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Rafael Costa Ebling

PATOGÊNESE EXPERIMENTAL DE PARAPOXVÍRUS BOVINO 2 EM BEZERROS, E POXVÍRUS SUÍNO COMO MODELO PARA INATIVAÇÃO VIRAL EM CARCAÇAS

Santa Maria, RS
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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária**.

Orientador: Prof. Dr. Eduardo Furtado Flores

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Aprovado em 31 de março de 2022:

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Santa Maria, RS
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DEDICATÓRIA

À minha família, dedico este trabalho como forma de agradecimento e retribuição pelo incansável esforço em fornecer a melhor educação possível que poderiam desejar aos seus filhos.

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RESUMO

PATOGÊNESE EXPERIMENTAL DE PARAPOXVÍRUS BOVINO 2 EM BEZERROS, E POXVÍRUS SUÍNO COMO MODELO PARA INATIVAÇÃO VIRAL EM CARCAÇAS

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ORIENTADOR: Eduardo Furtado Flores

Os poxvírus de animais apresentam importância sanitária e econômica como patógenos, mas também tem sido utilizado como vetores vacinais, imunoestimulantes e como modelos para outros vírus. Esta tese é composta por dois estudos envolvendo vírus da família *Poxviridae,* com enfoques diferentes. No primeiro projeto, estudou-se a patogenia da infecção pelo Parapoxvírus bovino *2* (*Pseudocowpox virus*, PCPV) em bezerros. Já no segundo, utilizou-se o Poxvírus suíno (*Swine poxvirus*, SPV) como modelo para inativação viral em carcaças. No experimento 1, bezerros de 3 – 4 meses de idade foram inoculados pela via intranasal (n=6) ou no plano nasal (n=2) com um isolado de PCPV que excretou o vírus até o dia 13 pós-inoculação (pi), mas não desenvolveram sinais locais ou sistêmicos até o dia 20pi. Entre os dias 28-34pi, no entanto, sete (7/8) bezerros inoculados desenvolveram um curso clínico assíncrono, com o desenvolvimento de número variável de lesões pápulo-pustulosas, erosivo-fibrinosas e crostosas no focinho, em alguns casos estendendo-se aos lábios e gengiva. Em alguns animais, as lesões coalesceram, formando extensas placas fibrinóticas/necróticas e crostosas cobrindo quase inteiramente o focinho. O curso clínico durou de 8 a 15 dias e diminuiu progressivamente após o dia 42pi. O DNA viral foi detectado por PCR em suabes coletados de lesões de seis animais entre os dias 34 e 42pi. O exame histológico de fragmentos coletados das lesões do focinho de dois bezerros afetados (dia 36pi) revelou hiperplasia epidérmica acentuada e hiperceratose ortoceratótica e paraceratótica grave, coberta por crostas espessas. A epiderme apresentava áreas multifocais de necrose coalescente de queratinócitos e leve degeneração vacuolar multifocal. Microabscessos ocasionais também foram observados na epiderme. Amostras de soro de bezerros coletadas no dia 50 pi apresentaram neutralização parcial do vírus em baixas diluições (1:5, 1:10 e 1:20), indicando soroconversão. No estudo 2, investigou-se a viabilidade do SPV em carcaças suínas pelo método de AGB (*above ground burial*), como modelo para o vírus da peste suína africana (*African swine fever virus*, ASFV). Para isso, o SPV foi inoculado por via intrafemoral em 90 carcaças de suínos adultos. Amostras de medula óssea foram coletadas e testadas periodicamente ao longo de 12 meses. No estudo *in vitro*, inóculos de Senecavírus A (SVA), vírus da diarreia viral bovina (BVDV) e SPV foram homogeneizados com material de medula óssea e mantidos a 21-23ºC por 30 dias. Em ambos os estudos, a viabilidade do vírus foi avaliada por isolamento viral, enquanto a presença de ácido nucleico viral foi avaliada por qPCR. No estudo de campo, o SPV permaneceu viável em 11 (55%) amostras de medula óssea coletadas apenas no dia 7; posteriormente apenas o DNA viral foi detectado. Estima-se que a inativação viral completa tenha ocorrido ao redor do dia 11pi. Os testes *in vitro* revelaram uma resistência variável dos vírus estudados. A viabilidade foi estimada em 80, 118 e 28 dias para SPV, SVA e BVDV, respectivamente. Desta forma, no primeiro estudo observou-se um curso clínico tardio e severo associado com a inoculação de PCPV em bezerros, pouco comum em infecções experimentais pelos parapoxvírus bovinos. No segundo estudo demonstrou-se que a técnica AGB foi eficaz na inativação de SPV em curto prazo. Assim, considerando-se que taxa de inativação do SPV é comparável à do ASFV em estudos de campo, essa técnica pode ser

explorada para futuros estudos com o ASFV. Além disso, este estudo contribui para a compreensão da cinética de inativação de vírus em condições específicas, o que é fundamental para projetar e aplicar contramedidas em caso de violação de biossegurança em locais de gerenciamento de mortalidade animal.

Palavras-chave: poxvírus animais, pseudovaríola, patogenia, inativação viral.

ABSTRACT

EXPERIMENTAL PATHOGENESIS OF BOVINE PARAPOXVIRUS 2 IN CALVES AND SWINE POXVIRUS AS A MODEL FOR VIRAL INACTIVATION IN CARCASSES

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Animal poxviruses present sanitary and economic importance as disease agents, but they have also been used as vaccine vectors, immunostimulants and as models for other viruses. This Thesis is composed of two studies involving *Poxviridae* viruses, with different approaches. In the first study, the pathogenesis of bovine Parapoxvirus 2 (*Pseudocowpox virus*, PCPV) infection was studied in calves. In the second study, swine poxvirus (*Swine poxvirus*, SPV) was used as a model for viral inactivation in carcasses. In study 1, 3-4 month old calves inoculated intranasally (n=6) or in the nasal plane (n=2) with a PCPV isolate replicated and shed virus until day 13 post-inoculation (pi) , but did not develop local or systemic signals until day 20pi. On days 28-34pi, however, seven (7/8) inoculated calves developed an asynchronous clinical course, with development of few to multiple papulopustular, erosive-fibrinous, and scaly lesions on the snout, in some cases extending to the lips and gum. In some animals, the lesions coalesced, forming extensive fibrinotic/necrotic and scaly plaques covering almost the entire snout. The clinical course lasted from 8 to 15 days and progressively decreased after day 42pi. Viral DNA was detected by PCR in swabs collected from lesions of six animals between days 34 and 42pi. Histological examination of fragments collected from the snout lesions of two affected calves (day 36pi) revealed marked epidermal hyperplasia and severe orthokeratotic and parakeratotic hyperkeratosis covered by thick crusts. The epidermis showed multifocal areas of coalescing necrosis of keratinocytes and mild multifocal vacuolar degeneration. Occasional micro-abscesses were also observed in the epidermis. Sera from calves inoculated at 50pi showed partial neutralization of the virus at low dilutions (1:5, 1:10 and 1:20), indicating seroconversion. In study 2, the viability of SPV in swine carcasses was investigated by the AGB (*above ground burial*) method, as a model for the *African swine fever virus* (ASFV). For this, SPV was inoculated intrafemorally in 90 carcasses of adult pigs. Bone marrow samples were collected and tested periodically over 12 months. In the in vitro study, inoculate of Senecavirus A (SVA), bovine viral diarrhea virus (BVDV) and SPV were mixed with bone marrow material and kept at 21-23°C for 30 days. In both studies, virus viability was assessed by viral isolation, while the presence of viral nucleic acid was assessed by qPCR. In the field study, SPV remained viable in 11 (55%) bone marrow samples collected on day 7 alone; later only viral DNA was detected. Complete viral inactivation is estimated to have occurred around day 11pi. The in vitro tests revealed a variable resistance of the studied viruses. Viability was estimated at 80, 118 and 28 days for SPV, SVA and BVDV, respectively. Thus, in the first study, a late and severe clinical course associated with PCPV inoculation in calves was observed, uncommon in experimental infections by bovine parapoxviruses. In the second study, the AGB technique was shown to be effective in the short-term inactivation of SPV. Thus, considering that the rate of inactivation of SPV is comparable to that of ASFV in field studies, this technique can be explored for future studies with ASFV. Furthermore, this study contributes to the understanding of the kinetics of virus inactivation under

specific conditions, which is critical to design and apply countermeasures in case of biosecurity breach in animal mortality management sites.

Keywords: animal poxviruses, pseudocowpox, pathogenesis, viral inactivation.

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LISTA DE ABREVIATURAS E SIGLAS

SV	Setor de Virologia
PCPV	Pseudowpoxvirus (vírus da pseudo-varíola)
SPV	Poxvírus suíno
BPSV	Vírus da Estomatite Papular Bovina
BVDV	Vírus da Diarreia Viral Bovina
SVA	Seneca Vírus A
ASFV	Vírus da Peste Suína Africana
PSA	Peste Suína Africana
PSC	Peste Suína Africana
PPVs	Parapoxvírus
ORFV	Vírus ORF de ovinos e caprinos – Ectima contagioso
ICVT	Internacional Committee on Taxonomy of Viruses
FMDV	Vírus da Febre Aftosa – *Foot and Mouth Disease Virus*
PVNZ	Vírus do Cervo Vermelho da Nova Zelândia – *Parapoxvirus of red deer in New Zealand* (Red deerpox virus)
VACV	Vírus da Vaccinia
OPXV	Orthopoxvirus

SUMÁRIO

1 INTRODUÇÃO

As doenças hemorrágicas e vesiculares em animais de produção podem estar associadas a um grupo de vírus que possuem relevância sanitária, econômica e de saúde pública, além de constituírem diagnóstico diferencial para a febre aftosa. Dentre esses, destacam-se alguns agentes zoonóticos que, ocasionalmente, afetam trabalhadores rurais, como ordenhadores e pessoas que tem contato próximo com animais infectados. A febre aftosa é considerada a doença vesicular mais importante, pois causa embargo econômico para o país que exporta produtos de carne bovina, causando perdas significativas (ALONSO et al., 2020).

O parapoxvírus bovino 2 [*Pseudocowpox virus* (PCPV) ou vírus da pseudo-varíola bovina] é um dos dois parapoxvírus (PPVs) de bovinos, juntamente com o vírus da estomatite papular bovina (*Parapoxvirus bovis 1* - BPSV). Duas outras espécies virais, o vírus da Orf de ovinos e caprinos (*Parapoxvirus ovis* - ORFV) e o parapoxvírus do cervo vermelho da Nova Zelândia (*Parapoxvirus of red deer in New Zealand* - PVNZ), completam o gênero *Parapoxvirus*, subfamília *Chordopoxvirinae* da família *Poxviridae* (ICTV, 2020).

Os PPVs são vírus epiteliotrópicos, distribuídos mundialmente e associados com doenças cutâneas não sistêmicas, vesiculares e eruptivas em mamíferos domésticos e selvagens, especialmente ruminantes (FLEMMING; MERCER, 2007). Infecções naturais pelo PCPV geralmente cursam com lesões pápulo-vesico-pustulosas nos tetos e úbere de vacas leiteiras e lesões focinho-orais em bezerros que se alimentam de vacas afetadas (ALONSO et al., 2020). Espécies de PPV (PCPV, BSPV e ORFV) exibem um espectro estreito de hospedeiros e podem ocasionalmente ser transmitidas para humanos, causando lesões localizadas nas mãos, geralmente chamadas de nódulo do ordenhador ou pseudovaríola (BÜTTNER; RZIHA, 2002).

Casos naturais ou surtos de doenças associadas ao PCPV têm sido descritos em todo o mundo, geralmente associados a lesões mucocutâneas leves e autolimitantes em bovinos (LEDERMAN et al., 2014) (VELAZQUEZ-SALINAS et al., 2018). Além disso, casos atípicos (BLOMQVIST et al., 2018) e infecções mistas com outros poxvírus também foram descritos (LAGUARDIA-NASCIMENTO et al., 2017). Os impactos sanitários e econômicos da infecção por PCPV para os bovinos permanecem incertos, provavelmente devido à sua ocorrência esporádica/rara e curso clínico leve. No Brasil, vários casos/surtos de doenças associadas ao PCPV (e outros PPVs também)

foram relatados nas últimas décadas, afetando vacas leiteiras, bezerros e, ocasionalmente, humanos (ALONSO et al., 2020). Em algumas regiões brasileiras, as infecções por PPV em bovinos leiteiros são relativamente frequentes e causam algumas perdas econômicas a esses rebanhos, representando um risco ocupacional para os seres humanos (CARGNELUTTI et al., 2012). Uma questão importante em relação à doença associada ao PPVs reside na sua semelhança com outras doenças vesiculares de bovinos, necessitando de diagnóstico diferencial, principalmente da febre aftosa.

A maioria dos relatos de PCPV publicados até o momento descreve casos/surtos clínicos, frequentemente associados à identificação do agente por microscopia eletrônica e/ou por técnicas moleculares. Aqui descrevemos uma infecção experimental de PCPV em bezerros, hospedeiros naturais do vírus. Os resultados aqui apresentados contribuem no esclarecimento da biologia da infecção por PCPV e sugeram aspectos importantes da patogênese do vírus que podem ser relevantes para o reconhecimento, controle e prevenção da doença.

Uma vez evidenciada a importância de diagnóstico diferencial de doenças de alto impacto econômico, temos também uma segunda enfermidade, a peste suína africana (PSA ou *African swine fever* - ASF). A PSA é uma doença que tem ocasionado perdas drásticas para a suinocultura de vários países. A disseminação do vírus da PSA pela Ásia, Europa e, recentemente, na América Central gerou um alerta significativo para a indústria global de carne suína. Em recente relatório da FAO (2019), os surtos de PSA em curso resultaram no abate de mais de seis milhões de animais em vários países.

A doença é causada pelo *African swine fever virus* (ASFV), gênero *Asfivirus*, pertencente à família *Asfarviridae.* Esse agente possui semelhanças estruturais (arquitetura e composição dos vírions, tipo e topologia do DNA genômico) e biológicas com vírus da família *Poxviridae*, dentre eles o poxvírus suíno (*Swinepox virus* - SPV) (DIXON et al., 2013). Embora permaneça uma questão de debate, a ordem provisória de *Megavirales* conteria, entre outras famílias, as famílias *Asfarviridae* e *Poxviridae* (ANDRÉS et al., 2020; ICTV, 2020).

Devido à PSA ser uma doença emergente de grande importância na suinocultura, o uso do SPV como substituto/modelo do ASFV em alguns estudos pode ser alternativa para determinados aspectos da PSA em países livres da PSA, já que além de compartilhar certas semelhanças morfológicas e biológicas, esses vírus possuem semelhanças com relação à resistência ambiental (SMITH, 2007). Dessa forma, devido ao grande impacto e à impossibilidade de se trabalhar com o vírus causador da PSA em

território livre desta doença, optou-se por utilizar o SPV como substituto, que possui distribuição mundial e apresenta similaridades na replicação e resistência ao meio ambiente em relação ao ASFV.

A inoculação do SPV foi realizada por via intrafemoral, devido ao nível de calcificação do fêmur, sendo uma das estruturas mais tardias a se decompor e, portanto, permitiu a recuperação da amostra ao longo dos 12 meses do projeto. Na sequência, um segundo estudo, porém *in vitro*, onde foi avaliado a viabilidade de SPV, Senecavírus A (SVA) e vírus da diarreia viral bovina (BVDV) em tecidos de medula óssea enriquecidos, mantidos a 21-23ºC por um período de 30 dias. Dessa forma, conseguimos isolar os fatores ambientais e avaliar um possível interferência da medula óssea no vírus, dando suporte para a avaliação um novo modelo de inativação viral a campo.

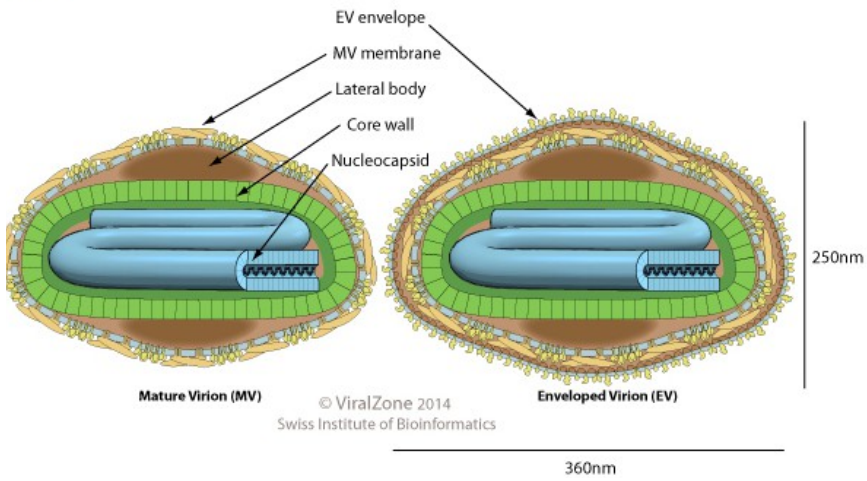
2 REVISÃO BIBLIOGRÁFICA

2.1 Parapoxvírus bovino 2

2.1.1 Propriedades gerais

O PCPV é o agente de uma doença vesico-papular erosiva das superfícies mucocutâneas de bovinos (BÜTTNER; RZIHA, 2002), sendo um dos dois PPVs de bovinos, juntamente com o BPSV. Juntamente com o vírus Orf e o PVNZ completam o gênero *Parapoxvirus,* subfamília *Chordopoxvirinae, família Poxviridae* (ICTV, 2020). Os parapoxvírus (FIGURA 1) são vírus grandes, envelopados, em forma de tijolo, com genoma de DNA de fita dupla. O genoma linear é flanqueado por sequências de repetição terminal invertida (*inverted terminal repeat* - ITR) que são covalentemente fechadas em suas extremidades (ICTV, 2020).

Figura 1. Estrutura do *Parapoxvirus*



Estrutura de vírions do *Parapoxvírus:* possuem formato ovóide, envelopados, com 220 – 300 nm de comprimento e 140 – 170 nm de largura. Fonte:https://viralzone.expasy.org/150

A membrana de superfície exibe filamentos e existem duas partículas virais infecciosas distintas: o vírus maduro intracelular (*intracellular mature virus* - IMV) e o vírus envelopado extracelular (*extracellular enveloped virus* - EEV) (Figura 1).

A densidade flutuante de vírions está sujeita a influências osmóticas: em tampões diluídos é de cerca de 1,16 g cm^{-3}, em sacarose cerca de 1,25 g cm^{-3} e em CsCl e tartarato de potássio cerca de 1,30 g cm^{-3}. Os vírions tendem a se agregar em solução com alto teor de sal. A infectividade viral de alguns membros do *Poxviridae* é resistente à tripsina. Alguns membros são resistentes ao éter. Geralmente, o vírion é sensível a detergentes comuns, formaldeído, agentes oxidantes e temperaturas superiores a 40°C. A membrana da superfície do vírion é removida por detergentes não iónicos e reagentes redutores de sulfidrilo. Os vírions são relativamente estáveis em condições secas à temperatura ambiente; eles podem ser liofilizados com pouca perda de infectividade (FENNE, 2017).

As proteínas constituem cerca de 90% do peso das partículas. Os genomas codificam 150–300 proteínas dependendo da espécie; cerca de 100 diferentes proteínas estão presentes nos vírions. As partículas de vírus contêm muitas enzimas envolvidas na transcrição de DNA. Os vírions envelopados possuem polipeptídeos na bicamada lipídica, que envolve a partícula.

Em geral, as proteínas conservadas essenciais para a replicação do vírus em cultura (polimerases e outras enzimas e proteínas estruturais) são codificadas na região central do genoma, enquanto as proteínas menos conservadas e não essenciais envolvidas nas respostas vírus-hospedeiro são codificados nas regiões terminais do genoma. Por exemplo, o vírus canaripox codifica 51 proteínas da família de repetição da anquirina (FENNE, 2017). Os lipídios constituem cerca de 4% do peso das partículas. Os vírions envelopados contêm lipídios, incluindo glicolipídios, que podem ser lipídios celulares modificados. Os carboidratos constituem cerca de 3% do peso das partículas. (FLEMMING; MERCER, 2007).

2.1.2 Impacto econômico e sanitário

Os impactos sanitários e econômicos da infecção por PCPV para o gado permanecem amplamente incertos, provavelmente devido à sua ocorrência esporádica/rara e curso clínico leve. No Brasil, vários casos/surtos de doenças associadas ao PCPV (e outros PPVs também) foram relatados nas últimas décadas, afetando vacas leiteiras, bezerros e, ocasionalmente, humanos (CARGNELUTTI et al., 2012; DE SANT'ANA et al., 2013; LAGUARDIA-NASCIMENTO et al., 2017; ALONSO et al., 2020).

A infecção pelo poxvírus está distribuída em todo o mundo e afeta principalmente vacas leiteiras, mas apresenta importância sanitária e econômica limitada na maioria dos países. Em alguns laticínios, no entanto, a infecção pode assumir alguma importância econômica devido às más condições de higiene. O agente geralmente é introduzido nos rebanhos por meio de animais infectados e se dissemina lentamente entre os animais. A transmissão dentro dos rebanhos ocorre por contato direto e indireto. A rota direta inclui a amamentação de bezerros e a indireta através de moscas, equipamentos de ordenha e manejo inadequados (MUNZ, E; DUMBELL, 1994).

Provavelmente devido à sua ocorrência esporádica e curso clínico leve, a infecção por PCPV tem chamado pouca atenção e as publicações e, consequentemente, o conhecimento sobre a biologia e patogênese da infecção por PCPV são escassos. Em algumas regiões brasileiras, as infecções por PPV em bovinos leiteiros são relativamente frequentes e causam algumas perdas econômicas a esses rebanhos, representando um risco ocupacional para os seres humanos (LAGUARDIA-NASCIMENTO et al., 2017). Além disso, a importância da infecção pelos PPV e outras

doenças vesiculares reside principalmente na sua natureza vesicular, exigindo diagnóstico diferencial imediato da febre aftosa e no risco de saúde pública e falta de conhecimento do sistema de saúde em identificar a lesão (ALONSO et al., 2020).

2.1.3 Patogenia

Infecções por PPVs têm sido descritas em todo o mundo e estão associadas a doenças mucocutâneas não sistêmicas em mamíferos domésticos e selvagens, especialmente ruminantes. Infecções naturais por PCPV geralmente cursam com lesões vesico-papulares nos tetos e úbere de vacas leiteiras e lesões focinho-orais em bezerros que se alimentam de vacas afetadas. Espécies de PPVs geralmente apresentam uma faixa estreita de hospedeiros, mas podem ocasionalmente ser transmitidas para humanos, causando lesões localizadas nas mãos. A infecção humana pelo PPV é geralmente ocupacional, afetando ordenhadores ou outras pessoas em contato com animais afetados – causando lesões chamadas de "nódulo de ordenhador" ou pseudo-varíola (ALONSO et al., 2020) .

As infecções por PCPV são diagnosticadas pela avaliação clínica das lesões da pele e mucosas juntamente com microscopia eletrônica ou análises imuno-histoquímicas, isolamento e/ou detecção de genoma viral por reação em cadeia da polimerase (*polymerase chain reaction* - PCR). As infecções são geralmente autolimitadas após 1 a 6 semanas, mas no caso de superinfecções bacterianas podem resultar em lesões ulcerativas e necrosantes graves. Sinais clínicos são mais comumente observados em bezerros de 2 a 10 meses de idade (YAEGASHI et al., 2013).

No Brasil, vários casos/surtos de doença associada de PPVs foram relatados nas últimas décadas, afetando vacas leiteiras, bezerros e, ocasionalmente, o homem. A doença geralmente é benigna, com lesões que progridem através do estágio papular para formar pústulas em poucos dias. Essas lesões dão origem a úlceras e, posteriormente, tornam-se espessas formando crostas sobrejacentes, que se descamam por volta de 4 a 6 semanas. Tais casos podem ser consideradas com lesões menos sérias, ou até mesmo auto limitantes, mas também no entanto, se a infecção viral for acompanhada por uma coinfecção bacteriana, como estafilococos, estreptococos ou corinebactérias, a taxa de mortalidade pode se aproximar de 90% (FLEMING et al., 2017).

Casos naturais ou surtos de doenças associadas a infecções por PCPV têm sido descritos em vários países, geralmente associados a lesões mucocutâneas leves e autolimitadas em bovinos, como foi o caso de estudo retrospectivo de todos os casos de

poxvírus diagnosticados e notificados em bovinos no Distrito Federal (DF), entre 2015 e 2018, onde envolveu 385 fazendas, abrigando um total de 23.963 bovinos dos quais 2.467 (10,29%) foram examinados. Noventa e três animais apresentaram lesões sugestivas e/ou compatíveis com poxvírus. Cinquenta e dois casos foram diagnosticados como associados a poxvírus (47 e 5 por vigilância ativa e passiva, respectivamente): 27 como vaccínia bovina (BV), 9 como (PCPV), 8 como BPSV, 5 como coinfecção (PCPV e BPSV) e 3 como um parapoxvírus não identificado (ALONSO et al., 2020).

Há casos considerados atípicos, onde foi descrito um surto de pseudocowpox com um quadro clínico incomum em um rebanho leiteiro de 80 vacas em *free-stall*. Aproximadamente 90% das vacas apresentaram vesículas, erosões, pápulas e crostas na vulva e na mucosa vaginal. A análise histológica dos tecidos da biópsia indicou uma infecção viral primária, embora não especificada. A microscopia eletrônica de transmissão revelou partículas de parapoxvírus em tecidos e materiais vesiculares, as análises confirmaram a ausência de outras causas potenciais de vulvovaginite pustulosa, como herpesvírus bovino 1 e *Ureaplasma diversum*. Suspeita-se que uma escova de vaca rolante tenha sido o fômite (BLOMQVIST et al., 2018).

Em casos de infecções mistas, há relato de detecção de DNA de PPV em amostras de leite de vacas afetadas, indicando que o vírus pode estar presente no leite e potencialmente contaminando produtos lácteos associados ou não ao Orthopoxvirus (OPXV). Além das lesões causadas pelo contato direto, a presença de 2 ou mais espécies de poxvírus no leite mostrou que o efeito das doenças exantemáticas zoonóticas na saúde pública e na pecuária é relevante e não pode ser negligenciado (REHFELD et al., 2018).

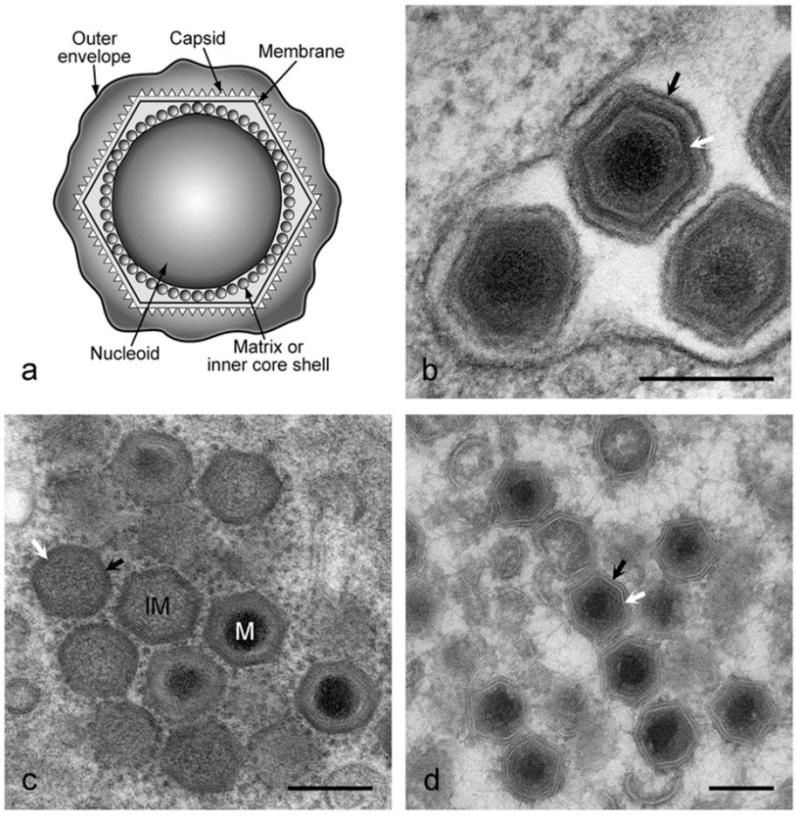
Há relatos de infecção de outras espécies animais, que teve como principal objetivo detectar BSPV, PCPV e VACV, onde 89 amostras foram recebidas no Laboratório Oficial do Ministério da Agricultura do Brasil, onde surtos de poxvírus foram detectados em nove estados: Amazonas, Bahia, Mato Grosso do Sul, Mato Grosso, Minas Gerais, Pará, Roraima, São Paulo e Tocantins. A maioria das amostras foi positiva para apenas um vírus nos testes moleculares, mas houve um número significativo de coinfecções por PCPV/BSPV, PCPV/VACV, BSP/VACV e PCPV/BSPV/VACV (LAGUARDIA-NASCIMENTO et al., 2017).

2.2 Poxvírus suíno como modelo para ASFV

2.2.1 Vírus da peste suína africana (ASFV)

Os vírions do PSA consistem em estruturas centrais de nucleoproteína, de 70 a 100 nm de diâmetro, envoltas por uma camada lipídica interna e um capsídeo icosaédrico, de 170 a 190 nm de diâmetro, que por sua vez é circundado por uma camada lipídica externa contendo envelope. O capsídeo exibe simetria icosaédrica (T = 189-217) correspondendo a 1892-2172 capsômeros. Cada capsômero tem 13 nm de diâmetro e aparece como um prisma hexagonal com um orifício central; a distância intercapsomérica é de 7,4–8,1 nm. Uma membrana interna envolve o núcleo que reveste o nucleoide contendo ácido nucleico (SALAS; ANDRÉS, 2013). Os vírions envelopados extracelulares têm um diâmetro de 175–215 nm (Figura 2).

Figura 2. Estrutura do *Asfivirus*



(a) Diagrama do vírus da peste suína africana mostrando nucleóide, camada central interna, membrana interna, capsídeo e envelope externo. (b) imagem por microscopia eletrônica (ME) de vírions extracelulares. A seta preta mostra o envelope externo, a seta branca mostra o capsídeo. Barra = 200 nm. (c) imagem de ME de vírions intracelulares. IM = vírion imaturo, M = vírion maduro. A seta preta mostra a proteína do capsídeo, a seta branca mostra a membrana do vírus. Barra = 200 nm. (d) imagem ME de vírions intracelulares. A seta preta mostra a proteína do capsídeo, a seta branca mostra a membrana do vírus. Barra = 200 nm. Fonte: Pippa Hawes, The Pirbright Institute, Reino Unido.

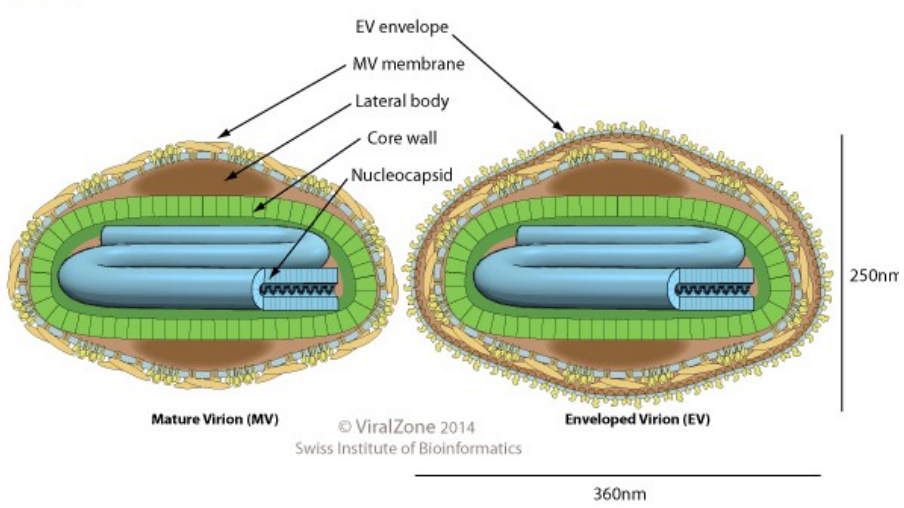
Os vírions são sensíveis ao éter, clorofórmio e desoxicolato, e são inativados a 60°C em 30 minutos, podendo manter sua viabilidade por anos a 20°C ou 4°C. A infectividade é estável em uma ampla faixa de pH. Alguns vírus infecciosos podem manter-se viáveis ao tratamento em pH4 ou pH13. O vírus é sensível a alguns desinfetantes (formaldeído a 1% por 6 dias, NaOH a 2% por 1 dias); onde os parafenilfenólicos são muito eficazes, assim como à irradiação (ALONSO et al., 2018).

O genoma consiste em uma única molécula de dsDNA linear, covalentemente fechado, de 170-194 kbp. As sequências finais estão presentes como duas formas de flip-flop que são invertidas e complementares uma em relação à outra, e adjacentes a ambas as extremidades estão arranjos idênticos de unidades de 2,1 kbp repetidas diretamente. As sequências nucleotídicas completas de 18 isolados foram determinadas. Estes incluem o isolado Ba71V adaptado para cultura de tecidos (ASFV-BA71V) e 17 isolados de campo da Europa e África (CHAPMAN et al., 2008; DE VILLIERS et al., 2010, DANZETTA et al., 2020; PORTUGAL et al., 2015; CHAPMAN et al., 2011).

2.2.2 Poxvírus suíno

O poxvírus suíno (SPV), do gênero *Suipoxvirus*, pertence à família *Poxviridae*. Essa família inclui a subfamília *Chordopoxvirinae*, na qual também estão os gêneros como *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus* e *Yatapoxvirus*, assim como subfamília *Entonopoxvirinae*, compreendendo gêneros como *Alphaentompoxvirus*, *Betaentompoxvirus*, *Deltaentompoxvirus* e *Gammaentompoxvirus* (FIGURA 3) (BECKER; MOYER, 2007; ICTV, 2020).

Figura 3. Estrutura do *Suipoxvirus*



Estrutura do vírions do SPV: são vírions envelopados, em forma de tijolo, com cerca de 300x250x200nm, com DNA genômico de cerca de 175 kbp de tamanho com repetições terminais invertidas de cerca de 5 kbp, assim como *Asfivirus*. Fonte: https://viralzone.expasy.org/154

Os vírions são produzidos em dois tipos de partículas virais infecciosas distintas: o vírus maduro intracelular (IMV) e o vírus envelopado extracelular (EEV) (FENNE, 2017). A densidade flutuante do vírion está sujeita a influências osmóticas: em tampões diluídos é de cerca de 1,16 g cm^{-3}, em sacarose cerca de 1,25 g cm^{-3} e em CsCl e tartarato de potássio cerca de 1,30 g cm^{-3}. Os vírions tendem a se agregar em solução com alto teor de sal. A infectividade de alguns membros é resistente à tripsina. Alguns membros são insensíveis ao éter. Geralmente, a infectividade do vírion é sensível a detergentes comuns, formaldeído, agentes oxidantes e temperaturas superiores a 40°C. A membrana da superfície do vírion é removida por detergentes não iónicos e reagentes redutores de sulfidrilo. Os vírions são relativamente estáveis em condições secas à temperatura ambiente; eles podem ser liofilizados com pouca perda de infectividade (ICTV, 2020).

O genoma é uma única molécula linear de dsDNA covalentemente fechada, com 130-375 kbp de comprimento. Os ácidos nucleicos constituem cerca de 3% do peso das partículas. As proteínas constituem cerca de 90% do peso das partículas. Os genomas codificam 150–300 proteínas dependendo da espécie; cerca de 100 proteínas estão presentes nos vírions. As partículas de vírus contêm muitas enzimas envolvidas na transcrição de DNA ou modificação de proteínas ou ácidos nucleicos. Os vírions envelopados possuem polipeptídeos codificados por vírus na bicamada lipídica, que envolve a partícula. Os entomopoxvírus podem ser ocluídos por uma proteína estrutural

principal codificada pelo vírus, a esferoidina. Da mesma forma, os cordopoxvírus podem estar dentro de corpos de inclusão novamente consistindo em uma única proteína (a proteína ATI de inclusão do tipo A). Em geral, as proteínas conservadas essenciais para a replicação do vírus em cultura (polimerases e outras enzimas e proteínas estruturais) são codificadas na região central do genoma, enquanto as proteínas menos conservadas e não essenciais envolvidas nas respostas vírus-hospedeiro (imunomoduladores, antiapoptóticos proteínas, etc.) são codificados nas regiões terminais do genoma. Várias grandes famílias de proteínas são codificadas dentro dos *Poxviridae*, em alguns casos com muitos membros (ICTV, 2020).

Devido ao grande impacto e à impossibilidade de se trabalhar diretamente com o ASFV em território livre desta doença, optou-se por trabalhar com o SPV, que é de distribuição mundial e apresenta similaridade de replicação, além de possuir resistência ao meio ambiente similar em relação ao *Asfivirus*.

2.3 MANEJO DE CARCAÇAS SUÍNAS EM LARGA ESCALA

O principal objetivo da deposição de carcaças é a inativação de agentes infecciosos, assim como a contenção do impacto ambiental devido à decomposição das carcaças. Tal problema não se restringe apenas ao setor agrícola, mas também a e outros setores da economia. O impacto econômico é difícil de calcular, pois vão desde custos diretos associados à vigilância, testes, avaliações, despovoamento, descarte, desinfecção e impactos no comércio internacional, assim como custos indiretos, que incluem os efeitos no consumidor, impactando na economia como um todo. Mudanças nos níveis de importação de produtos de origem animal fazem com que esse prejuízo seja ainda maior, o que faz pensar que o impacto econômico total de um surto provavelmente é subestimado (FLORY et al., 2017).

O descarte de carcaças por meio de depósitos de decomposição animal de baixa profundidade (AGB) já tem sido implementado com sucesso na inativação viral em carcaças ovinas (FLORY et al., 2017), porém surtos de doenças de animais de grande porte representam um desafio maior. As técnicas utilizadas até para conter os surtos como o de febre aftosa no Reino Unido em 2001 (TAYLOR, 2002), Taiwan (HSEU; CHEN, 2017), Japão (HAYAMA et al., 2012), Coréia do Sul (KIM; KIM, 2012), assim como os casos no Brasil em 2005 (CARVALHO et al., 2014), onde as valas seguem uma dimensão entre 3,5 a 4 metros de profundidade, 3 metros de largura e comprimento

de acordo ao número de carcaças, agravam-se pelo ponto de vista público e sanitário, já que é significativa a preocupação em relação a contaminação de águas subterrâneas e demora na decomposição de carcaças quando enterradas nessa profundidade.

Os esforços de erradicação de doenças na Coréia do Sul resultaram na destruição de 20% do total de bovinos do país e na criação de 4700 locais de sepultamento (KIM et al., 2015). Esse enterro generalizado de carcaças resultou em preocupações sobre impactos ambientais maciços associados a esta atividade. Embora as investigações para caracterizar os impactos reais dessa atividade estejam em suas fases iniciais, muitos temem que os impactos ambientais, incluindo a contaminação do suprimento de água potável, durem por décadas (FLORY et al., 2017).

A falta de planos detalhados e específicos de descarte de carcaças, como parte de uma estratégia abrangente em resposta a surtos, pode resultar em atrasos nos esforços de sua erradicação. Um estudo de simulação de surto de febre aftosa na Califórnia concluiu que o atraso na resposta à detecção de 7 para 22 dias aumentou o número médio de rebanhos em quarentena de 680 para 6200 dias, bem como aumentou o custo médio do impacto econômico de US$ 2,3 bilhões para US$ 69 bilhões (CARPENTER et al., 2011).

Um estudo publicado pelo Departamento de Saúde do Reino Unido analisou o potencial impacto na saúde de vários métodos de descarte de carcaça (LONDON:DEPARTMENT OF HEALTH, 2001). Foram analisados os riscos específicos presentes durante os esforços de descarte de carcaça de febre aftosa e as vias associadas. O enterro profundo foi classificado como o método de eliminação de maior risco, com muitas vias de exposição diferentes, incluindo contato direto, solo e água contaminada, migração de gases para os edifícios além de que os riscos potenciais podem facilmente contaminar as fazendas e a água potável local.

Outro exemplo de doença infectocontagiosa que está em segundo lugar na lista de maiores desastres da produção animal (atrás apenas da Febre Aftosa) é a Peste Suína Africana (PSA), que apresentou recentemente um foco na República Dominicana, o qual foi diagnosticado no Laboratório de Diagnóstico de Doenças Exóticas do Departamento de Agricultura dos Estados Unidos (USDA). Além dos casos mais recentes, a doença já se faz presente em países como Georgia, Rússia, Belarus, Ucrânia, República Tcheca, Estônia, Hungria, Letônia, Lituânia e Romênia (NETHERTON et al., 2019), assim como na China, que já se espalhou para países vizinhos, incluindo, Mongólia, Vietnã, Camboja, Laos e Coreia do Norte, podendo ter consequências ainda

mais desastrosas, já que a região é responsável por importante parte da produção mundial (SÁNCHEZ-CORDÓN et al., 2018).

Existem várias opções disponíveis para gerenciar altas mortalidades de diferentes espécies de animais de produção, como descarte em superfície, enterro, queima/incineração, renderização ou compostagem. O descarte em superfície envolve deixar os animais mortos na superfície da terra para se decompor pela exposição natural aos elementos ambientais em um local isolado. Embora exija pouca preparação, é apenas uma opção viável onde há vastas extensões de terra isolada, mas com consequentes problemas como transmissão de odores desagradáveis e parasitas, assim como conflitos com vizinhos e contaminação do suprimento de água dos demais animais. Já o enterro é uma opção cara, exigindo a escavação de grandes fossas e a subsequente contenção, gerenciamento e tratamento, com monitoramento contínuo para garantir que o lixiviado não polua as águas subterrâneas (EAMENS et al., 2011).

A queima/incineração massiva de carcaças, na propriedade ou fora dela, é geralmente impraticável, pois são necessárias grandes quantidades de material combustível. Além disso, a queima pode levar a emissões de poluentes químicos e partículas que podem afetar a qualidade do ar e a saúde pública. Por outro lado, a renderização que foi usada com sucesso no gerenciamento de carcaças de gado durante o surto de febre aftosa no Reino Unido, envolve o transporte dos animais potencialmente infecciosos por grandes distâncias até que chegue às instalações onde há os biodigestores, assim como envolve um grande custo e disponibilidade e capacidade para obtenção dessa tecnologia (EAMENS et al., 2011).

O AGB (*Above ground burial*) envolve a disposição de carcaças de animais em linhas, dentro de um sistema de enterro superficial no solo, e deixado *in situ* para se decompor. Essa técnica tem o potencial de resolver alguns dos problemas logísticos, práticos e ambientais associados a outras opções disponíveis para gerenciar a mortalidade ou destruição de animais de produção decorrentes de doenças exóticas ou desastres naturais. Se realizado corretamente, este sistema fornece contenção imediata da carcaça e cria temperaturas suficientes para inativar grande parte dos microrganismos presentes nas carcaças e nas fezes contaminadas (WILKINSON, 2007).

Como o AGB envolve um enterro superficial, apresenta menos ameaças às águas subterrâneas rasas ou lençóis freáticos profundos, reduzindo potenciais problemas de biosseguridade, assim como também pode ser elaborado rapidamente no local, usando máquinas agrícolas e materiais disponíveis na unidade de produção/fazenda. Assim,

minimizam-se atrasos associados às opções que exigem o transporte de carcaças para instalações ou locais de descarte (GLANVILLE et al., 2004).

Os riscos e benefícios do AGB no gerenciamento de um grande número de carcaças animais em condições climáticas de acordo cada região são incertos e, dessa forma, devem ser testados. Deste modo, o presente trabalho visou validar a eficácia desse sistema em larga escala, utilizando o poxvírus suíno (SPV) como modelo de um surto de mortalidade de suínos por PSA. Tais procedimentos práticos de campo ainda não foram realizados e existem procedimentos operacionais ainda não publicados. Essa falta de conhecimento é vista como um grande impedimento ao uso desta técnica em uma possível ocorrência de um evento de surto de doença infectocontagiosa, principalmente de origem viral.

3 ARTIGO 1

Late development of pustular, erosive lesions in the muzzle of calves inoculated with *Pseudocowpox virus*.

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Abstract

We studied the pathogenesis of *Pseudocowpox virus* (PCPV), a zoonotic parapoxvirus associated with mucocutaneous lesions in cattle. Inoculation of calves with PCPV isolate SD 76-65 intranasally (n=6) or transdermally in the muzzle (n=2) resulted in virus replication and shedding up to day 13 post-infection (pi). No local or systemic signs were observed in inoculated calves up to day 20pi, when the clinical monitoring was discontinued. However, from days 28 to 34 pi, seven (7/8) inoculated calves underwent an asynchronous clinical course characterized by development of a few (one or two) to countless papulo-pustular, erosive-fibrinous and scabby lesions in the muzzle, in some cases extending to the lips and gingiva. In some animals, the lesions coalesced, forming extensive fibrinotic/necrotic and scabby plaques covering almost entirely the muzzle. The clinical course lasted 8 to 15 days and spontaneously subsided after day 42pi. Infectious virus and/or viral DNA were detected in swabs collected from lesions of 5/8 animals between days 34 and 42pi. Histological examination of fragments collected from the muzzle lesions of two affected calves (day 36pi) revealed marked epidermal hyperplasia and severe orthokeratotic and parakeratotic hyperkeratosis, covered by thick scabs. The epidermis showed multifocal areas of keratinocyte coalescing necrosis and mild multifocal vacuolar degeneration.

Sera of inoculated calves at 50pi showed partial virus neutralization at low dilutions, demonstrating seroconversion. The delayed and severe clinical course associated with virus persistence in lesions are novel findings and contribute for the understanding of PCPV pathogenesis.

Key words: pseudocowpox, parapoxvirus, PCPV, PPV, pathogenesis, calves.

Highlights

Delayed clinical course of pseudocowpox infection in calves

Severe pseudocowpox in experimentally infected calves

Persistence of pseudocowpox in lesions of experimentally infected calves

1. Introduction

Pseudocowpox virus (*Parapoxvirus bovis 2,* PCPV) is the agent of a vesiculo-papular, erosive disease of muco-cutaneous surfaces of cattle [1]. PCPV is one of the two *parapoxviruses* (PPVs) of cattle, along with bovine papular stomatitis virus (*Parapoxvirus bovis 1,* BPSV). Orf virus of sheep and goats (*Parapoxvirus ovis,* ORFV) and *Parapoxvirus of red deer in New Zealand* (PVNZ) complete the genus *Parapoxvirus* of the family *Poxviridae* [2]. Parapoxviruses are large, brick-shaped, enveloped viruses with a linear double-stranded DNA genome of ~134 kb in length [1, 3].

Bovine PPVs are epitheliotropic viruses that cause infection of the epidermis and mucosal surfaces, producing lesions that progress through the stages of macules, papules, vesicles/pustules, erosions and, finally, scabs [1]. Natural PCPV infections usually course with papulo-vesiculo-pustular lesions in the teats and udder of milking cows and muzzle-oral lesions in calves feeding on affected cows [4, 5, 6]. Individual PPV species (e.g. PCPV, BSPV and ORFV) display a narrow host range and may be occasionally transmitted to human, causing localized lesions on the hands usually called *milker's nodule* or *pseudocowpox* [1, 4].

Natural cases or outbreaks of disease associated with PCPV have been described throughout the world, usually associated with mild, self-limiting muco-cutaneous lesions in cattle [7-10]. Nonetheless, atypical cases [11] and mixed infections with other poxviruses have been described as well [6, 12, 13]. The sanitary and economic impacts of PCPV infection for the livestock remain largely uncertain, probably due to its sporadic/rare occurrence and mild clinical course. In Brazil, a number of cases/outbreaks of disease associated with PCPV (and other PPVs as well) have been

reported in the last decades, affecting dairy cows, calves and, occasionally, humans [5, 6, 12-17]. In some Brazilian regions, PPV infections in dairy cattle are relatively frequent and cause some economic losses to these herds, posing an occupational risk to humans [5, 6, 12-18]. An important issue regarding to PPV-associated disease lies on its similarity with other vesicular diseases of cattle, requiring differential diagnosis, especially from foot and mouth disease (FMD).

Most reports of PCPV published to date describe clinical cases/outbreaks, frequently associated with the identification of the agent by electron-microscopy and/or by molecular techniques. Herein we describe an experimental infection of PCPV in susceptible calves, the natural host of the virus. The results presented here shed light on the biology of PCPV infection and reveal important aspects of the virus pathogenesis that might be relevant for recognition, control and prevention of the disease.

2. Materials and methods

2.1. *Virus and cells* – Pestivirus-free Madin-Darby bovine kidney cells (MDBK, ATCC-CCL22) were used in all procedures of virus amplification, quantification, isolation and virus-neutralizing (VN) assays. Cells were maintained in minimal essential medium (MEM) supplemented with 10% equine serum, antibiotics and anti-fungics. The PCPV isolate SD 76-65 (isolated in North America in 1976) was used for animal inoculation at passage # 7. The inoculum consisted of the supernatant of MDBK cells inoculated with the isolate, containing a titer of $10^{7.3}$ mean tissue culture infectious doses/mL ($TCID_{50}$/mL).

2.2. *Animal inoculation, monitoring and sample collection* – Ten 3 to 4-months-old seronegative male Holstein calves were used in the study. The animals had been weaned

at approximately 20 - 30 days of age. Calves were inoculated with 4 mL (2 in each nostril) of a viral suspension containing $10^{7,3}$ $TCID_{50}$/mL of PCPV isolate SD 76-65. The intranasal (IN) (n = 6) or transdermal route (TD, n = 2) were used. TD inoculation was performed by swabbing the viral suspension (4 mL) over the muzzle after slight scarification with a hard sponge. Two calves were inoculated IN with cell culture medium (MEM) and served as mock-infected controls.

Following virus inoculation, experimental animals were monitored in a daily basis for local (nose, muzzle, respiratory) and systemic signs (appetite, alertness, body temperature) up to day 20 post-infection (pi). The absence of clinical signs by day 20pi led to the discontinuation of the daily monitoring and sample collection. Monitoring was resumed after day 28pi and sample collection after day 34 up to day 50 pi. Swabs for virus isolation (immersed in 2mL of MEM) were collected from the inoculated sites up to day 20 pi and then, at different time points depending on the development of lesions (days 34, 36, 38, 40, 42 and 50 pi). Samples obtained during and after the clinical course (days 34 to 50pi) were also submitted to DNA extraction for PCR. Whole blood for investigation of viremia was collected every two days up to day 20 pi. Blood for serology was collected at days 0 and 50 pi.

All animal procedures were performed under veterinary supervision and according to the recommendations of the Brazilian Committee on Animal Experimentation (COBEA). The experiment protocol was approved by an Institutional Committee on Ethics on Animal Experimentation (CEUA/UFSM Nº 7209040618).

2.3 *Sample processing* - For virus isolation, the swabs were vortexed, drained and briefly centrifuged at low speed. The supernatants were submitted to three passages of four days each in MDBK cells. Samples negative for cytopathic effect (cpe) at the

end of the third passage were considered negative for virus. The infectivity in the original samples was quantitated by limiting dilution and the titers were expressed as $Log_{10}TCID_{50}$/mL.

Buffy coats obtained during early infection (days 1 to 20 pi) and swabs obtained from lesions (days 34 to 42pi) were submitted to DNA extraction for PCR. Total DNA was extracted from swabs (300 µL of MEM) or buffy coats (100 µL) by phenol-chloroform protocol and subjected to PCR amplification, using primers designed to detect the B2L gene of PPVs: PPP1 forward (5′-3′GTCGTCCACGATGAGCAG) and PPP4 reverse (5′-3′TACGTGGGAAGCGCCTCGCT) according to methodology described previously [5, 19]. Total DNA extracted from MDBK cells infected with PCPV isolate SV285/11 [5] was used as positive control and DNA extracted from mock-infected MDBK cells as negative control. Amplicons obtained from different animals at different days were subjected to DNA sequencing to confirm their identity.

Serum samples collected at days 0 and 50 pi were submitted to virus-neutralizing (VN) assays to detect neutralizing antibodies to PCPV. For this, two-fold dilutions of sera were incubated with 100 - 200 $TCID_{50}$ of isolate SD 76-65 for 2h, followed by addition of a suspension of MDBK cells and incubation of the plates at 37 °C and 5% CO_2. Tests were read at 96h by microscopic examination of cell monolayers for cytopathology. The antibody titers were considered as the reciprocal of the highest serum dilution capable of preventing the production of cytopathology.

Biopsies of muzzle lesions were collected from two calves (#78 and 88) at day 36pi. Punched 5 mm biopsies were collected and fixed in 10% buffered formalin, embedded in paraffin, sectioned to 5µm, stained with hematoxylin and eosin (H&E) and submitted to microscopic examination according to routine protocols of LPV/UFSM.

3. Results

3.1. Virological findings

Infectious virus was isolated from swabs collected from the inoculation sites of inoculated animals up to day 13 pi (Table 1). Virus detection was continuous in most animals and intermittent in others. Virus quantitation by limiting dilution showed an increase in GMTs from day 2 to 6 - 8pi, decreasing progressively thereafter (Figure 1). No infectivity was detected in secretions collected at days 14 and 17pi. Specimens collected at day 20pi, when the swab collection was discontinued, were not available for virus isolation.

Upon the development of lesions, infectious virus and/or viral DNA were detected in swabs obtained from lesions of 5/8 animals at day 34 pi (#76, 78, 79, 85 and 88) ; 2/8 at day 36 pi (#76 and 82); 2/8 at day 38 pi (#76 and 78) and from 1/8 at day 42 pi (#88) (Table 2). In general, virus detection correlated with moderate to severe lesions (# 78, 79, 82, 85 and 88) but was also positive in calf #76, which remained healthy throughout the monitoring period. Virus isolation in all samples was only achieved after the 2nd or 3rd passage in MDBK cells, indicating the presence of low titers. Hence, virus quantitation was not performed in these samples. No viral DNA (or infectious virus) were detected after day 42 pi up to day 50 pi when the monitoring was discontinued. These results demonstrated the persistence of the virus in lesions up to day 42pi.

PCR performed in DNA extracted from buffy coats collected every two days up to day 20 pi were negative for viral DNA. Likewise, no infectious virus or viral DNA were detected in specimens collected from control calves throughout the experiment.

3.2. Clinical monitoring

No local or systemic signs were observed in inoculated calves up to day 20 pi, when the daily clinical examination was discontinued. Clinical examination was resumed after day 28 pi, when one or two small hyperemic foci (0.2 to 0.3 cm) were noticed in the muzzle of calves #78 and #82. From day 28 to 34-36 pi, similar lesions were progressively observed in other animals. The lesions progressed in number and severity affecting an increasing number of animals and reaching a peak between days 38-40 pi, when 7/8 inoculated animals had developed mild to severe lesions in the muzzle. In some cases, these lesions extended to the lips and gingiva (2/8). Three calves (#78, 82 and 88) developed severe and disseminated lesions, covering the entire surface of the muzzle extending to the lips (Figure 2, Table 2). Two calves (#79 and 85) presented a moderate number of lesions, some including the lips and gingiva; and two (#80 e 84) presented one of two papulo-erosive lesions in the muzzle, close to the nasal opening and/or in the upper lip. Table 2 presents the summary of the clinical observations.

Lesions started as small circular and diffuse hyperemic foci (0.1 to 0.3 cm), ranging from a few lesions to multiple/countless affected spots. As they increased in size, most of these hyperemic/papular lesions soon became ulcerated and/or flat with a yellowish aspect due to fibrin deposition. Prominent vesicles were not a consistent finding in many animals. In two animals, large vesicles (0.3 – 0.6 cm) were observed on the anterior face of the muzzle. In some cases, coalescence of individual lesions gave rise to ulcerated plaques/regions covered with fibrin. In three animals (#78, 82 and 88), numerous lesions (0.2 to 0.5 cm), initially hyperemic/papular and subsequently flat, ulcerated and yellowish covered the entire muzzle (Figure 2). Ulcerated/fibrin covered lesions rapidly dehydrated and formed scabs, usually surrounded by light halos with reduced hyperemia with wrinkling edges. The next stage was the contraction of the crusts/scabs and eventual healing. Two calves developed large circular or ovoid lesions

on the bottom lips (>1 cm), with flat surface and brownish color (probably necrosis) and discrete margins. In general, most lesions regressed completely after days 40 – 42 pi such as only healing tissues were observed in two calves at day 50 pi. Individual clinical courses lasted from 8 to 15 days. No lesions were observed in the nasal mucosa of inoculated calves. Animal #76 and the two control calves (#83, 87) remained healthy throughout the monitoring period.

3.3. Histopathology

Histological examination of fragments collected from the muzzle lesions of two affected calves (# 78 and 88, day 36 pi) revealed a marked and diffuse epidermal hyperplasia, containing a severely thickened stratum corneum, characterizing orthokeratotic and parakeratotic hyperkeratosis (Figure 3A). Thick keratin-rich scabs containing degenerated neutrophils were observed over the epidermis (figure 3B). Multifocal areas of coalescing necrosis of keratinocytes were observed (figure 3B). A neutrophilic – and occasionally fibrinosuppurative – inflammation was observed within these areas, with occasional micro abscess formation (Figure 3C). Additionally, mild vacuolation of keratinocytes was occasionally seen surrounding these areas, however, ballooning degeneration was not observed. Mild lymphoplasmacytic inflammation was observed within the superficial dermis (near the dermoepidermic interface and surrounding blood vessels) (Figure 3D). No viral inclusion bodies were observed.

3.4. Serology

VN assays performed with sera collected from inoculated animals at day 50pi showed partial neutralization of virus infectivity at the lower serum dilutions (1:5, 1:10 and 1:20). The partial neutralization was characterized by the development of a few (one or two) small foci of cytopathic effect (cpe) in the cell monolayer, contrasting with the complete destruction of the monolayer in wells in which the virus was incubated

with sera collected prior to virus inoculation and with sera from negative control animals.

4. Discussion

Our study demonstrated a late development of papulo-pustular-erosive and scabby lesions in the muzzle, but also in the lips and gingiva, in animals inoculated with PCPV isolate SD 76-65. Virus replication was detected during early infection (up to day 13pi) and infectious virus and/or viral DNA were detected in material collected from lesions of 5/8 animals, from days 36 to 42pi. In addition, histological changes compatible with poxvirus-induced pathology were observed in lesions of two calves at day 36pi. In this scenario, virus replication and viral spread from the nasal mucosa to epithelial and/or in subjacent layers of the muzzle were likely responsible for the pathology.

The epidemiology, biology and pathogenesis of bovine PPVs (including PCPV) are poorly understood and the few reports in the literature deal with clinic-pathological description of cases or outbreaks, occasionally accompanied by molecular and/or electron microscopic identification of the agent [6, 7, 8, 11, 17]. To our knowledge, no experimental pathogenesis studies are available in the literature. In Brazil, the emergence of PPV-associated disease in cattle in the last years, occasionally associated with human cases [13, 15, 16] has stimulated some interest in their epidemiology and pathogenesis.

Our findings add novel information on the pathogenesis of PCPV (and PPVs as well): first, broken or damaged skin may favor but is not a requisite for the establishment of infection, as believed previously [1]. In our study, animals inoculated by the IN route (without scarification) were efficiently infected and developed moderate to severe lesions. The lesions developed by the IN group were even more severe than

those in calves inoculated transdermally in the muzzle. Second, lesions may develop at a distance from the primary sites of virus inoculation. In fact, calves inoculated IN developed extensive and severe lesions in the muzzle. Interestingly, no changes were observed in the nasal mucosa, the site of virus inoculation. These findings demonstrated a pronounced tropism of the virus for the stratified squamous epithelium of the muzzle and, secondly, for the epithelium of the lips and gingiva, where lesions were observed in some animals. In natural infections, PCPV lesions usually develop in the udder and teats of milking cows and in the muzzle-oral region of calves suckling on affected cows [4, 5, 6].

The late onset of lesions was somewhat surprising since it differed markedly from the descriptions of experimental infections with ORFV, in which inoculated sheep usually develop lesions as early as at days 3-4 pi [20, 21]. On the other hand, the delayed onset of clinical disease may find some parallel with natural BPSV infections [24]. At least two scenarios may be envisioned to explain the late development of lesions in the inoculated calves. First, the late onset of the lesions might reflect a slow virus replication and gradual spread to nearby areas of the muzzle and/or a delayed pathology in the muzzle epithelium. In this case, the late pathology/lesions would reflect the dynamics of virus replication, spread and mechanisms of tissue injury. Alternatively, the development of lesions at a late stage may have been triggered by some stressful condition imposed to the animals late after virus inoculation. Indeed, the discontinuation of clinical monitoring (day 20 pi) was followed by a marked change in food quality/characteristics, in which the calves were moved into a pen with an abundant and rough native grass. It is possible that abrasions produced by the rough grass in the muzzle – associated with the stress caused by the food change - may have triggered the development of lesions. Virus isolation and/or viral DNA detection demonstrated the

association of PCPV with the lesions. Accordingly, no lesions were observed in control animals maintained in a separate pen with similar grass. In this sense, BPSV disease has been shown to emerge in healthy animals after stressful conditions, independently from recent virus transmission [22, 23, 24]. The occurrence of clinical BPSV disease approximately 30 days after the introduction of healthy calves in a BL-3 containment reinforced the hypothesis that subclinical PPV carriers may be triggered to develop the disease under some stressful conditions (24).

The duration of the clinical course in inoculated calves (around 8-15 days) was similar to what has been described in natural cases of PCPV and BPSV infection [5, 22, 24] and shorter than that observed in lambs inoculated with ORFV (18-28 days) [20, 21]. Likewise, the nature and aspect of the lesions produced by PCPV inoculation were similar to what has been described in natural cases of PCPV and BPSV infection [5, 22, 24], with the exception of late proliferative lesions, not observed in our study. Rather, PCPV produced flat papule-pustular and fibrinotic/erosive lesions that subsided without undergoing a proliferative stage. A similar outcome has been described for natural cases of BPSV infection in calves [24]. Thus, in spite of their similarities in molecular and biological aspects, the pathogenesis and clinical presentation of bovine and ovine PPVs (e.g. PCPV, BPSV and ORFV) may present differences.

Viral persistence (or long-term replication) in muco-cutaneous surfaces has been described for ORFV and suggested for BPSV [24]. Lambs inoculated with ORFV isolates in the labial commissures shed virus through lesions for up to 30 days [21] or longer [20]. In another study, ORFV DNA was detected in the labial commissures of experimentally infected lambs for up to 50 days pi, much longer after the resolution of lesions (Martins et al., unpublished). Indeed, the isolation of BPSV from oral secretions

of healthy calves has suggested that cattle subclinically infected may serve as reservoirs of PPVs [25]. The occurrence of BPSV disease in calves that had been introduced healthy into a BL-3 containment reinforced the hypothesis of healthy carriers [24]. This hypothesis if further supported by cases of BPSV disease in calves submitted to experimental surgical and/or therapeutical procedures [22, 26]. In the present study, infectious virus was detected early in infection and, in low levels, associated with lesions of 5/8 animals at days 36, 38 and 42 pi. Thus, PPVs are apparently able to persist for a relatively long period in muco-cutaneous epithelium, probably by counteracting the host immune response. Virus persistence may have epidemiological implications in natural infections, since it would favor virus dissemination through prolonged exposure/transmission to susceptible animals. Unfortunately, our experiment was discontinued at day 50pi, precluding a more extended follow up on virus persistence.

No viremic spread was detected in inoculated animals, reinforcing most observations that PPV replication is largely restricted to muco-cutaneous surfaces surrounding the sites of virus entry without significant systemic spread [1, 4]. A transient viremic spread of a parapoxvirus has been reported in a cow treated with gamma interferon but it seems more an exception than a rule [26].

In summary, our study adds important information on the pathogenesis of PCPV infection. Notably, the marked viral tropism for the muzzle epithelium and the delayed clinical course, associated with a prolonged virus replication and/or stress-induced triggering of clinical disease are important contributions for biology and pathogenesis of PPVs. In addition, our study provided a detailed follow-up and description of the clinical course of the disease, which is missing in most clinical reports published to date.

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Table 1. Virus detection in nasal or muzzle secretions of calves inoculated with PCPV intranasally (IN) or transdermally (TD) in the muzzle.

Animal/ route	Virus shedding[a]													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
76-IN	≤1,81[b]		-	3,11	2,81	4,11	4,31	-	-	-	≤1,8	-	-	-
78-IN	-	1,98	2,31	3,81	3,51	3,98	3,64	3,11	-	-	2,11	-	≤1,81	-
79-IN	-	2,11	2,81	4,11	3,98	4,51	3,64	3,64	2,81	-	-	-	-	-
80-TD	≤1,81	-	2,51	2,11	3,64	4,31	4,11	3,51	2,88	2,98	-	-	-	-
82-TD	-	-	3,11	4,31	2,64	3,31	2,81	-	-	2,31	-	-	-	-
84-IN	-	2,31	3,11	2,81	2,81	3,81	3,51	3,98	2,88	3,11	2,11	2,31	≤1,81	-
85-IN	≤1,81	2,51	2,11	2,81	3,31	3,51	3,31	-	-	3,31	1,88	1,98	-	-
88-IN	≤1,81	-	-	-	2,11	-	-	2,31	-	-	-	1,98	-	-
83-IN[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
87-IN[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GMT[d]	nc[e]	2,27	2,81	3,82	3,42	4,07	3,83	3,65	2,86	3,05	1,99	2,11	nc	na

[a] Virus titers are expressed $Log_{10}TCID_{50}$/mL of cultu ıedium (2mL) used to harvest the swabs collected from e respective sites of ino ıation. [b] Infectious virus was detected by virus isolation after 2nd or 3rd passage in tissue culture, corresponding to a titer of ≤ 1,8 $Log_{10}TCID_{50}$/mL. [c]Mock-infected controls. [d]Geometric mean titer. [e]Not calculated. Na = not applicable.

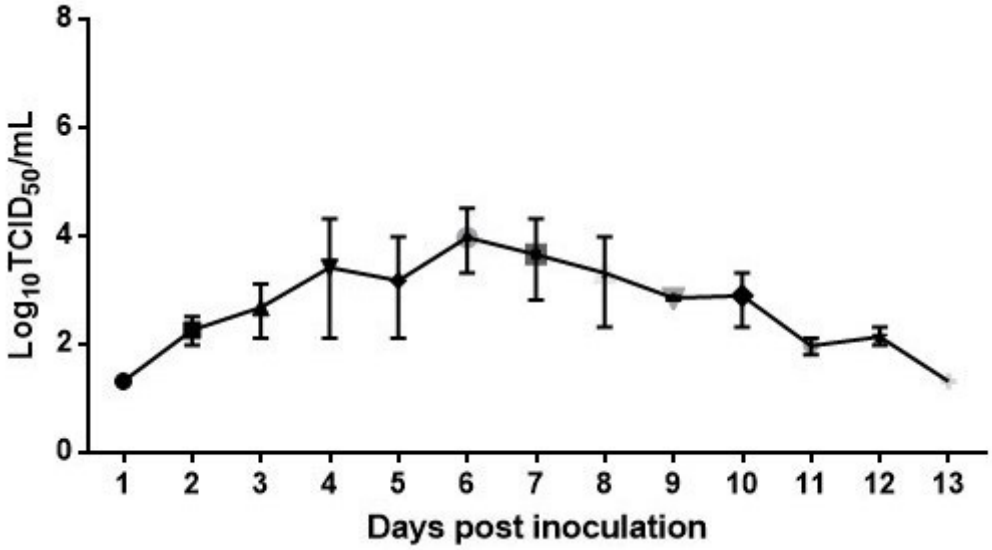
8
6
4
2
0
Log10TCID50/mL
1
2
3
4
5
6
7
8
9
10
11
12
13
Days post inoculation

Figure 1. Mean virus titers present in secretions of calves inoculated with PCPV intranasally (IN) or transdermally (IN) in the muzzle. Table 2. Clinical findings and detection of virus or viral DNA in lesions (or inoculation sites) of calves inoculated with PCPV intranasally (IN) or transdermally (TD) in the muzzle.

Calf /route of inoculation	Clinical course/lesions	PCR	Virus isolation
78-IN	Very severe. Developed countless lesions, disseminated over the muzzle and extending to the upper and lower lips. Some lesions coalesced, forming fibrinotic plaques. Large brownish (necrotic), flat lesions developed in the lower lip. Started around days 28 - 30; peaked around day 36 pi and rapidly resolved by day 42.	+ (days 34, 38 pi)	+ (days 34 and 38)
88-IN	Severe. Multiple lesions disseminated over the muzzle, some coalesced forming ulcerated, fibrinotic plaques. Some lesions in the upper, lower lips and gingiva. Lesions started by days 28 – 30, peaked by days 38 - 40 pi, regressing promptly thereafter.	+ (days 34, 42 pi)	+ (day 34pi)
85-IN	Moderate to severe. An early, ulcerated and scabby lesion was observed in the muzzle, close to the nasal opening at day 34 pi. From day 36 onwards, several lesions developed in the muzzle reaching a peak of severity by days 38 - 40, reducing drastically by day 42 and completely disappearing by day 50 pi.	+ (day 34 pi)	-
82-TD	Moderate to severe. Lesions started around day 28 pi and reached a peak between days 30 and 32 pi. At day 34 pi there were multiple ulcerated/fibrinotic lesions, which regressed promptly between days 34 and 40 pi.	+ (day 36 pi)	+ (day 36pi)
79-IN	Moderate. Several lesions developed between days 28 – 30 pi. Ulcerated and fibrinotic lesions were observed at day 34 pi. One large lesion in the upper lip and other in the transition between the muzzle and skin. Acute clinical course between days 28 – 32 pi.	+ (day 34 pi)	+ (day 36pi)
84-IN	Mild. Presented a single, convalescent lesion in the muzzle at day 34 pi, regressing almost entirely by day 38 pi. Reached a peak of intensity between days 28 and 30 pi.	- [b]	-
80-TD	Mild. Presented a large hyperemic foci at the nasal opening entry at day 34 pi, and two additional small foci in the muzzle. Intensity peaked between days 34 and 36 and was almost resolved at day 38 pi. A small hyperemic foci appeared at day 40 pi.	-	-
76-IN	No lesions were observed.	+ (days 34, 36, 38 pi)	+ (days 34, 36, 38pi)
83, 87 IN - C[b]	No lesions were observed	-	-

[a] PCR negative
[b] MEM-inoculated controls

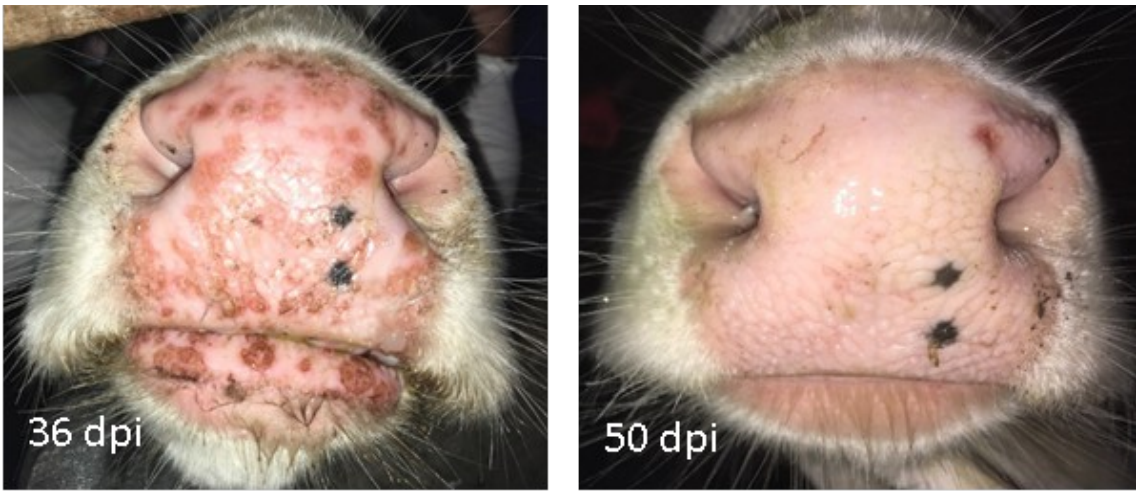
36 dpi
50 dpi

Figure 2**.** Muzzle of calf #78 inoculated with *Pseudocowpox virus* (PCPV) by the intranasal route, at days 36 and 50 post-infection (pi). At day 36 pi, countless lesions at different stages, ranging from hyperemic foci to ulcerated/fibrinotic and scabby lesions were observed. Large flat brownish areas, probably necrotic, were observed in the lower lip. At day 50 pi only a reminiscent cicatricial lesion was still present near the left nare opening.

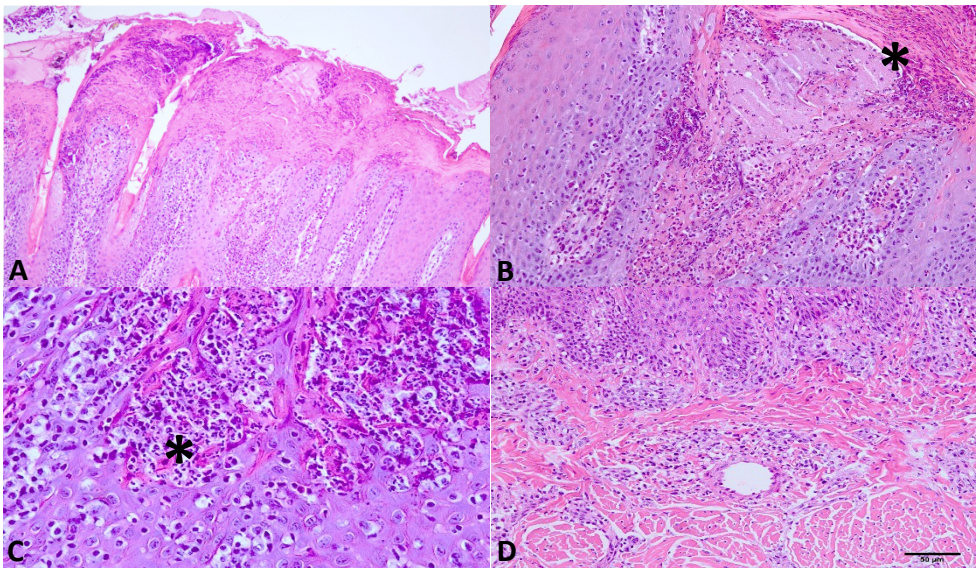

Figure 3. Histologic findings in fragments of the muzzle of calves # 88 and 78 collected at day 36pi. 3A – Hyperplasic epidermis, parakeratotic hyperkeratosis and scab (Obj 10x); 3B – Epidermal degeneration and necrosis within the hyperplastic epidermis. The area is infiltrated by fibrinosuppurative exudate and covered by a thick scab (*) (Obj 20x). 3C – Outer stratum spinosum and granular layer. Epidermal neutrophilic infiltrate with microabscess formation (*) (Obj 40x). 3D. Mild lymphoplasmacytic inflammation within the superficial dermis, near the dermoepidermic interface and surrounding a blood vessel (Obj 20x).

4 ARTIGO 2

Virus viability in spiked swine bone marrow tissue submitted to the above ground burial method and under *in-vitro* conditions

(Artigo puplicado na *Transboundary and Emergence Disease*, fevereiro de 2022 de 2021)

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Abstract

The emergence of high consequence animal diseases requires managing significant mortality. One desirable aspect of any carcass management method is the ability to contain and inactivate the target pathogen. The above-ground burial (AGB) technique was recently developed and posed as an alternative carcass management method. Here, we investigate the viability length of swinepox virus (SwPV), as a surrogate model for African Swine Fever Virus (ASFV) in swine carcasses submitted to the AGB method. For this, SwPV was inoculated intrafemorally in 90 adult swine carcasses. Bone marrow samples were recovered and tested periodically throughout 12 months. Additionally, an *in-vitro* study assessed the inactivation rate of SwPV, Senecavirus A (SVA), and Bovine Viral Diarrhea Virus (BVDV). Viruses were mixed with bone marrow material and maintained at 21-23ºC for 30 days. In both studies, virus viability was assessed by virus isolation, while the viral nucleic acid presence was assessed by qPCR. In the field study, SwPV remained viable only in 11 (55%) bone marrow samples collected on day 7, and only viral DNA was detected afterward. Inactivation was estimated to have occurred by day 11. The *in-vitro* testing revealed a variable tenacity of the studied viruses. The viability length was estimated to be 80, 118, and 28 days respectively, for SwPV, SVA, and BVDV. Overall, these findings indicate that the AGB technique was effective in short-term inactivating SwPV. Additionally, the SwPV inactivation rate is comparable to ASFV under field studies and poses a potential model for preliminary ASFV inactivation studies with reduced biosecurity requirements. Moreover, this study contributes to understanding the inactivation kinetics of viruses under specific conditions, which is critical toward designing and applying countermeasures in case of biosecurity breaches in sites managing animal mortality.

Introduction

The emergence and spread of the African Swine Fever Virus (ASFV) in Asia, Europe, and recently in Central America is a significant disruptor to the global pork industry. The introduction of ASFV or any high consequence animal disease affecting livestock into major producing areas requires the euthanasia and safe disposal of an immense number of animals (Moennig, 2000). For instance, a 2019 report estimates that the ongoing ASFV outbreaks resulted in over six million pigs being slaughtered in several countries (FAO, 2019).

Carcass disposal methods available for large-scale depopulation during high consequence animal disease outbreaks are limited. This is especially challenging for carcasses of adult pigs, cattle, or other large animals. Despite being a common carcass disposal technique among the available options, deep burial is an expensive option, requiring the excavation of deep trenches and a constant contamination risk for groundwater (Moennig, 2000; Eamens et al., 2011). Rendering was successfully used to manage livestock carcasses during the foot-and-mouth virus (FMDV) outbreak in the United Kingdom in 2001 (Taylor, 2002). However, it involves transporting infectious animals or carcasses over long distances to the biodigesters location, increasing chances for virus dissemination. In addition, composting may be restricted due to space limitations, scarcity of carbon sources, or lack of functioning static compost piles during the winter months (Schwarz and Bonhotal, 2015; Pepin et al., 2020). This is especially critical in subtropical, temperate, and subpolar regions with significant pork production.

Two critical aspects of carcass disposal methods are to limit the environmental impact and to provide containment and inactivation of the target pathogen (FAO, 2019). The newly developed Above Ground Burial (AGB) technique poses as a potential

alternative for carcass management (Miller and Flory, 2018). The method involves placing the animal carcasses in shallow trenches, over a layer of organic compost, like wood shavings, and covering the carcasses with a thin layer of soil (Flory et al., 2017). The superficial burial method allows for the effective aerobic decomposition of the tissues by the active microbiome found in the soil's upper layers (Flory et al., 2017). The technique also has the potential to solve some of the logistical, practical, and environmental problems associated with other carcass management options (Flory et al., 2017).

However, despite the benefits of the AGB method, the relative superficial location of the infected tissues increases the vulnerability to biosecurity breaches due to extreme environmental conditions, like heavy rain and flooding. In addition, it facilitates the carcass access to scavengers. Oral transmission by infected tissues is a concern for high-impact animal diseases viruses, including ASFV and Classical Swine Fever Virus (CSFV) (Edwards, 2000; Cowan et al., 2015; Blome et al., 2020; Zani et al., 2020). Therefore, it is critical to understand the level of infectious virus remaining in decomposing tissues over time toward the rational development and application of countermeasures during biosecurity breaches. However, there is a paucity of data related to the time-course evaluation of infectious viruses in decomposing tissue, especially in bone marrow, which contain cells that support the replication of relevant viruses, including ASFV, CSFV, and FMDV (Gomez-Villamandos et al., 2003; Gómez-Villamandos et al., 2003; Fischer et al., 2020; Stenfeldt et al., 2020).

To further build knowledge on the inactivation rate of viruses in swine carcasses during decomposition, two projects were conducted: a field study inoculated swinepox virus (SwPV), as a surrogate for ASFV, into the medullary cavity of 180 femurs from 90

cull sow carcasses and evaluated the viral infectivity and viral DNA presence for 12 months. ASFV is the sole virus in the *Asfarviridae family*. Although it remains a matter of debate, the tentative order of *Megavirales* would contain, among other families, the *Asfarviridae and Poxviridae* families (Iyer et al., 2006; Andrés et al., 2020). The selection of SwPV as an ASFV model was based on the structural similarities with ASFV, and the increased resistance members of this family demonstrate in environmental conditions (Smith, 2007) and due to its endemic status in the United States. The inoculation of SwPV intrafemorally was opted due to the calcification level of the femur. It is one of the latest structures to decompose and therefore allowed sample recovery over the 12-month long project. The second study was conducted using an *in-vitro* system, and the viability of SwPV, Senecavirus A (SVA), and Bovine Viral Diarrhea Virus (BVDV) was assessed in spiked bone marrow tissues maintained at 21-23ºC over a 30 days period.

Material and Methods

Viruses and cells

The SwPV (NVSL catalog number 002-PDV) was amplified in PK-15 cells (porcine kidney), whereas the and SVA strain Hawaii (Kindly provided by Drs Lager and Buckley – USDA ARS) was propagated in swine testis (ST) cells. The BVDV strain Singer (NVSL catalog number 140-BDV) was amplified in Madin-Darby Bovine Kidney (MDBK) cells. Cell lines were cultured at 37°C with 5% CO_2 in MEM medium (Corning) supplemented with 10% fetal bovine serum (Seradigm), 2 mM l-glutamine (Corning), 1% Antibiotic-Antimycotic 100X (Gibco), and gentamicin (50 µg/ml; Corning).

When the cytopathic effect was observed in more than 90% of the monolayers, the flasks were submitted to a freeze-thaw cycle, followed by centrifugation at 1200 × *g* for 5 min. The clarified viral stock was titrated using limiting dilution assay, and the titer was calculated using the Reed and Muench (1938) method. Viral stocks were titrated in triplicates and stored at -80°C until use. The viral stocks' titers were $10^{6.8}$, $10^{9.5}$, and $10^{7.5}$ tissue culture infectious dose ($TCID_{50}$/ml) for SwPV, SVA, and BVDV.

Virus isolation and titrations

For the virus isolation (VI) assay, spiked bone marrow samples were centrifuged for 5 min, at 1200 × *g* and the supernatant was diluted (1:20) in PBS. The diluted samples were inoculated in 24-well plates about 70% confluent using the appropriate cell line for each virus as described above. Samples were submitted to 5 passages of 3 to 6 days each. The original material of the VI positive samples were subsequently submitted to virus titration assay using limiting dilution assay, and the titer calculated using the Reed and Muench (1938) method.

Field study design

To evaluate the viability of the SwPV in decomposing swine bone marrow tissues under a filed depopulation training, two trenches were constructed (denominated West and East trench). A total of 100 cull sows, 50 for each trench, with an average weight of 200 kg, were used. The project was approved by the Institutional Animal Care and Use Committee, protocol number VM19-19. The animals were separated into two groups of 50, one week apart. Euthanasia was conducted with a penetrating captive bolt. The animal death was confirmed by the absence of a heartbeat, absence of a corneal reflex, and cyanosis of mucous membranes. After the animal death was confirmed, the

carcasses were transported to the burial site. Carcasses were placed in lateral decubitus position over a layer of about 30 cm of woodchips. The 50 carcasses covered about 30 m in length of each trench. The trenches were about 2 m wide and 0.6 m deep.

Inside the trench, the medial aspect of the femur was accessed. Femurs from 5 carcasses in each trench were collected on day 0 and served as negative controls during subsequent tests. For the remaining 45 carcasses in each trench, a drill was used to create a passage (22 mm in diameter) to the bone marrow. A portion of bone marrow was removed to facilitate the inoculation of 10 mL of SwPV with a $10^{6.8}$ $TCID_{50}$/ml titer. Subsequently, a stainless-steel plug was placed to seal the bone passage.

A longitudinal incision was made in the abdominal wall of the carcasses to prevent gas accumulation during the decomposition. Two Onset HOBO U12 temperature data loggers were used to measure temperatures in each trench. Each logger was composed of eight channels. The temperature probes were placed on the 12^{th}, 24^{th}, 36^{th}, and 48^{th} carcass on top of the chest cavity and inside the abdomen. The temperature for each probe was recorded hourly.

Ten inoculated femurs were harvested per trench on days 7, 14, 21, 28, and around months 2, 3, 6, and 12 post-inoculation (pi) to evaluate virus viability. A total of 10 inoculated femurs were collected on day 0 in each trench and used as positive controls during subsequent testing. The pelvic portion of the carcasses was exposed for sample collection, femurs were identified, the plug was removed, and the bone marrow content was aspired using a syringe. The samples remained in a liquid-to-viscous state in most femurs approximately up to day 60 post-inoculation, and between 2 and 5ml were collected per femur. After day 60, or in the absence of a liquid sample, 10ml of PBS pH 7.2 (Corning®) was used to rinse the internal femur surface and then recovered as the sample. Finally, the samples were aliquoted in microtubes and immediately stored

in an isothermal box with ice for subsequent laboratory testing. The samples were submitted to viral isolation, titration, and qPCR assays.

The length of SwPV viability in inoculated femurs was estimated using a linear regression model. The model considered the logarithm concentration of the initial virus titer retrieved from two syringes containing the SwPV inoculum and two inoculated femur samples collected at day 0. The titers from these samples were about $10^{6.5}$ $TCID_{50}$/ml. The regression model also considered that the detection limit of the virus titration was $10^{1.8}$ $TCID_{50}$/ml.

SwPV inoculum biosafety

The SwPV virus stock used to inoculate the bone marrow in the field study was tested for adventitious viruses for biosafety purposes. For this, the sample was sequenced using an Illumina platform. The virus was amplified, and an aliquot of 50 ml was purified as previously described (Paim, Bauermann et al., 2021). The DNA library was prepared with 1 ng of purified DNA using the Illumina Nextera XT DNA Library Preparation Kit and sequenced using the Illumina iSeq 100 System with Illumina Reagent Kit V1 (2 x 150 paired-end reads). The data was *de novo* assembled on BaseSpace Cloud (Illumina) with the metaSPAdes genome assembler (Version 3.0). Assembled contigs were examined for similarities to viral nucleotide using Blastn in a database created from the RefSeq virus database (taxid: 10239). The analyses were conducted using Geneious Prime software (version 2020.2.1). To assemble the SwPV genome, the viral contigs were mapped against a reference SwPV genome using Geneious Prime app as previously described (Paim, Maggioli et al., 2021).

Additionally, the virus stock was tested for Bovine Respiratory Syndrome Virus (BRSV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Senecavirus

A (SVA), and Swine Influenza Virus (SIV) using qPCR. The tests were performed following standard diagnostic protocols at the Oklahoma Animal Disease Diagnostic Laboratory (OADDL).

SwPV DNA extraction and qPCR

According to the manufacturer's instructions, viral DNA was extracted from BM samples using the MagMAX Viral RNA / DNA kit (Life Technologies) in an automated nucleic acid extractor (King Fisher Purification System, Thermo Fisher Scientific). The used forward and reverse primers were respectively the PoxF 5'-TCAGTACATCCAATTGTCAAGGA-3', PoxR 5'-CTGGCTAAATAGAATGAGTGAAACG-3', and the probe [6FAM]ACTTCCAGAAACGAGTAATCCTTACAAGAC[BHQ-2] (Integrated DNA Technologies). The SwPV genome retrieved in this study was used to design the qPCR assay using the PrimerQuest Tool (Integrated DNA Technologies). The conditions of the qPCR were: 40 cycles of 50ºC for 30 sec, followed by 95ºC for 10 sec and 62.5ºC for 30 sec. The assay was performed on Applied Biosystems 7500 thermocycler.

***In-vitro* study design**

An *in-vitro* system was used to evaluate the viability of SwPV, SVA, and BVDV in decomposing bone marrow kept at room temperature (21-23ºC). The SVA and BVDV, respectively, belong to the same viral families of FMDV and CSFV. Both FMDV and CSFV have tropism to hematopoietic cells and are frequently identified in bone marrow tissues of infected animals (Gómez-Villamandos et al., 2003; Stenfeldt et al., 2020). Although the SwPV has no tropism for hematopoietic cells, it was included in the *in-vitro* study to evaluate any possible effect of the bone marrow matrix to directly inactivating the SwPV and impacting the results of the field study. For the *in-vitro* study,

bone marrow tissue from the femurs of pigs older than 4 months was collected in a biosafety cabinet. About 500 mg of tissue was mixed with 500 ml of viral stock in microtubes. As controls, microtubes containing only virus suspensions were prepared. After inoculation, control and spiked samples were collected at days 0, 2, 7, 15, 20, and 30. Three microtubes containing spiked samples and three control microtubes were collected and independently titrated for each time point. The titer of the viral triplicates for each sampling point was used in a linear regression model to evaluate the virus viability decay. The analyses were conducted using GraphPad Prism (Version 9.2.0).

Results

AGB study - SwPV inoculum biosafety

Following metagenomics sequence and specific qPCR assays, no other viral pathogen was identified in the SwPV inoculum used in the AGB field study. Metagenomics of the SwPV inoculum allowed us to retrieve the near-complete genome sequence of the SwPV used in the current study. The average coverage for the genome was 194×. The genome has over 146,456 base pairs in length and is about 99.93%, similar to the isolate 17077-99 (GenBank accession number AF410153.1). A total of 150 open reading frames were predicted. The sequence was deposing in GenBank under the accession number MZ682626.

AGB study – Carcasses temperature monitoring, SwPV viability and viral DNA detection

Temperature monitored by probes placed in the carcasses demonstrated that during the first 10 days of the study, the average daily temperatures were above 33ºC in both trenches and gradually decreased up to day 14 (Fig. 1 and Tab. 1). The temperature

in the West trench trended higher, especially from day 4 to day 8, with an average temperature between 1.5ºC and 1.9ºC higher than the East trench.

Infectious SwPV was isolated in cell culture in 11 samples (three from the West trench and eight from the East trench) on day 7. The viral cytopathic effect was noted only on the fourth passage and suggested a low titer remaining. This was subsequently confirmed by virus titration, in which all virus isolation positive samples had titers below the assay threshold ($10^{1.8}$ $TCID_{50}$/ml). No sample with an infectious virus was identified in samples collected after day 7. Linear calibration regression estimated that 1 TCID50 was achieved in about 11 days. Due to sample limitations, a confidence interval could not be provided because no estimate of variability was available.

The qPCR testing detected SwPV DNA in 93% of 160 bone marrow samples collected (Fig. 2). Eight of the negative samples were in the West trench, whereas three were in the East trench. The Cq values of positive samples during the study demonstrate the stability of SwPV DNA under the AGB conditions. Additionally, the consistently positive results validated the sample recovery procedure from femurs throughout the study.

***In-vitro* study - Virus viability in bone marrow samples**

Using an *in-vitro* system, the viability of SwPV, BVDV, and SVA was periodically assessed in virus spiked bone marrow tissue kept at 21-23ºC for 30 days. Variable levels of virus inactivation were observed among the studied viruses in the spiked bone marrow matrix and the viral control solution (Fig. 3A-F). The SwPV viability in spiked bone marrow decreased from about $10^{4.5}$ $TCID_{50}$/ml on day 0 to $10^{3.0}$ $TCID_{50}$/ml on day 30. The analyses of SwPV titer decay using the regression model demonstrated a coefficient of determination (R^2) of 0.95, and the X-intercept point

(virus inactivation) was estimated to be 80 days (63 to 114 days with 95% confidence interval). Similar to SwPV, SVA remained viable over the study period. In bone marrow spiked samples, the titer decreased from $10^{9.5}$ to $10^{6.5}$ $TCID_{50}$/ml over the 30 days. SVA regression model indicated an R^2 of 0.89 with an estimated virus inactivation of 118 days, ranging from 83 to 223 days with a 95% confidence interval. Conversely, BVDV tenacity in bone marrow was reduced compared to SwPV and SVA. BVDV titer in bone marrow samples ranged from $10^{6.0}$ $TCID_{50}$/ml to undetectable viable virus on day 30. However, on day 20, the samples had a titer of $10^{1.7}$ $TCID_{50}$/ml. The inactivation was estimated to be achieved in 28 days. The regression model determined a R^2 of 0.92, and the inactivation of BVDV was estimated to range from 22 to 40 days considering a 95% confidence interval. Comparing the viral titers from bone marrow spiked samples to the virus solution used as control, the bone marrow extends the virus viability for the SVA. The estimated viability for the control samples was estimated in 83, 29, and 24 days respectively, for SwPV, SVA, and BVDV. The R^2 for the control groups were respectively 0.85, 0.89, and 0.85. When considering a 95% confidence interval, the viability was estimated in 57 to 158 days, 22 to 46 days, and 17 to 44 days for SwPV, SVA, and BVDV.

Discussion

The emergence and spread of high-impact animal diseases create the need for prompt actions toward pathogen containment and eradication, which in most cases will require depopulation of affected herds and safe carcass management. The current alternatives for carcasses management during large depopulation events are limited. Among the options, the recently developed AGB method was previously tested during a sheep mortality event caused by Peste des Petits Ruminants Virus (PPRV) and in

chickens infected with Avian Influenza Virus (Miller et al., 2016; Miller and Flory, 2018). However, these studies have not evaluated the viability length of these viruses in the carcasses. In our field study, the viability of SwPV, as a surrogate for ASFV was evaluated. While the SwPV DNA was consistently retrieved in most of the samples throughout the one-year sampling period, viable virus was only detected in samples collected at day 7 after inoculation.

Statistical analyses suggested that virus inactivation was achieved around day 11 post-inoculation in the specific conditions of this study. These results align with the findings from an ASFV field project conducted in Lithuania (Zani et al., 2020). In that study, ASFV naturally infected wild boar carcasses were buried using the AGB system, and carcasses were excavated in various locations. By the time of the bone marrow sampling, carcasses were buried for 18 to 440 days and none of the samples yielded positive infectious virus (Zani et al., 2020). Another study using bone marrow tissues of pigs naturally infected with ASFV only detected infectious virus at samples kept at room temperature on day 0 and no viable virus was detected after the first week of the study (Fischer et al., 2020). Although, ASFV DNA was detected for up to two years on tested samples (Fischer et al., 2020). Conversely, ASFV was viable in the bone marrow of Parma ham for 94 days (McKercher et al., 1987). Differences in the initial viral titer in the samples may affect the viability length. For instance, in the study conducted by Fischer et al, 2020, there were significant higher titers of ASFV in bones rich with red marrow compared to bones rich in fat marrow (yellow marrow). This is likely related to the concentration of ASFV permissive cells in the different types of marrow.

The temperature monitoring in the carcasses demonstrated slightly lower temperatures (1.5 ºC to 1.9 ºC) in the East trench compared to the West trench. It is important to note that carcasses in the two trenches were buried 7 days apart. The

temperature difference in the trenches are likely associated with whether conditions, however, intrinsic conditions to each trench may also be involved. Although comparison of the inactivation rate between the two trenches was out of the scope of the present study, 73% of the virus positive samples were from the East trench. Whereas it is impossible to strictly correlate these results with the carcass temperature, previous studies have demonstrated that minor temperature variations significantly impact viral survival (Edwards, 2000).

It is conceivable that factors associated with specific environmental conditions in each mortality management site, including weather, soil biochemical characteristics, and local insect activity, will create unique factor combinations influencing virus inactivation (Benninger et al., 2008; Heaton et al., 2014; Fischer et al., 2020; Zani et al., 2020). Additionally, it is well described that temperature, pH, and the matrix containing the virus will directly affect virus inactivation (Depner et al., 1992; Farez and Morley, 1997; Edwards, 2000; Cowan et al., 2015; Fischer et al., 2020). Additionally, different organs may promote variable levels of virus tenacity, and the matrix effect may prolong viability for months (Edwards, 2000; Fischer et al., 2020). In addition to the microenvironment of each carcass management site, the inactivation rate may vary based on the characteristics of each virus (Dee et al., 2018).

Whereas it is impossible to fully replicate under laboratory settings the environmental factor affecting carcass decomposition under natural conditions, the evaluation of SwPV viability using the *in-vitro* system suggests that the SwPV viability could be prolonged under lower temperatures. Over the 30-day period, viable SwPV was isolated from spiked bone marrow samples. Importantly, the *in-vitro* method demonstrated no significant direct effect of bone marrow tissue in inactivating the SwPV, or promoting significant interference in viable virus recovery. Similarly, high

levels of infectious SVA have been observed in the bone marrow spiked samples. SVA has demonstrated high tenacity in previous studies under various combinations of temperature, humidity, and matrices (Dee et al., 2018; Caserta et al., 2021). Like SVA, FMDV is also a member of the *Picornaviridae* family. A study with FMDV demonstrated viability in bone marrow infected samples maintained at 4ºC for up to 7 months (Cottral, 1969), and viral titers over 10^3 $TCID_{50}$/ml were observed in the bone marrow of carcasses stored at 4ºC for 77 days (Stenfeldt et al., 2020).

The BVDV has been used as a CSFV model in inactivation studies, and under specific conditions, demonstrated similar tenacity to CSFV (Depner et al., 1992). The decreased tenacity of BVDV (estimated in 28 days) compared to the other studied viruses has been demonstrated (Depner et al., 1992; Edwards, 2000; Cowan et al., 2015). Studies have shown a drop of 2.7 logs in the CSFV titer in 3 days at room temperature (Kubin, 1967). On the other hand, the virus demonstrated prolonged stability at 4ºC, with a drop of 1.6 logs in 4 days (Kubin, 1967). Another study showed that at 21ºC in neutral pH the half-life of CSFV virus was 50 h, compared to 7 h at 37ºC (Depner et al., 1992). In organs of pigs infected with CSFV, the viability was estimated at 32 h, 36 h and 148 h at 25ºC respectively, in lymph node, fat, and muscle (Cowan et al., 2015).

These results further validated the potential use of the AGB method in the US. The technique is now outlined as an approved method by the USDA-APHIS to be used during animal mortality emergencies (available at https://www.aphis.usda.gov/animal_health/emergency_management/downloads/agb-emergency-policy.pdf). Whereas SwPV may be a suitable surrogate for preliminary testing of virus viability as a model for ASFV, time course studies using AGB and ASFV infected pigs are needed to support these findings. Additionally, this study

extended the knowledge of virus viability in the decomposing bone marrow and may provide critical information toward rational design of countermeasures in cases of biosecurity breaches in sites managing animal mortality.

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Figure legends

Fig. 1. Carcass temperature monitoring. Data showing the temperature in the carcasses during the first two weeks after carcass burial. The carcasses were buried in the West trench on September 4th, whereas on the 11th for the East trench. The temperature was monitored using HOBO probes placed in the abdomen and on top of the chest area in four carcasses in each trench. The temperature was recorded hourly, and the presented results are the daily average. Bars represent the standard error of the mean.

Fig. 2. Swinepox virus qPCR in samples collected during the field study. Eight sampling points were conducted from week 1 (W1) to month 12 (M12). The samples are shown below the dotted line (Cq of 40) are negative samples. The bars represent the average of the cycle threshold (Ct) of the positive samples and include the standard error of the mean.

Fig. 3. Linear regression of viral titers retrieved from bone marrow spiked samples and controls. Swinepox virus (A-B), Senecavirus A (C-D), and Bovine viral diarrhea virus (E-F). The average titer for the independent triplicate testing is presented (triangle), and the bars represent the standard error of the mean.

Fig. 1

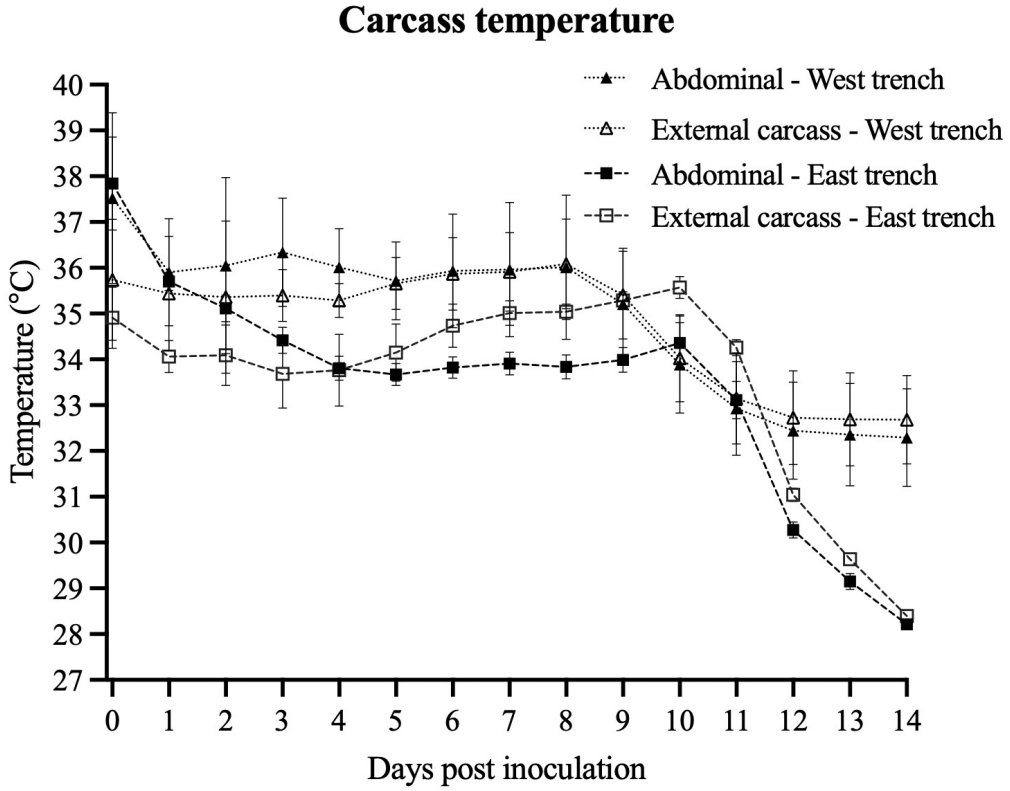
Carcass temperature
Abdominal - West trench
External carcass - West trench
Abdominal - East trench
External carcass - East trench
Temperature (°C)
40
39
38
37
36
35
34
33
32
31
30
29
28
27
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days post inoculation

Fig. 2

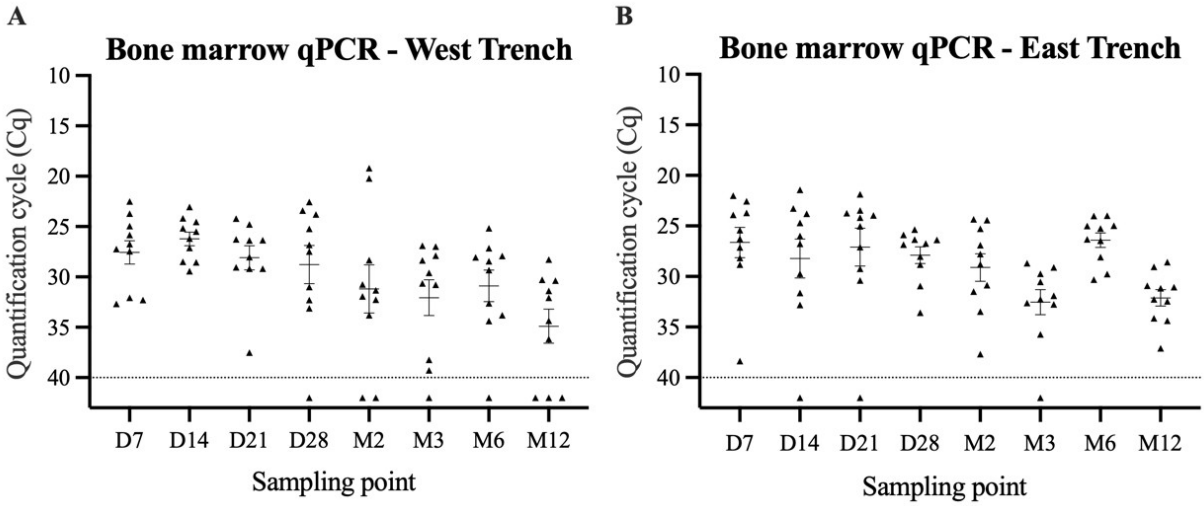
A
Bone marrow qPCR - West Trench
Quantification cycle (Cq)
10
15
20
25
30
35
40
D7
D14
D21
D28
M2
M3
M6
M12
Sampling point
B
Bone marrow qPCR - East Trench
Quantification cycle (Cq)
10
15
20
25
30
35
40
D7
D14
D21
D28
M2
M3
M6
M12
Sampling point

Fig. 3

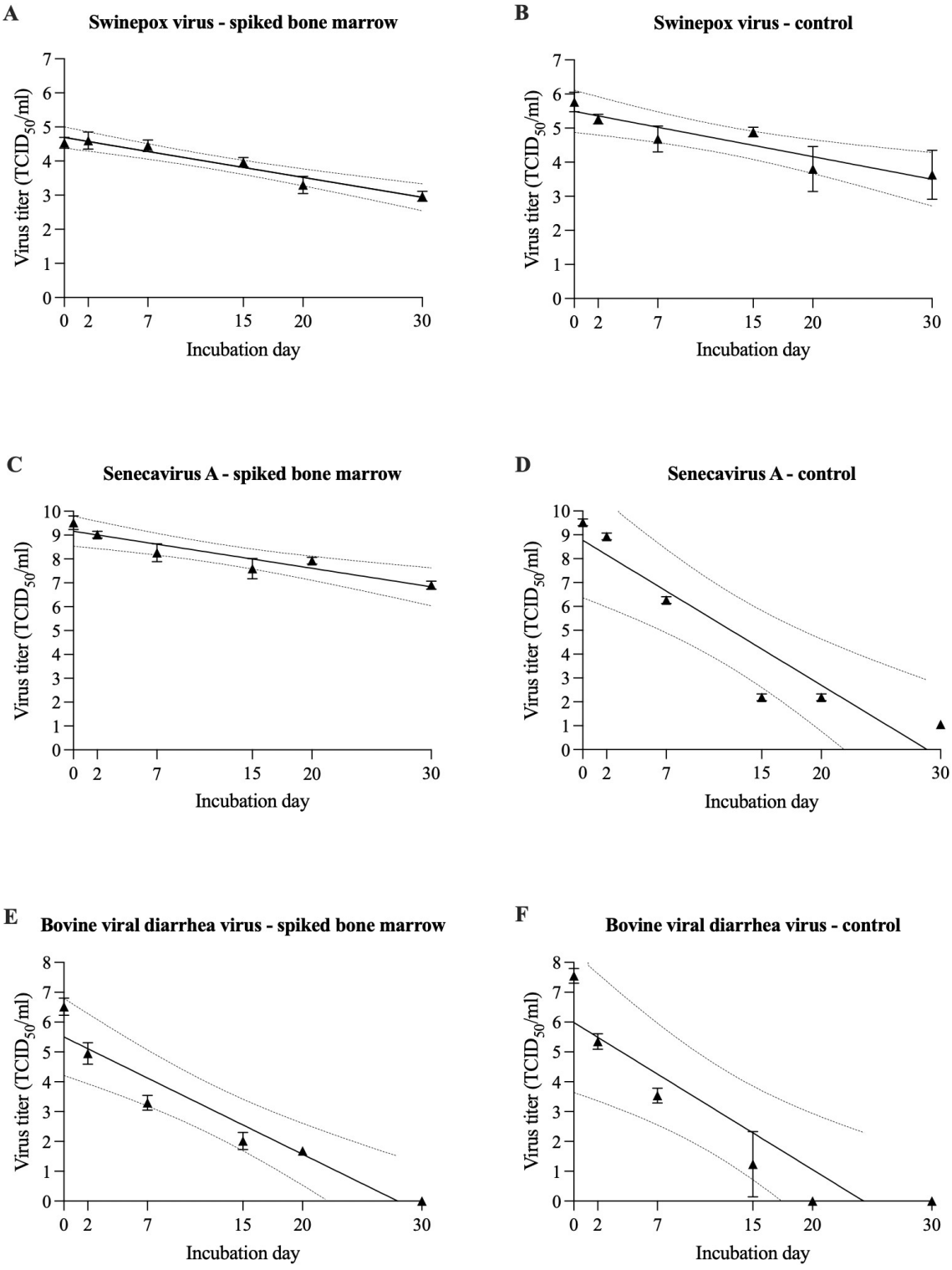

Table legends

Table 1. Air temperature during the first 2 weeks of the study for each trench. The maximum (Max temp) and minimum (Min temp) air temperatures (◦C) were obtained from the Lake Carl Blackwell Mesonet environmental monitoring station located less than 1 km from the study location. Data are available at http://www.mesonet.org

Tab. 1

West trench				East trench			
Day	Date	Max temp	Min temp	Day	Date	Max temp	Min temp
Day 0	9/4/19	33.3	18.9	Day 0	9/11/19	32.8	20.6
Day 1	9/5/19	34.4	20.0	Day 1	9/12/19	28.9	20.0
Day 2	9/6/19	35.6	18.3	Day 2	9/13/19	27.8	18.9
Day 3	9/7/19	35.6	20.0	Day 3	9/14/19	32.8	18.3
Day 4	9/8/19	34.4	19.4	Day 4	9/15/19	34.4	19.4
Day 5	9/9/19	33.9	21.1	Day 5	9/16/19	33.3	20.0
Day 6	9/10/19	33.9	22.2	Day 6	9/17/19	33.3	20.6
Day 7	9/11/19	32.8	20.6	Day 7	9/18/19	32.8	20.6
Day 8	9/12/19	28.9	20.0	Day 8	9/19/19	32.2	20.0
Day 9	9/13/19	27.8	18.9	Day 9	9/20/19	28.9	21.1
Day 10	9/14/19	32.8	18.3	Day 10	9/21/19	30.6	21.1
Day 11	9/15/19	34.4	19.4	Day 11	9/22/19	28.9	18.9
Day 12	9/16/19	33.3	20.0	Day 12	9/23/19	30.6	16.1
Day 13	9/17/19	33.3	20.6	Day 13	9/24/19	29.4	21.1
Day 14	9/18/19	32.8	20.6	Day 14	9/25/19	31.1	19.4

5 DISCUSSÃO

Em estudo de inoculação experimental do PCPV, foi demonstrado um desenvolvimento tardio de lesões. A replicação viral foi detectada durante a infecção precoce e o vírus infeccioso e/ou DNA viral foram detectados em material coletado de lesões de 5/8 animais, dos dias 36 a 42pi. Além disso, alterações histológicas compatíveis com patologia induzida por poxvírus foram observadas em lesões de dois bezerros no dia 36pi. Nesse cenário, a replicação do vírus e a disseminação viral da mucosa nasal para o epitélio e/ou nas camadas subjacentes do focinho provavelmente foram os responsáveis pela patologia.

Até o momento do estudo, não havia relatos de patogênese experimental disponíveis na literatura. Nossos achados adicionam novas informações sobre a patogênese do PCPV, onde lesões primárias podem ser fatores predisponentes para a infecção, mas não é um requisito necessário (BÜTTNER; RZIHA, 2002). Assim como as lesões podem se desenvolver em regiões distantes do local da inoculação do vírus, demonstrando que o vírus apresenta um tropismo pelo epitélio escamoso estratificado do focinho, seguido do epitélio dos lábios e gengiva. Diferente do que se observa em infecções naturais, quando geralmente se desenvolvem nas tetas/úbere das vacas leiteiras e plano nasolabial dos bezerros lactentes (CARGNELUTTI et al., 2012; DE SANT'ANA et al., 2013; LEDERMAN et al., 2014).

O início tardio das lesões diferiu de infecções experimentais com ORFV, quando ovelhas desenvolveram lesões nos primeiros 3-4pi (SCHMIDT et al., 2012; MARTINS et al., 2014). Por outro lado, vai ao encontro de estudos de doença clínica em infecções naturais por BPSV (DAL POZZO et al., 2011). Essa condição tardia pode ser explicada de duas formas distintas, primeiro, o início das lesões resultado de uma replicação lenta do vírus somado a disseminação gradual para áreas próximas do epitélio do plano nasolabial, refletindo assim a dinâmica da replicação do vírus. Alternativamente, o desenvolvimento tardio pode ter sido desencadeado por alguma condição estressante imposta aos animais quando os animais foram transferidos para um piquete com abundante pastagem nativa e abrasiva, reforçando hipótese que portadores sub-clínicos do PPV podem desencadear lesões sob condições estressantes (DAL POZZO et al., 2011).

Ao trabalharmos no contexto de inativação viral com outro membro da família dos PPVs, o SPV foi utilizado como substituto do vírus da PSA em estudo de campo, através da inoculação intrafemural em suínos adultos, o qual foi possível ser recuperado na maioria das amostras ao longo do período amostral de um ano, mas foi viável apenas

em amostras coletadas no dia 7pi, onde sugere-se que a inativação do vírus foi alcançada por volta do dia 11pi nas condições específicas deste estudo, indo de acordo a achado de projeto de campo de vírus da PSA usando carcaças de javalis naturalmente infectados usando o mesmo sistema AGB com intuito de inativação viral (ZANI et al., 2020).

Considerando a incapacidade de replicar em laboratório o fator ambiental que afeta a decomposição da carcaça em condições naturais, a avaliação da viabilidade do SPV usando o sistema *in vitro* sugere que a viabilidade do SPV pode ser prolongada so temperaturas mais baixas. Ao longo do período de 30 dias, o SPV viável foi isolado de amostras de medula óssea enriquecidas. O que deve ser ressaltado também é que o método *in vitro* não demonstrou efeito direto significativo do tecido da medula óssea na inativação do SPV ou na promoção de uma interferência significativa na recuperação do vírus viável.

Da mesma forma, altos níveis de SVA infeccioso foram observados nas amostras de medula óssea. O SVA demonstrou alta semelhança em estudos anteriores sob várias combinações de temperatura, umidade e matrizes (DEE et al., 2018; CASERTA et al., 2021). Assim como o SVA, o vírus da febre aftosa, que também é membro da família *Picornaviridae*, demonstrou viabilidade em amostras infectadas de medula óssea mantidas a 4ºC por até 7 meses (COTTRAL, 1969), e títulos virais acima de 10^3 TCID50/ml foram observados na medula óssea de carcaças armazenadas a 4ºC por 77 dias (STENFELDT et al., 2020).

Ao testar a inativação viral do BVDV, que tem sido utilizado como modelo de CSFV, sob condições específicas, demonstrou semelhança ao CSFV (DEPNER; BAUER; LIESS, 1992). A viabilidade diminuída do BVDV (estimada em 28 dias) em comparação com os outros vírus estudados foi demonstrada (DEPNER; BAUER; LIESS, 1992; EDWARDS, 2000; COWAN et al., 2015). Estudos mostraram uma queda de 2,7 logs no título de CSFV em 3 dias à temperatura ambiente (KUBIN, 1967). Por outro lado, o vírus demonstrou estabilidade prolongada a 4ºC, com queda de 1,6 logs em 4 dias (KUBIN, 1967). Outro estudo mostrou que a 21ºC em pH neutro a meia-vida do vírus CSFV foi de 50 h, comparada a 7 h a 37ºC (DEPNER; BAUER; LIESS, 1992). Em órgãos de suínos infectados com CSFV, a viabilidade foi estimada em 32 h, 36 h e 148 h a 25ºC, respectivamente, em linfonodo, gordura e músculo (COWAN et al., 2015).

6 CONCLUSÃO

Os estudos apresentados nesta Tese permitem concluir que:

O estudo com PCPV acrescenta informações importantes a respeito de sua patogênese deste agente. O tropismo viral acentuado para o epitélio nasolabial e o curso tardio, associado a uma replicação prolongada e/ou desencadeamento de doença clínica induzida por estresse, são contribuições importantes para o conhecimento da biologia e patogênese dos PPVs.

O método AGB teve seu potencial validado na inativação do SPV, sendo que este pode ser um substituto para testes preliminares de viabilidade do vírus da PSA. O estudo ampliou o conhecimento da viabilidade do vírus na medula óssea em decomposição e pode fornecer informações críticas para projetos de contramedidas em casos de alta mortalidade animal.

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