente, encontrados no líquido de lavagem, atestando que, em embriões adequadamente manipulados, é altamente improvável que os vírus sejam capazes de atravessar a ZP intacta e provocar efeito deletério no desenvolvimento embrionário, indicando a efetividade do processo de lavagem.

De acordo com as diretrizes descritas pela Sociedade Internacional de Transferência de Embriões (IETS), os embriões devem ser lavados com tripsina, de 10 a 12 vezes para remover o BoHV-1 (Stringfellow, 1998). Ademais, é recomendável que as doadoras e as receptoras apresentem ciclos normais e sejam examinadas com relação à saúde geral e

reprodutiva. Os programas de transferência de embriões preconizam que os rebanhos de doadoras e receptoras sejam avaliados quanto à possibilidade da ocorrência de doenças infecciosas (Mapletof e Stookey, 1998).

A lavagem e/ou tratamento com tripsina dos embriões é justificado pela facilidade de incorporação no protocolo de produção de embriões, pelo baixo custo e pela efetividade demonstrada após exposição *in vitro* a diferentes patógenos bovinos (Stringfellow e Givens, 2000c).

Em contrapartida, outros autores discordam da eficácia da lavagem com tripsina. Stringfellow e Givens (2000c) descreveram que há patógenos infecciosos, dentre eles o BoHV-1, que podem permanecer associados a embriões bovinos produzidos *in vitro*, mesmo após exposição à tripsina. Estes autores relataram que embriões produzidos *in vivo* e expostos ao BoHV-1 não apresentaram mais o agente viral após exposição à tripsina. Porém, o vírus permaneceu associado aos embriões produzidos *in vitro*. Eles destacam a relativa ineficácia dos procedimentos de lavagem, o que gera preocupações legítimas.

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3. HIPÓTESES

1) O *bovine herpesvirus* 1 (BoHV-1) é capaz de realizar replicação nos tecidos uterino, tubárico e ovariano de vacas infectadas naturalmente e sem sintomatologia clínica.

2) Os complexos *cumulus*-ovócitos (COCs) de vacas soropositivas são sítios de replicação do vírus em condições naturais de infecção.

3) O BoHV-1 é capaz de penetrar a zona pelúcida intacta de ovócitos bovinos co-incubados com o vírus por período de uma e 24 horas.

4. OBJETIVOS

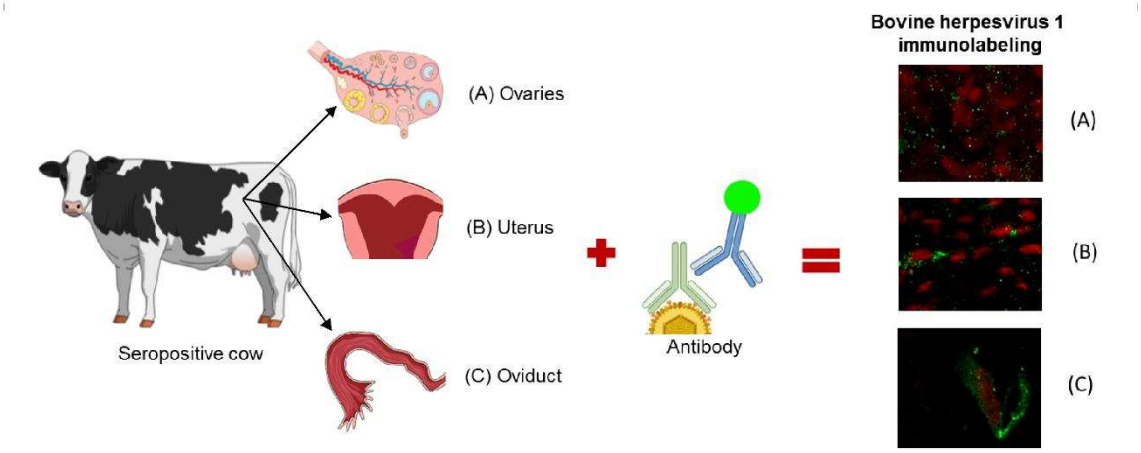
1) Verificar a presença do bovine herpesvirus 1 (BoHV-1) nos tecidos uterino, tubárico e ovariano de animais infectados naturalmente e sem sintomatologia clínica da doença, por meio de ensaios de imunofluorescência utilizando microscopia confocal de varredura a laser.

2) Investigar a presença do BoHV-1 em complexos *cumulus*-ovócitos (COCs) de vacas soropositivas e assintomáticas, em condições naturais de infecção, por meio de ensaios de imunofluorescência utilizando microscopia confocal de varredura a laser.

3) Investigar a eficiência da zona pelúcida intacta de ovócitos bovinos co-incubados com o vírus, por período de uma e 24 horas, como barreira à infecção viral.

5. ARTIGO I - Detection of bovine herpesvirus 1 in genital organs of naturally infected cows

Graphical abstract



5.1 Abstract

Bovine herpesvirus 1 (BoHV-1) is a causative agent of respiratory diseases in cattle, and infection with BoHV-1 can cause reproductive failure. There are few studies regarding infections in natural conditions in the reproductive organs of bovine animals. In this context, this study investigated the presence of BoHV-1 in the uterus, oviducts, and ovarian tissues of naturally infected cows. The three genital structures were evaluated for the presence or absence of BoHV-1 by immunofluorescence assay using confocal scanning laser microscopy. Blood and genital organ samples of 75 cows unvaccinated against BoHV-1 were used. Fragments of uterus, oviduct, and ovarian tissue were processed and analyzed by confocal scanning laser microscopy. Neutralization by antibodies was observed in 54.7% (41/75) of the serum samples tested. BoHV-1 were detected in the uterus of all the seropositive cows. The oviducts contained BoHV-1 in 73.2% of the samples and the ovaries contained BoHV-1 in 58.5% of the samples from seropositive animals. The presence of the virus was not observed in any of the genital organs of seronegative animals. There was no correlation between the antibody title and

the detection of BoHV-1 in positive tissue in the different genital organs or with the number of infected structures per animal. The detection of BoHV-1 in 100% of the uterus samples from seropositive cows suggests that this organ may be a source of infection for the fetus, resulting in abortion. Further studies on the mechanism by which BoHV-1 infects the fetus via the uterine route should be performed.

Key words: Abortion, Bovine Herpesvirus 1, Uterus.

5.2 Introduction

Bovine herpesvirus 1 (BoHV-1) belongs to the *Herpesvirales* order, *Herpesviridae* family, *Alphaherpesvirinae* subfamily and *Varicellovirus* genus [1]. BoHV-1 is the causative agent of Infectious Bovine Rhinotracheitis (IBR) and infectious pustular vulvovaginitis, in addition to having the ability to cause temporary infertility, embryonic death, and abortions [2, 3].

BoHV-1 is widely distributed throughout herds worldwide [4, 5]. It is an economically important veterinary pathogen, causing economic losses estimated at an average financial expense of US$ 379.00 per cow [6].

BoHV-1 can result in latent infections in the neuronal ganglia, like the other *Alphaherpesvirinae* viruses, which, following the primary infection, causes the animals to become lifelong carriers and potential disseminators [7, 8]. The number of animals in the latent infection phase tends to be significantly higher than those with clinical symptoms [9]. Host immunosuppression leads to BoHV-1 recrudescence following viral re-excretion episodes, making it easy to transmit and difficult to control [10, 11].

BoHV-1 infection can cause damage directly in the uterus, oviducts and ovaries, leading to endometritis, oophoritis, oviduct injuries, infertility, and abortions [2, 12]. A recently reported study revealed that the Cumulus-oocyte complexes (COCs) from naturally-infected cows without clinical signs of infectious bovine rhinotracheitis (IBR)

may present the BoHV-1 in the cytoplasm of cumulus cells. These findings could be related to the reproductive failures commonly shown in BoHV-1 infected cows [13].

Although this viral agent has been known to cause abortions, information on the mechanisms involved in the dissemination of the virus from the respiratory system or, when reactivated, from the trigeminal and sacral ganglia to infecting the developing fetus in the uterus is still unclear [14].

Some studies in *in vitro* conditions involving BoHV-1 in cells derived from the reproductive organs have already been performed [15, 16, 17]. With regards to studies about reproduction, placenta from cows that were infected with BoHV-1 at the beginning of the third month of pregnancy contained the virus [18]. BoHV-1 DNA was observed in placental endothelial cells and cotyledons from infected cows that underwent abortion [14, 19].

Nevertheless, studies under natural conditions and infection in the reproductive tissues are poorly reported in the literature. In addition, the mechanisms of BoHV-1 dissemination from the cow to the fetus should be studied. In this context, this study aimed to investigate the possible presence of BoHV-1 in the uterus, oviduct and ovarian tissue of naturally infected cows. The three genital structures were evaluated to determine the presence or absence of the BoHV-1 by immunolocalization using confocal scanning laser microscopy.

5.3 Material and Methods

5.3.1 Sample collection

The samples were collected in a slaughterhouse located in the municipality of Muriaé, Minas Gerais (Brazil, geographic coordinates: 21°8'59"S 42°25'36"W). Samples of blood and genital organs from 75 cows unvaccinated against BoHV-1 were collected after slaughter. Blood samples were collected with vacutainer tubes at the time of

bleeding. All the experimental procedures were conducted in accordance with the ethical principles adopted by the National Council of Animal Experimentation with definitive authorization from the Ethics Committee on Animal Use of the Federal University of Vicosa under protocol n° 94/2015.

5.3.2 Experimental design

Blood was processed to separate the serum, which was stored at -20°C for the virus neutralization assays. Fragments of uterus, oviduct and ovarian tissues were removed in cross-sections made with surgical scalpel blade support. A fragment of each ovary (right and left) was removed and added in the same identified microtube so that the two fragments were processed as a pool, since they were both included in one treatment. The fragments from the uterus and oviduct tissue were selected from the cranial, medial, and caudal portion, randomly selected on the right or left side and also processed as a pool of the three portions for each animal.

The fragments were prepared for confocal scanning laser microscopy assay. Thus, they were fixed in a 4% paraformaldehyde solution (Sigma-Aldrich, St. Louis, USA) and 0.4% picric acid (Sigma-Aldrich, St. Louis, USA) in 0.1M sodium phosphate buffer solution, pH 7.2 for a period of 2 hours and stored in phosphate buffered saline (PBS) solution, pH 7.4 at 4°C.

5.3.3 Cells and viruses

The “Los Angeles” (LA) BoHV-1 strain sample was replicated in Madin-Darby bovine kidney (MDBK) cells cultured in monolayers. The cells were multiplied and maintained at 37°C and 5% CO_2 atmosphere using minimum essential medium (MEM, Sigma-Aldrich, St. Louis, USA) plus 0.4 mg/L streptomycin (Sigma-Aldrich, St. Louis, USA) and 1.6 mg/L penicillin (Sigma- Aldrich, St. Louis, USA), and supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, USA). The viruses were titrated

by the Tissue Culture Infective Dose method ($TCID_{50}$) according to the Reed and Muench method [20].

5.3.4 Virus neutralization assay

Virus neutralization assays were performed as described by House and Baker [21], with the addition of 100 $TCID_{50}$/50 μL of the LA BoHV-1 strain to the serum dilutions of each sample. After incubation of the serum-virus for 1 hour at 37°C in a CO_2 incubator, 50 μL of MDBK cell suspension was added at the concentration of 300,000 mL^{-1} cells. The reading of the tests was performed after 72 hours of incubation by monitoring the cytopathic effect. The neutralizing activity of the anti-BoHV-1 antibody was expressed as being the geometric mean of the observed title values. Positive and negative reference samples were used as control.

5.3.5 Immunolocalization of BoHV-1

Fixed fragments were transferred to PBS with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. They were then incubated overnight with monoclonal primary antibody (VMRD, Washington, USA), IgG2b isotype, specific for BoHV-1 gC glycoprotein (1:100). Afterward, the fragments were washed in PBS and overnight incubated with anti-IgG secondary antibody conjugated with fluorescein isothiocyanate – FITC (490 nm – excitation; Sigma-Aldrich, St. Louis, USA) (1:200).

After further washings, the samples were incubated with the TO-PRO™-3 Iodide (642 nm exciton; Waltham, USA), a nucleic acid coloring, for 30 minutes. The samples were prepared on an identified microscopy slide with Mowiol (Sigma-Aldrich, St. Louis, USA) and analyzed on Zeiss 510 META confocal scanning laser microscope.

The argon laser (488 nm - excitation) was used to excite the FIT-C fluorescence and the HeNe laser (633 nm - excitation) was used for the TO-PRO™ - 3 Iodide

excitation. The images were obtained using the 40× and 63× objectives with the Zeiss LSM Image Browser software.

5.3.6 Statistical analysis

The dichotomous qualitative variable (presence or absence of viral protein) of each treatment was compared in contingency tables and analyzed by the chi-square test at a 5% probability [22]. Pearson's correlation tests were performed between neutralizing antibody titles and detection of viral structural proteins in the genital organs.

5.4 Results

We analyzed a total of 450 samples of genital organs from 75 cows (three different organs/cow and the respective control) by CSLM technique. We observed in the tested serum samples 54.7% (41/75) of BoHV-1-seropositive cows. All animals that had neutralizing antibodies to BoHV-1 had uterus BoHV-1 positive as shown on Table 1. No genital organ samples from the 34 seronegative animals had virus presence.

Table 1. Detection of BoHV-1 viral structural proteins in bovine genital organs

Genital organs	N (seropositives)	Presence of BoHV-1	
		N	%
Uterus	41	41	100[a]
Oviduct	41	30	73.2[b]
Ovaries	41	24	58.5[b]

[a,b] Values with different superscript letters indicate difference ($P < 0.01$) by the Chi-square test.

There was no correlation found between the title of antibodies and the percentage of BoHV-1 detected in the different genital organs or with the number of infected organs per animal. Samples of uterus, oviduct and ovarian tissue showing BoHV-1 are shown in Fig 1.

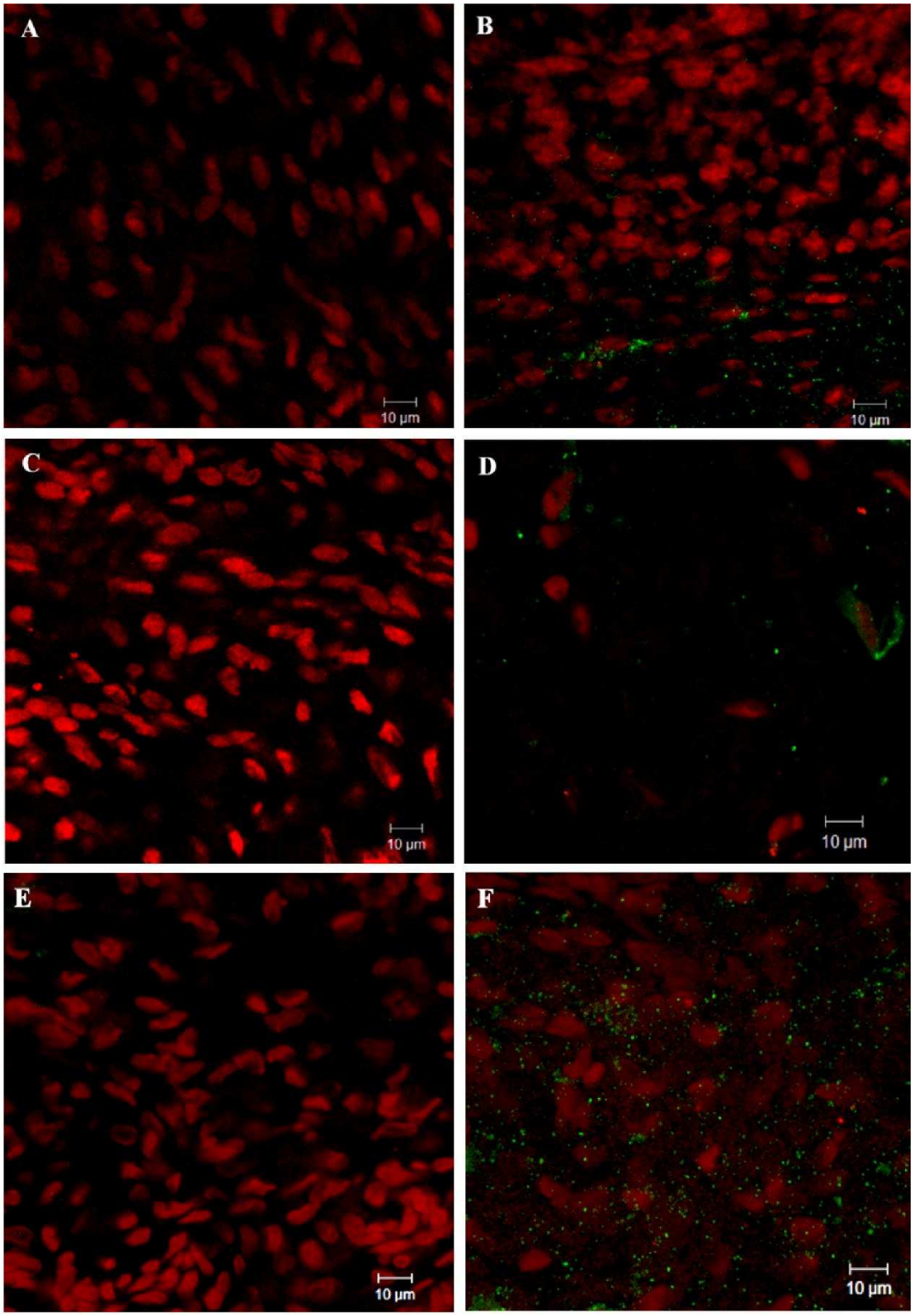


Figure 1. Detection of BoHV-1 images obtained by confocal scanning laser microscopy. (a) negative control uterine tissue, (b) uterine tissue containing BoHV-1 viral structural proteins, (c) negative control oviduct tissue, (d) oviduct tissue positive for BoHV-1, (e) negative control ovarian tissue, and (f) ovarian tissue positive for BoHV-1. The presence of the virus was proven by the green fluorescence (FITC) and the DNA of the cells, which are stained in red (TO-PRO^{TM} 3-iodide).

5.5 Discussion

In this study we detected BoHV-1 in the uterus, oviduct and ovaries of tissue samples obtained from cows. We used a monoclonal primary antibody that binds to the C glycoprotein of the BoHV-1 viral envelope that enables marking of viral structural proteins. The findings suggest that these reproductive structures can be targets for active viral replication, once glycoprotein C is not expressed during latent phase of infection.

Although BoHV-1 has been known to cause abortion, the mechanisms of transmission from cow to fetus remain unclear. In this context, the findings of this study enable us to show that the uterus, oviduct, and ovarian tissues could be targets of viral replication and a potential source of viral dissemination to the embryo and fetus.

It is important to emphasize that all seropositive animals showed the presence of BoHV-1 in the uterine tissue. The epitheliotropic nature of BoHV-1 and reports of BoHV-1 DNA found in the placenta [14, 19, 23] could provide support for the hypothesis that the uterus is a viral replication target and indicate that the dissemination would come from the uterine tissue to the placenta resulting in fetal infection and abortion. Denis et al. [24] describes that there are BoHV-1 migrating from the infected cells to the adjacent ones even in the presence of neutralizing antibodies in the extracellular medium.

The virus could reach the genital organs through semen, by natural mating using an infected bull, or by artificial insemination. There is evidence that BoHV-1 may be present in the seminal plasma of infected bulls and associated with spermatozoa [25, 26]. Once infected, positive bulls without clinical symptoms can shed the virus in the semen during episodes of viral reactivation, and the presence of BoHV-1 does not affect sperm motility [27, 28].

Notwithstanding the fact that the BoHV-1 is one of the infectious agents for which bulls should be evaluated before becoming as semen donors (according to the World Organization for Animal Health), Brazilian legislation does not establish that tests for

BoHV-1 are required among the minimum sanitary requirements for the domestic production and commercialization of bovine semen [29]. Furthermore, according to Chapman et al. [30] and Van Der Engelenburg et al. [31], studies have verified the resistance of BoHV-1 to cryopreservation.

BoHV-1 has already been isolated from the uterine content of cows mated naturally by a known seropositive bull [32]. In another study, Parsonson and Snowdon [33] observed chronic endometritis and lesions in the oviduct of cows inseminated with BoHV-1 positive semen. Endometrial biopsies indicated the development of chronic necrotic endometritis in cows inseminated with semen infected with BoHV-1 and histopathological changes were also observed in the oviduct [12].

Oviducts accounted for 73.2% of the samples infected with BoHV-1 in this study. This organ is covered by a simple epithelium, which under hormonal influence undergoes cyclic changes related to the estrous cycle. In the presence of progesterone, loss of ciliated epithelium occurs, and secretory cells tend to lose their biosynthetic structures [34, 35]. This could explain the visualization of discrete markings in this organ, Fig 1 (d).

The infection occurs through the hematogenous route in the ovaries. Van Der Maaten and Miller [36] observed ovarian lesions in cows injected intravenously with BoHV-1. The virus was recovered from the ovaries of all females, but not from the other genital organs. The authors concluded that BoHV-1 readily acquires access to the ovarian tissue through blood.

Another possible route of contamination of the genital organs would be the adherence of BoHV-1 to the oocyte zona pellucida (ZP) or even associated with the spermatozoa (which could represent a potential route for infection of an oocyte during fertilization), affecting the *in vitro* embryo production. The association of BoHV-1 with the sperm cell is mediated by the gC and gD viral glycoproteins, similar to the binding mechanisms of this virus to the host cell [26]. The importance of the capacity of the

BoHV-1 adherence to ZP was emphasized by several studies [37, 38, 39]. Therefore, in this other possible route of contamination of the genital organs, adhesion would allow BoHV-1 to contact the endometrial cells of the uterus and/or the oviduct to initiate the replicative cycle.

5.6 Conclusions

We have demonstrated that the detection of BoHV-1 in the uterine, oviduct and ovarian tissue has great significance for the field of animal reproduction. The findings suggest new insights regarding the BoHV-1 infection in reproductive tissue. The virus detection in the uterus in 100% of the seropositive cows suggests this organ as a fetal infection source to be implicated in playing a role in abortion.

5.7 Acknowledgements

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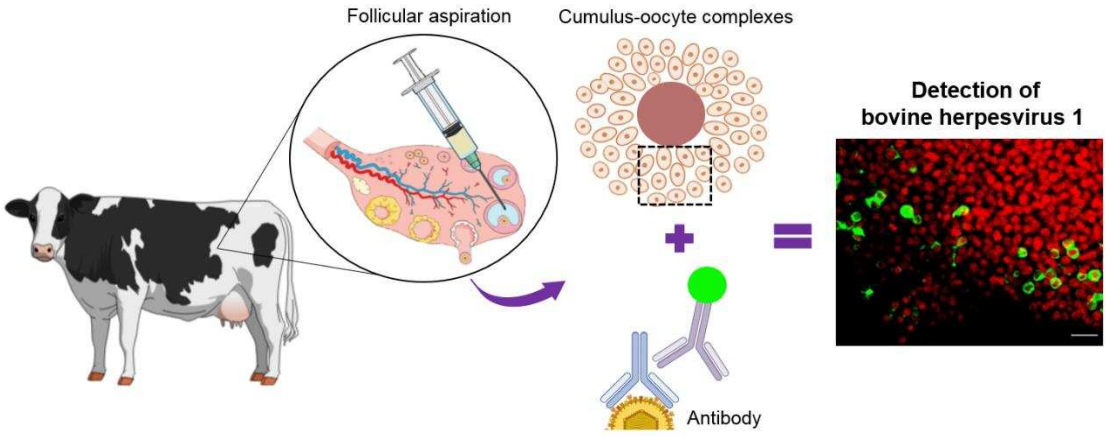
6. ARTIGO II – Short Communication: Detection of bovine herpesvirus 1 in *cumulus*-oocyte complexes of cows

ARTIGO PUBLICADO: Research in Veterinary Science, v. 120, p. 54-58, 2018. Doi: 10.1016/j.rvsc.2018.08.010.

Graphical abstract



Abstract

Bovine herpesvirus 1 (BoHV-1) is the causative agent of infectious bovine rhinotracheitis (IBR) and is also associated with reproductive failure. This study investigated the presence of BoHV-1 in *cumulus*-oocyte complexes (COCs) of naturally-infected cows without clinical signs of IBR. The presence of BoHV-1 in COCs was evaluated by immunofluorescence using confocal laser scanning microscopy. Blood samples and ovaries from 82 cows that had not been vaccinated against BoHV-1 were collected for serological analysis. COCs were divided into two pools: COCs derivate from seropositive cows and from seronegative cows. Then, the samples were processed for confocal microscopy analysis. The results indicated that 61% (50/82) of cows were seropositive for BoHV-1. A total of 719 COCs were obtained from the cows and processed. None of 276 COCs from the 32 seronegative cows presented BoHV-1. However, BoHV-1 was present in the cytoplasm of *cumulus* cells from 158 out of 443 COCs aspirated from the

seropositive cows. The detection of BoHV-1 in the COCs of seropositive cows suggests that the COCs of naturally-infected, asymptomatic cows may be infected with BoHV-1.

Keywords: Bovine herpesvirus, *cumulus* cells, immunolocalization, oocyte.

Bovine herpesvirus 1 (BoHV-1) causes infectious bovine rhinotracheitis (IBR) and pustular vulvovaginitis, which results in embryonic death, miscarriage, and temporary infertility (Muylkens et al., 2007). The animals that survive to IBR may carry the virus with no clinical signs. In addition, BoHV-1 may establish a latent infection in sensitive ganglia. Any factor that triggers host immunosuppression may result in a BoHV-1 recurrence, followed by episodes of virus re-excretion. This phenomenon facilitates transmission between animals and limits the disease control in the herd (El-Mayet et al., 2017; Jones et al., 2011).

Previous studies involving experimental infection with BoHV-1 in an *in vitro* embryo production system have reported that the presence of this herpesvirus in oocyte maturation and fertilization media impaired the *in vitro* fertilization and embryonic development (Bielanski et al., 1997; Guérin et al., 1990; Vanroose et al., 1999). Moreover, *cumulus* cells may serve as potential BoHV-1 replication sites (Tsuboi et al., 1992; Tsuboi and Imada, 1997; Vanroose et al., 1999). On the other hand, studies using *cumulus*-oocyte complexes (COCs) from naturally-infected animals are still scarce. . Indeed, the use of this animal model isextremely relevant as BoHV-1 virus has been detected in ovarian tissue (Pereira et al., 2015), oviductal epithelial cells (Bielanski et al., 1993), as well as follicular fluid (Weber et al., 2013) from seropositive naturally-infected cows. Particularly, there is no information to date about the presence of BoHV-1 in oocytes from naturally-infected cows. In this framework, the present study aimed to investigate the presence of BoHV-1 virus in COCs from naturally-infected cows by immunolocalization using confocal microscopy.

Blood samples and ovaries were collected from 82 cows that had not been vaccinated against BoHV-1, at different phases of the estrous cycle, after slaughter in a slaughterhouse located in the municipality of Muriaé, Minas Gerais, Brazil (21°8'59"S and 42°25'36"W). (All the experimental procedures were reviewed and approved by the Committee on the Ethics and Use of Animal Experiments of UFV (CEUA process number 94/2015).

The COCs were recovered by aspiration, and those with compact *cumulus* cell layers and homogeneous cytoplasm were fixed in paraformaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) at 4% and picric acid (Sigma-Aldrich, St. Louis, MO, USA) at 0.4% in 0.1 M sodium phosphate buffer pH 7.2 for 1 h. The samples were then stored in phosphate buffer saline (PBS) solution pH 7.4 at 4°C. After performing the serum neutralization tests, the COCs were divided into two pools: COCs derivate from seropositive cows and from seronegative cows. Then, the samples were processed for confocal microscopy analysis.

The Los Angeles BoHV-1 strain was grown in Madin-Darby bovine kidney (MDBK) cells and titrated using the tissue culture infective dose method ($TCID_{50}$), according to the method proposed by Reed and Muench (1938). The neutralization virus tests followed the technique described by House and Baker (1971), with modifications.

The COCs, prefixed and stored in PBS, were transferred to PBS with Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at 1% for 1 h. Then, the COCs were incubated overnight in anti-mouse primary monoclonal antibody (1:100; VMRD, Pullman, WA, USA) isotype IgG2b, specific for the gC glycoprotein of BoHV-1. The oocytes were washed in PBS and incubated with the IgG anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (FITC) (1:200; excitation at 490 nm; Sigma-Aldrich, St. Louis, MO, USA) overnight. After further washing, the oocytes were incubated with the nucleic acid dye TO-PRO-3 iodide (excitation at 642 nm; Invitrogen, Waltham, MA,

USA) for 30 min. The histological slides containing the samples were mounted with Mowiol (Sigma-Aldrich, St. Louis, MO, USA) and analyzed in a Zeiss 510 META confocal laser scanning microscope. Argon laser (excitation at 488 nm) was used for the FITC dye, and helium-neon laser (excitation at 633 nm) was used for the TO-PRO-3 iodide. The slides were analyzed under the confocal laser scanning microscope at a magnification of 40×. The negative control of each pool was treated with the same protocol, but without incubation with the monoclonal primary antibody.

Our results indicated that out of the 82 cows sampled, 50 cows were seropositive for BoHV-1, corresponding to a seroprevalence of 61%. A total of 719 COCs from the evaluated cows were processed. None of COCs (n=276) from the 32 seronegative cows was infected with BoHV-1 (Fig. 1A). However, BoHV-1 was present in the cytoplasm of the *cumulus* cells (Fig. 1B and C) in 158 out of 443 COCs collected from naturally-infected cows.

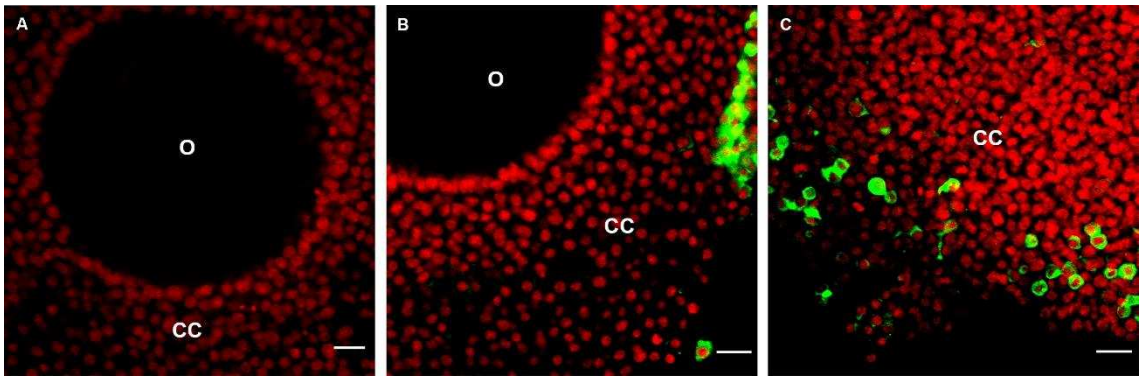


Fig. 1. Confocal images of the *cumulus*-oocyte complex (A) from seronegative cow with no bovine herpesvirus 1 (BoHV-1) labeling. (B, C) Immunolabeling of BoHV-1 (*green*) in the cytoplasm of *cumulus* cells (CC) from *cumulus*-oocyte complexes of seropositive cow. O: oocyte. Nuclei were stained with TO-PRO 3 iodide (*red*). Bar scale = 20 µm.

These results suggest that the *cumulus* cells of COCs from naturally-infected cows are BoHV-1 replication sites. To date, it was believed that genitals were infected only in the acute phase of this disease. The latter involves the presence of clinical signs in females, including fever, nasal discharge, and pustules and erosions in the vulva and

vagina (Muylkens et al., 2007). Bielanski and Duboc (1994) diagnosed fever and nasal discharge in cows experimentally infected with BoHV-1, and the virus was isolated from granulosa cells and oocytes, demonstrating that transmission occurs during the acute phase. To the best of our knowledge, this study is the first to detect BoHV-1 in the COCs of naturally-infected cows that did not show clinical signs of viral infection and, therefore, were asymptomatic for BoHV-1 infection.

Of note, the morphology of COCs observed herein was apparently normal, with several layers of compact *cumulus* cells and homogeneous cytoplasm. In fact, the morphology of this cell type is a critical parameter in *in vitro* embryo production (Costa, 1994). Previous studies described lysed *cumulus* cells when they were infected with BoHV-1 in vitro (Tsuboi et al., 1992; Tsuboi and Imada, 1997; Vanroose et al., 1999). The detection of BoHV-1 in *cumulus* cells of COCs from seropositive cows in this study, and the epitheliotropic nature characteristic of BoHV-1 (Babiuk et al., 1996) allow us to suggest that the virus reaches the follicles through endothelial vessels. It is known that ovarian tissue may be infected by BoHV-1 hematogenously (Pereira et al., 2015; Van Der Maaten and Miller, 1985). The ovarian parenchyma is composed of a smooth connective tissue rich in blood vessels. The latter nourishes primordial and primary follicles by passive diffusion, as there are no capillaries between granulosa cells. In this sense, individual capillary network is formed around each follicle (Robinson et al., 2009). Furthermore, the formation of preantral follicles results in an increase in vascularization, and approximately 40% of the proliferating cells in the theca interna are endothelial origin (Martelli et al., 2009).

During follicular development, the follicular fluid composed of plasma transudate and secretions from granulosa cells, accumulates and gives rise to the antral follicle. In this phase, the granulosa cells coating the oocyte are separated into two subtypes: *cumulus*

cells (linked to the oocyte) and wall cells (which form the follicle wall) (Gilchrist et al., 2004).

The hypothesis of BoHV-1 dissemination from ovarian tissues to endothelial cells and later to granulosa cells is supported by Denis et al. (1994), who found that BoHV-1 migrated from infected cells to adjacent cells, even in the presence of neutralizing antibodies in the extracellular medium.

Notably, bidirectional functional communication between *cumulus*-oocyte cells is an essential characteristic of mammalian reproduction. These cells provide the oocyte with transcripts necessary for oocyte competence and subsequent embryonic development (Gilchrist et al., 2016; Russell et al., 2016). Therefore, the presence of BoHV-1 in the *cumulus* cells in naturally infected-animals may negatively affect this communication. Additional studies are necessary to elucidate the reproductive failures due to viral infection, including temporary infertility and embryonic death.

In addition, ovulated COCs infected with BoHV-1 may establish infection in the epithelial cells of the oviduct by the contact of *cumulus* cells with oviductal epithelial cells. Edens et al. (2003) and Vanroose et al. (1999) observed that tubal cells co-cultivated with infected embryos became infected. This result is relevant because infected cells undergo degeneration and may lose their biological function, including the secretion of embryotrophic factors that support embryonic development (Ellington, 1991; Carolan et al., 1995).

In summary, the detection of BoHV-1 in the *cumulus* cells of naturally-infected, asymptomatic cows is important to the field of animal reproduction. Our findings suggest that the COCs of seropositive animals may be infected with BoHV-1, which could be related to the reproductive failures commonly shown in BoHV-1 infected cows.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this study.

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7. ARTIGO III – Bovine herpesvirus 1 can cross the intact zona pellucida of bovine oocytes after artificial infection

7.1 ABSTRACT

Bovine herpesvirus 1 (BoHV-1) is an important bovine pathogen responsible for causing respiratory diseases and reproductive problems. This study investigated the penetration capacity of BoHV-1 in oocytes after co-incubation with the virus for either 1 h or 24 h. Immunofluorescence assays in cumulus-oocyte complexes (COCs) and denuded oocytes (without the presence of cumulus cells) were performed and evaluated using confocal laser scanning microscopy. Blood samples and ovaries from BoHV-1 seronegative cows were used. The oocytes recovered were divided into two groups: Group I: COCs (n=312) and denuded oocytes (n=296) were experimentally infected with BoHV-1 and incubated for 1 h at 38.5 °C and 5% CO2. Group II: COCs (n = 425) and denuded oocytes (n = 405) were subjected to the same co-incubation protocol with BoHV-1, but for 24 h. The negative control of this two groups were respectively subjected to the same protocol except exposure to BoHV-1. To our knowledge, this study provides the first evidence of BoHV-1 detection within COCs and denuded oocytes exhibiting intact ZP when co-incubated with the virus for 24 h. Immunolocalization also confirmed the presence of BoHV-1 in the cytoplasm of cumulus cells of all COCs exposed to the virus in both incubation periods. In conclusion, the detection of BoHV-1 inside oocytes has a great meaning for the field of animal reproduction. In addition, the detection of BoHV-1 in different layers of cumulus cells demonstrate that these cells are sources of viral infection.

Keywords: *cumulus* cells, oocyte, zona pellucida.

7.2 INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is the causative agent of Infectious Bovine Rhinotracheitis (IBR) responsible for important reproductive disorders as endometritis, oophoritis, temporary infertility, embryonic death, and miscarriages [1,2]. It is an economically-significant veterinary pathogen, causing an estimated average financial loss of US$ 379.00 per infected cow [3]. Similarly to IBR, *alphaherpesvirinae* viruses can establish latent infections in neuronal ganglia after primary infection, making the animals lifelong carriers and potential disseminators [4-6]. Immunosuppression of the host leads to the re-emergence of BoHV-1 followed by episodes of viral re-excretion, making it easy to transmit and difficult to control [7,8].

Several studies were carried out to determine whether bovine embryos produced in vitro and exposed to BoHV-1 would compromise their initial development with contradicting results. Guérin et al. [9] observed that oocytes exposed to BoHV-1 showed reduced rates of in vitro fertilization and cleavage. In addition, Vanroose et al. [10] found that exposure of embryos to BoHV-1 during in vitro maturation resulted in significantly lower rates of blastocyst development. Makarevich et al. [11] emphasized that embryo exposure to BoHV-1 compromised embryonic development and also that 80% of the embryos stagnated during development and degenerated. On the other hand, Bielanski and Dubuc [12] found no effect on the initial development of embryos exposed to the virus. In all studies, the authors emphasized that the zona pellucida (ZP) was intact.

In this sense, previous studies have explored the effectiveness of the ZP as a barrier to viral infection. Zona pellucida is an extracellular matrix rich in glycoproteins, which separates the oocyte from *cumulus* cells. For instance, ZP may protect embryos against BoHV-1 infection during the early stages of development if its structure is intact [13-16]. Moreover, Vanroose et al. [10,14] concluded that BoHV-1 is not capable of transposing intact ZP of bovine oocytes and embryos. These authors observed that there was no

degeneration of the embryonic cells of embryos that were exposed to the virus and found that by removing the ZP, the BoHV-1 was able to replicate within the embryos. Vanroose et al. [15,16] emphasized the ZP efficiency as a barrier because no fluorescent microspheres of similar size to BoHV-1 were able to enter co-incubated zygotes in medium containing the microspheres. Conversely, Silva-Frade et al. [17] identified BoHV-5 within bovine oocytes via in situ hybridization assays, concluding that even intact ZP was ineffective in protecting against viral infection.

In this framework, the present study investigated whether BoHV-1 is able to transpose intact ZP of bovine oocytes with and without the presence of *cumulus* cells, exposed to the virus for either one or 24 h of co-incubation, using viral immunolabelling and laser scanning confocal microscopy.

7.3 MATERIAL AND METHODS

Sample Collection

Blood samples and ovaries were collected from cows that had not been vaccinated against BoHV-1, after slaughter in a slaughterhouse located in the municipality of Muriaé, Minas Gerais, Brazil (21°8'59"S and 42°25'36"W). Blood samples were collected with vacutainer tubes at the time of bleeding. All experimental procedures were conducted in accordance with the ethical principles adopted by the National Council of Animal Experimentation with definitive authorization from the Ethics Committee on Animal Use of the Federal University of Vicosa under protocol n° 94/2015.

Infected oocytes

The COCs were recovered by aspiration and those with cell layers of compact *cumulus*, homogeneous cytoplasm and whole zona pellucida were transferred to culture plates containing Talp-Hepes medium and morphologically classified according to Costa

et al. [18]. Part of the selected COCs were denuded (removed from the *cumulus* cells) according to the procedures described by Costa et al. [18]. Subsequently, oocytes from cows known to be seropositive were divided into two groups. Group I was composed of COCs (n = 312) and denuded oocytes (n = 296), which were transferred to drops containing 100 μL of maturation medium [19], experimentally infected with 10 μL of BoHV-1 virus $10^{4.3}$ $TCID_{50}$/mL [17] and incubated for 1 h at 38.5 °C and 5% CO_2. The negative control formed by COCs (273) and denuded oocytes (n = 310) were subjected to the same protocol except for exposure to BoHV-1. Group II was composed of COCs (n = 425) and denuded oocytes (n = 405) subjected to the same co-incubation protocol with BoHV-1 for 24 h. The negative control of this group consisted of 398 COCs and 425 denuded oocytes subjected to the same protocol except exposure to BoHV-1. The evaluation of COCs and denuded oocytes was performed with a stereomicroscope after the incubation period. Subsequently, COCs and denuded oocytes were subjected to the fixation protocol for the immunolocalization technique according to Queiroz-Castro et al. [20].

Cells and Viruses

The "Los Angeles" BoHV-1 strain sample was replicated in Madin-Darby bovine kidney (MDBK) cells cultured in monolayers. The cells were multiplied and maintained at 37°C and 5% CO_2 atmosphere using minimum essential medium (MEM, Sigma-Aldrich, St. Louis, USA) plus 0.4 mg/L streptomycin (Sigma-Aldrich, St. Louis, USA) and 1.6 mg/L penicillin (Sigma- Aldrich, St. Louis, USA), and supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, USA). The viruses were titrated by the Tissue Culture Infective Dose method ($TCID_{50}$) according to the Reed and Muench method [21].

Virus Neutralization Assay

Virus neutralization assays were performed as described by House and Baker [22], with the addition of 100 $TCID_{50}$/50 μL of the LA BoHV-1 strain to the serum dilutions of each sample. After incubation of the serum-virus for 1 h at 37°C in a CO_2 incubator, 50 μL of MDBK cell suspension was added at the concentration of 300,000 mL^{-1} cells. The reading of the tests was performed after 72 h of incubation by monitoring the cytopathic effect. The neutralizing activity of the anti-BoHV-1 antibody was expressed as the geometric mean of the observed values. Positive and negative reference samples were used as controls.

Immunolocalization of BoHV-1

After the incubation period and the stereomicroscope evaluation, all COCs and denuded oocytes were prepared for immunofluorescence assays using laser scanning confocal microscopy. The samples were incubated in anti-mouse primal monoclonal antibody (1: 100; VMRD, NW, Washington, USA), IgG2b isotype specific for the BoHV-1 gC glycoprotein, then in anti-mouse IgG conjugated secondary antibody with fluorescein isothiocyanate-FITC (1:200; 490 nm - excitation; Sigma-Aldrich, St. Louis, MO, USA). The nucleic acid dye used was TO-PRO ™ - 3 Iodide (642 nm - excitation; Waltham, Massachusetts, USA). Samples were mounted on slides identified with Mowiol (Sigma-Aldrich, St. Louis, MO, USA) and analyzed on Zeiss 510 META laser scan confocal microscope. For the FITC dye, the Argon laser (488 nm - excitation) was used and the HeNe laser was used for TO-PRO ™ - 3 Iodide. The procedures were performed according to Queiroz-Castro et al. [20]. The slides were analyzed under the confocal laser scanning microscope at a magnification of 20x or 40x.

7.4 RESULTS

Under light microscopy, it was observed that COCs and denuded oocytes not exposed to BoHV-1 (control) showed no morphological changes after incubation for one and 24 h. The COCs co-incubated with the virus in each group showed partial disintegration of *cumulus* cells, being more evident in group II. However, the denuded oocytes exposed to BoHV-1 for both one and 24 h did not present morphological differences for either exposure time. After the immunolabeling analysis, the presence of BoHV-1 was not detected in samples from the control group (Fig. 1A), in contrast to COCs co-incubated with the virus for 1 h. In these samples, BoHV-1 was detected in the cytoplasm of *cumulus* cells, most of which was located more peripherally to the oocyte ZP (Fig 1B). In the COCs co-incubated with the virus for 24 h, BoHV-1 was detected in the cytoplasm of the *cumulus* cells closest to the ZP and inside the oocyte (Fig 1C).

In denuded oocytes, no control samples showed the presence of BoHV-1 (Fig 1D). However, immunolabeling evidenced the presence of BoHV-1 outside the ZP with 1 h of incubation (Fig 1E) and inside denuded oocytes with 24 h exposure to BoHV-1 (Fig 1 F).

A primary monoclonal antibody that binds to glycoprotein C of the BoHV-1 viral envelope enables labeling of the viral structural proteins.

3D reconstruction of COCs and denuded oocytes were performed in confocal microscopy, from serial cuts of X micrometers. In these, it was possible to observe the presence of BoHV-1 inside the oocyte that presented *cumulus* cells (Fig 2). Similarly, BoHV-1 was also evidenced inside denuded oocytes, as well as on the ZP surface (Fig 3).

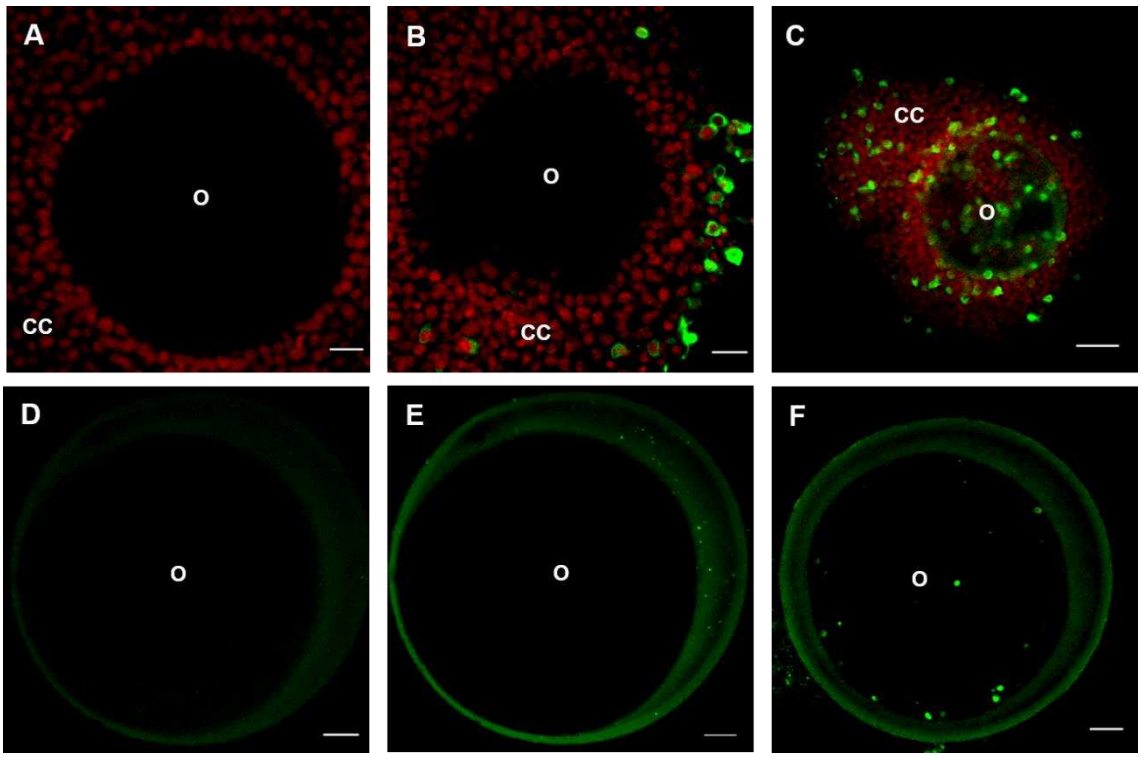

Figure 1. Experimental infection of bovine oocytes with BoHV-1 incubated for one and 24 h. (A) confocal image of COC negative control, (B) immunolabeling of BoHV-1 (green) in the cytoplasm of *cumulus* cells (CC) of co-incubated COCs for 1 h (Scale bar = 20 μm) and (C) immunolabelling of BoHV-1 in the cytoplasm of COC *cumulus* cells co-incubated for 24 h (Scale bar = 50 μm). O: oocyte. The nuclei of the *cumulus* cells were stained with TO-PRO 3 iodide (red). (D) denuded oocyte control. (E) denuded oocyte co-incubated with BoHV-1 for 1 h and (F) denuded oocyte co-incubated with BoHV-1 for 24 h (Scale bar = 20 μm).

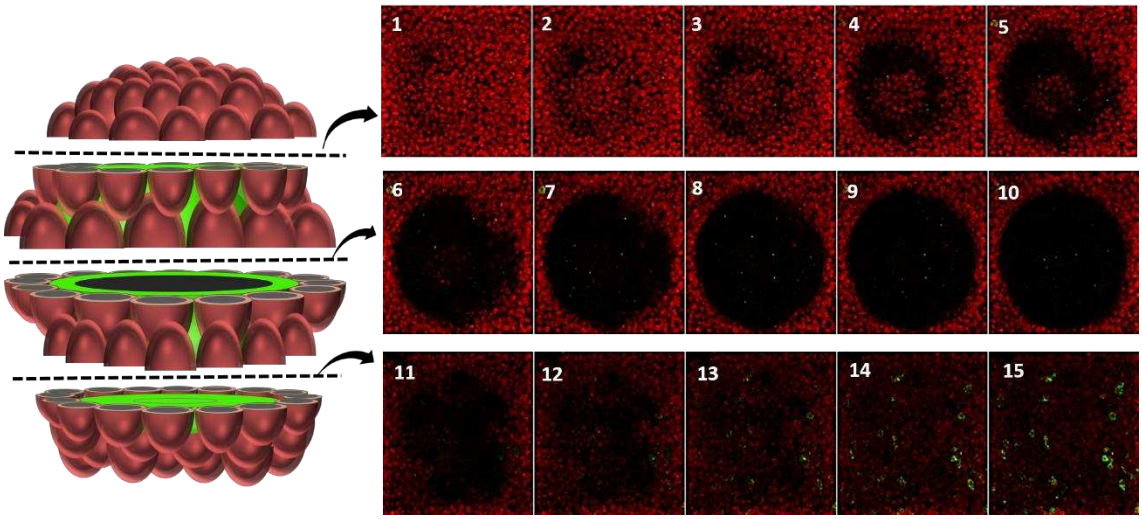

Figure 2. Reconstruction of the image of COC co-incubated with BoHV-1 for 24 h in a series of focal planes (step size of 1μm) evidencing the presence of BoHV-1 (green) inside the oocyte.

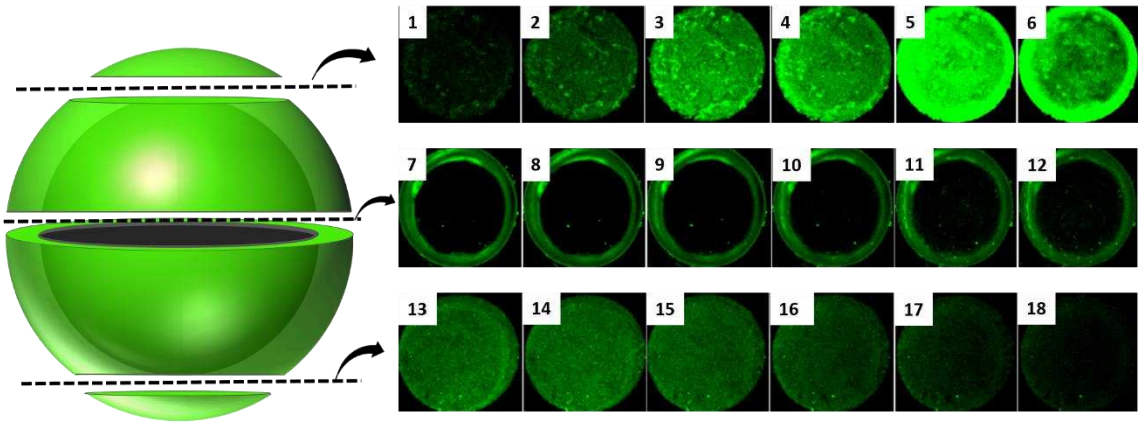

Figure 3. Image reconstruction of a denuded oocyte co-incubated with BoHV-1 for 24 h in a series of focal planes (step size of 1µm) evidencing the presence of BoHV-1 (bright green dots) within the oocyte and on the outer face of the zona pellucida.

7.5 DISCUSSION

This study provides the first evidence of the BoHV-1 presence within the COCs and denuded oocytes exhibiting intact ZP when co-incubated with the virus for 24 h. Hence, BoHV-1 can cross the intact ZP of bovine oocytes with and without the presence of *cumulus* cells exposed to the virus for 24 h of co-incubation. BoHV-1 was also detected in the cytoplasm of *cumulus* cells from all COCs that were co-incubated with the virus. However, within 1 h of incubation, BoHV-1 was limited to the more peripheral region of *cumulus* cells.

The detection of BoHV-1 in cells closest to the ZP and inside the oocyte from infected COCs after 24 h exposure can be attributed to the complete replicative cycle period of BoHV-1, with formation of new progenies in permissive cells occurring in approximately 18-20 h [2]. Therefore, the co-incubation of 24 h allowed the occurrence of a complete cycle with the formation of new progenies that were able to cross the integrated ZP.

In accordance with our findings, studying the BoHV-5, Silva-Frade et al. [17] reported that this virus was able to pass through the intact ZP of bovine oocytes.

Nevertheless, Vanroose et al. [11] concluded that intact ZP acts as an efficient barrier to entry of BoHV-1 into the bovine oocyte, however these investigators evaluated whether or not there was infection of the embryonic cells of the embryos exposed to the virus. It is noteworthy that bovine oocyte ZP presents approximately 1511 pores and these are large enough to allow the entry of BoHV-1 [13].

For the successful replication of a virus within a cell, there must be interactions between the virus and the host cell. Viral infection results in disordered cellular processes that can trigger apoptosis [23]. In addition, it is known that organisms use apoptosis as an antiviral defense, but many viruses can modulate the apoptotic pathways of the host cell [24,25]. It is believed that the virus's ability to prevent apoptosis prolongs infection and enables successful viral replication [26,27].

Silva-Frade et al. [28] concluded that BoHV-5 was able to suppress specific apoptotic pathways in infected bovine oocytes. However, BoHV-5 infection did not compromise the embryonic development as commonly described for BoHV-1 [10,11,14-16]. In bovine kidney (MDBK) cells, BoHV-1 was able to induce apoptosis slightly after penetration and then inhibited the apoptotic process [29]. The blocking of caspase activation by the virus itself increased BoHV-1 replication [30].

Many mechanisms involved in cell survival during viral infection remain unclear, however, the findings of the present study that demonstrated the presence of BoHV-1 within the bovine oocyte could provide explanations for BoHV-1 interference in the in vitro fertilization process and in the compromising of embryonic development mentioned in several studies [10,11,14,15,31].

BoHV-1 was also highlighted in the ZP layers, corroborating the findings of Vanroose et al. [12,13]. The persistence of ZP adherence even after trypsin washes as cautioned by the International Embryo Transfer Society (IETS) was presented in several studies [32-36].

Regarding COCs observation under light microscopy, a partial disintegration of the *cumulus* cells was observed which was more evident in the 24-h co-incubated group. This can be explained by cellular lysis resulting from viral replication, known as the cytopathic effect, characteristic of the herpes virus. The cytopathic effect was mentioned by Tsuboi and Imada [37] in oocytes of *cumulus* cells matured in vitro in the presence of BoHV-1. This effect was also observed by Vanroose et al. [11] by rounding of *cumulus* cells and concurrent failure to produce a confluent monolayer.

It is worth mentioning that between *cumulus* cells and oocytes there is a bidirectional communication that is an essential characteristic of mammalian reproduction. The *cumulus* cells provide the oocyte with nutrients, regulatory signals [38,39] and chemical components such as microRNAs that regulate the genes involved in oocyte maturation processes [40]. Transcripts needed for oocyte development may be passed to the oocyte from *cumulus* cells through communicating junctions [41] or straddling projections [42].

The premature rupture of communication between *cumulus* and oocyte cells affects the level of oocyte competence and the rate of viable blastocyst formation [43,44]. Thus, the replication capacity of BoHV-1 in these cells, which was observed in this study, negatively affects this communication and may compromise oocyte viability.

In conclusion, the detection of BoHV-1 within oocytes with or without the presence of *cumulus* cells is of great relevance to the field of animal reproduction. Additionally, the detection of cytoplasmic BoHV-1 in the different layers of *cumulus* cells demonstrates that these cells can act as sources of viral infection.

7.6 ACKNOWLEDGEMENTS

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8. CONCLUSÕES GERAIS

Os achados deste trabalho de tese são de grande importância para a área de reprodução animal. A detecção do BoHV-1 por meio da imunomarcação viral, com a microscopia confocal de varredura a laser, propiciou novos esclarecimentos quanto à interação do vírus com os órgãos genitais de vacas.

A detecção de partículas virais no útero, tubas uterinas e ovários revela esses órgãos como sítios de replicação do BoHV-1. A imunomarcação viral no útero em 100% das vacas infectadas naturalmente, sugere este órgão como fonte de infecção fetal para ocasionar o abortamento.

Adicionalmente, o BoHV-1 também foi evidenciado no citoplasma das células do *cumulus* de vacas soropositivas. Deste modo, em condições naturais de infecção, o vírus poderia afetar negativamente a comunicação existente entre as células do *cumulus* e o ovócito sugerindo novos esclarecimentos a respeito das falhas reprodutivas atribuídas a infecção por este vírus, como a infertilidade temporária e morte embrionária.

Este estudo também evidenciou a presença do BoHV-1 no interior de ovócitos co-incubados com o vírus por 24 horas, indicando que o BoHV-1 foi capaz de transpassar a zona pelúcida intacta de ovócitos bovinos com e sem a presença das células do *cumulus*.

APÊNDICE I

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 94/2015, intitulado "**Capacidade de contaminação celular pelo herpesvírus bovino 1 em estruturas ovarianas e detecção do agente em ovários, tubas uterinas e útero de animais soropositivos**", coordenado pelo professor Eduardo Paulino da Costa do Departamento de Veterinária, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado definitivamente em 19/03/2018.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 94/2015, named **"Cell contamination capacity bovine herpesvirus 1 in the ovarian structures and detection agent in ovaries, fallopian tubes and uterus of positive animals**", is in agreement with the a ctual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being definitive approved on March 19, 2018.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV