UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

VÍRUS VACCÍNIA ISOLADOS DE EQUINOS: PATOGENIA EM MODELOS ANIMAIS E ANÁLISE DE GENES DE VIRULÊNCIA

TESE DE DOUTORADO

Juliana Felipetto Cargnelutti

Santa Maria, RS, Brasil
2013

VÍRUS VACCÍNIA ISOLADOS DE EQUINOS: PATOGENIA EM MODELOS ANIMAIS E ANÁLISE DE GENES DE VIRULÊNCIA

Juliana Felipetto Cargnelutti

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração de Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
Doutor em Medicina Veterinária.

Orientador: Prof. Rudi Weiblen

Santa Maria, RS, Brasil
2013

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

A comissão examinadora, abaixo assinada,
aprova a Tese de Doutorado

VÍRUS VACCÍNIA ISOLADOS DE EQUINOS: PATOGENIA EM MODELOS ANIMAIS E ANÁLISE DE GENES DE VIRULÊNCIA

elaborada por
Juliana Felipetto Cargnelutti

Como requisito parcial para a obtenção do grau de
Doutor em Medicina Veterinária

COMISSÃO EXAMINADORA:

Rudi Weiblen, PhD.
(Presidente/Orientador)

Eduardo Furtado Flores, PhD. (UFSM)

Claudio Severo Lombardo de Barros, PhD. (UFSM)

Charles Fernando Capinos Scherer, PhD. (HIPRA)

Mário Celso Sperotto Brum, Dr. (UNIPAMPA)

Santa Maria, 28 de outubro de 2013.

Agradecimentos

Inicialmente, agradeço a Deus pela vida e por ter me dado saúde e força para viver todos os momentos e as oportunidades que me foram concedidas.

À Universidade Federal de Santa Maria (UFSM) e ao Programa de Pós-Graduação em Medicina Veterinária pela oportunidade de realizar o doutorado em um programa de excelência da CAPES.

À minha família, por todo apoio e pela confiança depositada em mim durante a minha vida acadêmica.

Ao Eduardo K. Masuda, por ser parte indispensável da minha vida, e por colaborar em diversas etapas desta tese.

Aos meus orientadores Rudi Weiblen e Eduardo Furtado Flores, pela oportunidade que me concederam de realizar o estágio curricular, o mestrado e, agora, o doutorado em Virologia Veterinária. Obrigada por todos os ensinamentos e conselhos, e por participarem do meu crescimento pessoal e profissional.

À querida professora Luciane Lovato, pela amizade e pelo carinho diário.

A todos os meus amigos e colegas do Setor de Virologia – UFSM, por toda colaboração na execução dos experimentos, nas organizações de laboratório, nos momentos de diversão e, também no companheirismo dos momentos tensos. É muito bom fazer parte desta equipe!

Ao Laboratório de Patologia Veterinária (UFSM) pelo auxílio no processamento de amostras, e ao Laboratório de Vírus da Universidade Federal de Minas Gerais pelo treinamento necessário para a manipulação do vírus vaccínia.

A todos os professores que tive desde o ensino fundamental até a pós-graduação, pelo entusiasmo e pelos ensinamentos passados, despertando em mim o interesse pela vida acadêmica.

Ao CNPq pelo auxílio financeiro.

E a todas as pessoas que de alguma forma contribuíram para a minha formação pessoal e profissional, meu muito obrigado!

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

VÍRUS VACCÍNIA ISOLADOS DE EQUINOS: PATOGENIA EM MODELOS ANIMAIS E ANÁLISE DE GENES DE VIRULÊNCIA

AUTOR: JULIANA FELIPETTO CARGNELUTTI
ORIENTADOR: RUDI WEIBLEN
Santa Maria, 28 de outubro de 2013.

Duas amostras de vírus vaccínia (VACV) geneticamente e fenotipicamente distintas foram isoladas de um mesmo animal em um surto de doença vesicular e exantemática em equinos no Rio Grande do Sul, e denominados Pelotas 1 (P1V) e Pelotas 2 (P2V). Esta tese descreve estudos realizados para investigar a patogenia dos isolados P1V e P2V em coelhos e cobaias, e analisar a sequência de genes potencialmente envolvidos no fenótipo desses isolados. O *Capítulo 1* relata a investigação da susceptibilidade dose-dependente de coelhos ao P1V e P2V. Os animais foram inoculados pela via intranasal (IN) com três doses ($10^{2,5}$ $DICC_{50}$, $10^{4,5}$ $DICC_{50}$ e $10^{6,5}DICC_{50}$/coelho) de cada um dos isolados. A inoculação resultou em enfermidade respiratória grave e morte na maioria dos coelhos, independente do isolado utilizado. Os sinais clínicos iniciaram nos dias 3 e 6 pós-inoculação (pi) e culminaram com a morte ou eutanásia dos animais, 5 a 10 dias pi. Viremia foi detectada em coelhos de todos os grupos. Anticorpos neutralizantes foram detectados em todos os animais que sobreviveram além do dia 9 pi. Pneumonia intersticial com broncopneumonia necrossupurativa e conteúdo líquido intestinal foram lesões observadas em animais inoculados com o P1V ou P2V que evoluíram para a morte ou foram motivo para a eutanásia *in extremis*. Esses resultados demonstram que P1V e P2V são virulentos para coelhos e não apresentam diferenças evidentes de patogenia nessa espécie. No *Capítulo 2* foi investigada a susceptibilidade de coelhos após inoculação de VACV pela via intradérmica (ID). Para isso, os coelhos foram inoculados com um dos isolados ou com ambos. Todos os coelhos inoculados apresentaram lesões de pele caracterizadas por hiperemia, pápulas, vesículas, pústulas e úlceras. Excreção viral foi detectada nas lesões cutâneas e também em amostras de pulmão e intestino de animais que morreram durante a fase aguda da infecção. Os resultados desta inoculação demonstraram que coelhos desenvolvem doença cutânea e sistêmica após a inoculação ID de P1V e P2V. Algumas evidências indicam que os coelhos co-infectados desenvolveram lesões mais severas do que na infecção simples. No *Capítulo 3,* investigou-se a susceptibilidade e o potencial de transmissibilidade dos isolados P1V e P2V por cobaias. Para isso, cobaias foram inoculadas pela via intranasal (IN) com uma mistura dos isolados P1V e P2V (10^{6} $DICC_{50}$/ml). As cobaias não apresentaram sinais clínicos, porém excretaram o vírus nas secreções nasais, desenvolveram viremia e soroconverteram para VACV. Apesar disso, o vírus não foi transmitido a sentinelas por contato direto, indireto (aerossóis) ou por água e alimentos contaminados com fezes deliberadamente infectadas com o vírus. No *Capítulo 4*, quatro genes (C7L, K2L, N1L e B1R) envolvidos no fenótipo do VACV foram amplificados por PCR, sequenciados e submetidos à análise molecular. Uma deleção de 15 nucleotídeos (nt) no gene K2L foi identificada no P2V. Essa mesma deleção também foi identificada em isolados brasileiros do VACV pertencentes ao genogrupo 1. Mutações pontuais foram identificadas nos genes K2L, C7L e N1L no P2V comparando-se com o P1V e cepas de referência do VACV. A análise molecular desses genes não permite associar essas deleções/mutações presentes no P2V com o fenótipo, mas sugere que a deleção de 15 nt no gene K2L possa ser utilizado como marcador molecular de isolados de VACV do genogrupo 1. Em resumo, os resultados obtidos nesses experimentos demonstram que: i. P1V e P2V produzem doença sistêmica e cutânea em coelhos, mas não diferem fenotipicamente nessas espécies; ii. cobaias são susceptíveis à infecção mista pelo P1V e P2V, mas aparentemente não transmitem o vírus com eficiência; iii. P1V e P2V apresentam algumas diferenças em genes de virulência, sendo que a deleção de 15 nt no gene K2L pode ser utilizada como marcador de genogrupos de VACV.

Palavras-chave: VACV, varíola bovina, coelhos, cobaias, sequenciamento.

ABSTRACT

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

VACCINIA VIRUS ISOLATED FROM HORSES: PATHOGENESIS IN ANIMAL MODELS AND SEQUENCE ANALYSIS OF VIRULENCE GENES

AUTHOR: JULIANA FELIPETTO CARGNELUTTI
ADVISER: RUDI WEIBLEN
Santa Maria, October, 28th, 2013.

Two vaccinia viruses (VACV) genetically and phenotypically divergent were isolated, in a mixed infection, from a horse lesion during an outbreak of vesicular and exanthematous disease in horses in Southern Brazil and termed Pelotas 1 (P1V) and Pelotas 2 (P2V). This thesis describes studies performed to investigate the pathogenesis of P1V and P2V infection in rabbits and guinea pigs, and to analyze the sequence of genes potentially involved in their phenotype. *Chapter 1* investigated the dose-dependent susceptibility of rabbits to P1V and P2V after intranasal (IN) inoculation. Groups of weaning rabbits were inoculated with three doses of each VACV isolate ($10^{2.5}$ $TCID_{50}$, $10^{4.5}$ $TCID_{50}$ e $10^{6.5}$ $TCID_{50}$/rabbit). The inoculation resulted in severe respiratory distress and death of most inoculated rabbits regardless the viral strain. Clinical signs started three to six days post-inoculation (pi) and culminated in death or euthanasia at days 5 to 10 pi. Viremia was detected in animals of all groups. All rabbits surviving the infection beyond day 9 pi developed neutralizing antibodies. Interstitial pneumonia, necrossupurative bronchopneumonia and diarrhea were observed in animals which died or were euthanized *in extremis*. These results demonstrate that P1V and P2V are virulent for rabbits and show no apparent differences in phenotype in this species. *Chapter 2* describes the investigation of the susceptibility of rabbits to intradermal (ID) inoculation to VACV, in single or mixed infection. All inoculated animals developed skin lesions characterized by hyperemia, papules, vesicles pustules and ulcers. Infectious virus was detected in cutaneous lesions, lungs and intestine of animals that died during acute infection. These results demonstrate that rabbits develop cutaneous disease and systemic infection after P1V and P2V ID inoculation. Apparently, co-infected animals developed lesions more severe than those submitted to single virus infection. In *chapter 3*, the susceptibility and the potential of transmission of P1V and P2V by guinea pigs were investigated. For that, guinea pigs were inoculated IN with both P1V and P2V (10^{6} $TCID_{50}$/ml). The guinea pigs did not showed clinical signs but developed viremia, shed virus in secretions and seroconverted to VACV. Nevertheless, the virus was not transmitted to guinea pig sentinels maintained in close contact or when exposed to food and feces contaminated with VACV. In *Chapter 4*, four genes involved in virus phenotype/virulence (C7L, K2L, N1L e B1R) were submitted to nucleotide sequencing and analysis. A 15 nucleotide (nt) deletion in K2L gene was identified in P2V. The same pattern of nucleotide deletion was also detected in other genogroup 1 Brazilian VACV isolates. Point mutations were identified in K2L, C7L and N1R genes from P2V isolates when compared to P1V and to a standard VACV strain. The molecular analysis of these genes would not allow the establishment of association between the sequences/genotype and phenotype. However, this analysis indicate that the 15 nt deletion in K2L gene may be used as a molecular marker for genogroup 1 Brazilian VACV isolates. In summary, the results obtained in these studies demonstrate: i. P1V and P2V produce systemic and cutaneous disease in rabbits but they do not exhibit evident differences in virulence for rabbits; ii. Guinea pigs are susceptible to mixed P1V an P2V infection but apparently do not effectively transmit the virus; iii. P1V and P2V present some sequence differences in virulence genes and that a 15 nt deletion in K2L gene may be used as a molecular marker to distinguish between VACV genogroups.

Key words: VACV, cowpox, rabbit, guinea pig, sequencing.

LISTA DE FIGURAS

CAPÍTULO 1

FIGURA 1 – Virus shedding, time course of the disease and serological response of rabbits inoculated intranasally with VACV P1V at three different doses.... 33

FIGURA 2 – Virus shedding, time course of the disease and serological response of rabbits inoculated intranasally with VACV P2V at three different doses………………………………………………………………… 34

FIGURA 3 – Survival rate of rabbits inoculated with three different doses of VACV P1V and P2V……………………………………………………………… 35

FIGURA 4 – Clinical signs and gross pathological changes observed in rabbits inoculated intranasally with three different doses of VACV P1V and P2V…………………………………………………………………… 36

FIGURA 5 – Mean of body weights of rabbits inoculated with VACV P1V and P2V at three different dose.……………………………………………………….. 37

FIGURA 6 – Virus titers in nasal secretions of rabbits inoculated intranasally with two VACV strains (P1V and P2V) at three different doses……………….…… 37

FIGURA 7 – Histological changes in the lungs of rabbits inoculated with three different doses of VACV P1V and P2V…………………………………… 38

CAPÍTULO 2

FIGURA 1 – Lesions in the ears in rabbits inoculated with Brazilian VACV isolates….. 53

FIGURA 2 – Secondary signs developed by rabbits inoculated with two Brazilian VACV isolates……………………………………………………….…...... 54

FIGURA 3 – Virus shedding from ear lesions of rabbits inoculated with two Brazilian VACV isolates…………………………………………….…..................... 54

FIGURA 4 – Gross and microscopic changes in the lungs of rabbits inoculated with two Brazilian VACV isolates……………………………………………… 55

CAPÍTULO 4

FIGURA 1 – Alinhamento parcial das sequências do gene K2L do P1V, P2V, isolados cepas vacinais de VACV 74

LISTA DE TABELAS

CAPÍTULO 1

TABELA 1 – Virus titers in lungs and gut, viral DNA in the blood and feces of rabbits inoculated with VACV P1V and P2V at three different doses…………… 32

CAPÍTULO 2

TABELA 1 – Virological, clinical and serological findings in rabbits inoculated intradermally with two Brazilian vaccinia virus isolates…………………. 51

TABELA 2 – Infectious virus and viral DNA in tissues and blood of rabbits inoculated intradermally with two Brazilian vaccinia virus isolates………………….. 52

CAPÍTULO 4

TABELA 1 – Funções dos genes analisados.. 73

TABELA 2 – Sequência dos iniciadores utilizados na amplificação dos genes N1L, K2L, B1R e C7L.. 73

SUMÁRIO

1 INTRODUÇÃO........ 10
2 REVISÃO DE LITERATURA........ 12
2.1 Agente, doença e diagnóstico........ 12
2.2 Epidemiologia, aspectos moleculares e infecções de outras espécies........ 14
2.3 Genes envolvidos na virulência, espectro de hospedeiro e fenotipia........ 17
3 CAPÍTULO 1 – VACCINIA VIRUSES ISOLATED FROM CUTANEOUS DISEASE IN HORSES ARE HIGHLY VIRULENT FOR RABBITS........ 20
Abstract........ 21
Introduction........ 21
Material and Methods........ 22
Results........ 24
Discussion........ 27
References........ 29
4 CAPÍTULO 2 – VACCINIA VIRUSES ISOLATED FROM SKIN INFECTION IN HORSES PRODUCED CUTANEOUS AND SYSTEMIC DISEASE IN EXPERIMENTALLY INFECTED RABBITS........ 39
Abstract........ 40
Introduction........ 40
Material and Methods........ 41
Results........ 43
Discussion........ 45
References........ 48
5 CAPÍTULO 3 – GUINEA PIGS EXPERIMENTALLY INFECTED WITH VACCINIA VIRUS REPLICATE AND SHED, BUT DO NOT TRANSMIT THE VIRUS........ 56
Abstract........ 57
Resumo........ 57
Nota........ 58
Committee for Ethics and Animal Welfare........ 61
References........ 61
6 CAPÍTULO 4 – ANÁLISE MOLECULAR DE GENES DE VIRULÊNCIA DE AMOSTRAS DE VÍRUS VACCÍNIA ISOLADAS DE EQUINOS........ 63
Resumo........ 64
Abstract........ 64
Nota........ 65
Referências........ 69
7 CONCLUSÕES........ 77
8 REFERÊNCIAS........ 79

1. INTRODUÇÃO

O vírus vaccínia (VACV) – um orthopoxvírus da família ***Poxviridade***, foi utilizado por muitos anos como vírus vacinal em programas de erradicação da varíola humana, devido à sua similaridade genética e antigênica com o vírus da varíola (VARV ou ***Smallpox virus***). Muitos anos após a erradicação da varíola do Brasil, casos de doença vesicular e exantemática em bovinos leiteiros e, ocasionalmente em ordenhadores, vêm sendo descritos em diversas regiões do país (DAMASO et al., 2000; LEITE et al., 2005; LOBATO et al., 2005; SANT'ANA et al., 2013). A doença tem sido considerada um problema importante de saúde pública e animal em diversas regiões leiteiras do Brasil e, embora o VACV tenha sido consistentemente identificado em bovinos (crostas, secreções, leite, fezes, soro), ordenhadores (líquido das vesículas e soro) e em fezes e tecidos de camundongos, a origem desses vírus permanece incerta (LOBATO et al., 2005; TRINDADE et al., 2006; ABRAHÃO et al., 2009a; SANT'ANA et al., 2013). Inicialmente, foi sugerido que esses surtos se originaram de um escape vacinal, e o vírus vacinal teria sido mantido na natureza, e eventualmente infectado animais susceptíveis (DAMASO et al., 2000; TRINDADE et al., 2007a). Porém, estudos genéticos subsequentes, contradizem a hipótese de escape vacinal (TRINDADE et al., 2007); por isso, a existência de reservatórios silvestres do VACV tem sido investigada como a provável fonte de infecção para os animais domésticos (ABRAHÃO et al., 2009a; MIRANDA et al., 2013).

No Rio Grande do Sul, o primeiro caso da infecção pelo VACV foi descrito em 2008 no município de Pelotas, acometendo equinos de diferentes idades e categorias (BRUM et al., 2010). As observações feitas nesse surto por Brum et al. (2010), representaram uma novidade em relação ao cenário brasileiro, uma vez que, até então somente infecções pelo VACV em bovinos, roedores e humanos haviam sido descritas (DAMASO et al., 2000; LOBATO et al., 2005).

A caracterização do agente envolvido no surto em equinos revelou a presença concomitante de duas espécies de VACV, divergentes fenotípica e genotipicamente. Esses vírus foram então denominados de Pelotas 1 (P1V) e Pelotas 2 (P2V) (CAMPOS et al., 2011). O P1V e P2V apresentam semelhanças com outros VACV isolados de animais no Brasil, e por isso foram classificados em genótipos diferentes. O P2V compartilha uma deleção de 18 nucleotídeos no gene codificante da hemaglutinina (HA), produz placas

pequenas em cultivo celular e é avirulento para camundongos. Esses achados são semelhantes aos verificados nos isolados brasileiros de VACV pertencentes ao genogrupo 1. Por outro lado, P1V não possui a deleção no gene da hemaglutinina, produz placas grandes em cultivo celular e está envolvido em enfermidade sistêmica severa em camundongos e, desta forma, P1V pertence ao genogrupo 2 (CAMPOS et al., 2011; KROON et al., 2011).

Embora a infecção experimental já tenha sido realizada em camundongos (CAMPOS et al., 2011), a patogenia dos isolados P1V e P2V em outros modelos animais (coelhos e cobaias) ainda não foi investigada. Além disso, genes potencialmente envolvidos no fenótipo ainda não foram completamente analisados em isolados de VACV de equinos. Considerando que, apesar desta doença já ter sido relatada em equinos, a infecção desta espécie com o VACV é incomum, pouco diagnosticada ou confundida com outras enfermidades cutâneas vesiculares. Com isso, estudos de patogenia e um maior conhecimento genético das cepas de VACV isoladas de equinos poderiam auxiliar no entendimento da biologia do vírus e levar a possíveis esclarecimento sobre a origem desse agente.

2. REVISÃO BIBLIOGRÁFICA

2.1 O agente, a doença e diagnóstico

O vírus vaccínia (VACV) pertence à família ***Poxviridae***, subfamília ***Chordopoxvirinae*** e gênero ***Orthopoxvirus*** (ESPOSITO & FENNER, 2001). A sua origem é desconhecida, e por muitos anos esse agente foi utilizado como vírus vacinal para a erradicação da varíola humana, devido sua alta reatividade sorológica cruzada com o agente desta enfermidade, o vírus da varíola (VARV) (BAXBY, 1977). No gênero ***Orthopoxvirus*** também estão classificados o VARV, e os vírus do cowpox (CPXV), monkeypox, ectromelia, camelpox, raccoonpox, skunkpox, taterapox e volepox (ICTV, 2012). Destes, no Brasil, até o presente, apenas o VACV vem sendo detectado.

O VACV é um vírus envelopado, com formato de tijolo, de dimensões aproximadas de 200 x 400 nm, e contém proteínas de superfície no formato de espículas. O genoma é constituído por uma molécula de fita dupla de DNA, com aproximadamente 200 kpb, e com extremidades fechadas, no formato de "grampo de cabelo". O genoma codifica proteínas essenciais e não essenciais para replicação viral *in vitro,* além de proteínas envolvidas na virulência e modulação da resposta imune do hospedeiro. Os genes localizados na região central do genoma são altamente conservados entre os gêneros e codificam proteínas essenciais para a replicação e morfogênese viral. As regiões terminais do genoma do VACV codificam proteínas envolvidas no espectro de hospedeiro, virulência, evasão do sistema imune e proteínas não-essenciais para a replicação *in vitro* (SMITH, 2007). Os poxvírus são complexos e codificam a maioria das proteínas necessárias para a síntese e modificação de RNAs mensageiros, replicação do genoma e morfogênese (MOSS, 2013).

Como os demais poxvírus, a replicação do VACV produz dois tipos de partículas: os vírus maduros intracelulares (MV) e os vírus envelopados extracelulares (EV). A forma EV se constitui em um MV com um envelope adicional. Assim, uma vez que a MV já possui um envelope, a EV apresenta dois envelopes recobrindo o capsídeo. Esta membrana adicional é adquirida no complexo de Golgi ou nos endossomas durante a morfogênese, e é modificada pela inclusão de diversas proteínas virais e celulares que são ausentes na MV (SMITH, 2007; MOSS, 2013). As proteínas estruturais, o genoma e as enzimas associadas com essas partículas estão localizados no interior do núcleo (*core*) viral. Entre o núcleo e a membrana do

MV, estão presentes dois corpos laterais, que possuem função ainda desconhecida (SMITH, 2007).

Embora os poxvírus possuam genoma DNA, a sua replicação ocorre inteiramente no citoplasma celular, em regiões denominadas “viroplasmas” ou “fábricas de vírus”, e se inicia entre 2 e 5 h após a infecção. A expressão gênica do VACV ocorre em três etapas: 1. logo após a penetração são expressos genes iniciais que codificam proteínas essenciais para a replicação do DNA viral e interações com o hospedeiro; 2. após a replicação do DNA são expressos genes intermediários; 3. e logo após a expressão dos genes intermediários são expressos os genes tardios, produzindo proteínas que são essenciais para a formação das partículas víricas e para o egresso viral (BULLER & PALUMBO, 1991).

Em medicina veterinária, o VACV tem importância principalmente em rebanhos bovinos leiteiros, onde é responsável por doença cutânea de caráter vesículo-pustular e ulcerativo, localizada principalmente nos tetos das vacas, predispondo à mastite (LOBATO et al., 2005, SANT'ANA et al., 2013). Vacas infectadas também servem de fonte de infecção para bezerros lactentes e ordenhadores (LEITE et al., 2005; TRINDADE et al., 2009). Assim, o VACV é um vírus zoonótico, responsável por lesões pustulares e ulcerativas, principalmente nas mãos e braços de ordenhadores, o que pode constituir em uma fonte de infecção para outros bovinos e humanos, uma vez que pessoas infectadas podem excretar o vírus nas lesões por algumas semanas (LOBATO et al., 2005).

Após a penetração do vírus na pele ou mucosas, as lesões iniciam com hiperemia que evolui para máculas, pápulas, vesículas, pústulas e crostas que muitas vezes dão origem a extensas áreas de ulceração (ESPOSITO & FENNER, 2001). O curso da infecção se estende por três a quatro semanas, podendo ser agravado por infecções bacterianas e parasitárias secundárias. Bezerros que mamam em tetos infectados podem desenvolver lesões ao redor das narinas e nos lábios (TRINDADE et al., 2007b). As lesões de pele causadas pelo VACV são muito semelhantes, e às vezes confundidas com as enfermidades causadas por CPXV, por herpesvírus bovino tipo 2 (BoHV-2) e pelos vírus da pseudovaríola e estomatite papular (ambos do gênero ***Parapoxvirus***) (SMITH, 2007; SCHATZMAYR et al., 2009; ABRAHÃO et al., 2010a). Embora a distribuição da infecção por CPXV seja restrita ao continente europeu (LEWIS-JONES, 2004), os vírus da pseudovaríola, da estomatite papular e da mamilite herpética circulam com frequência em rebanhos brasileiros (LOBATO et al., 2005; TORRES et al., 2009; ABRAHÃO et al., 2010a; CARGNELUTTI et al., 2012; SANT’ANA et al., 2012; CARGNELUTTI et al., 2013; SANT’ANA et al., 2013). Além do contato direto entre ordenhadores e bovinos infectados, a transmissão dessa enfermidade também pode ocorrer

por meio de equipamentos de ordenha, sendo que a penetração na pele do hospedeiro ocorre por soluções de continuidade pré-existentes, o que favorece a infecção dos lábios e gengivas de bezerros, no momento da amamentação (BULLER & PALUMBO, 1991; LOBATO et al., 2005).

O diagnóstico da infecção pode ser realizado clinicamente, mas a confirmação depende de exames laboratoriais, devido à semelhança das lesões produzidas por outros vírus envolvidos em doença cutânea (SMITH, 2007; ABRAHÃO et al., 2010a). Amostras de crostas (ou suabes) das lesões são maceradas e clarificadas por centrifugação para posterior inoculação na membrana corioalantóide de ovos embrionados (CAM) ou no cultivo celular. Após 48-72h podem ser observadas alterações em formato de pontos brancos (*pocks*) na CAM e efeito citopático nas células de cultivo, característicos de VACV. Outro método utilizado é a reação em cadeia da polimerase (PCR) utilizando oligonucleotídeos iniciadores (*primers*) específicos para VACV, com o objetivo de amplificar produtos da região do gene do fator de crescimento vascular (*vgf*) ou do gene da HA (ROPP et al., 1995; DAMASO et al., 2007; ABRAHÃO et al., 2010b). Testes de soroneutralização, para detectar anticorpos contra ***Orthopoxvirus*** podem auxiliar na confirmação do diagnóstico, mas são incapazes de diferenciar a espécie viral que induziu a resposta de anticorpos, uma vez que ocorre reação sorológica cruzada entre os membros pertencentes a este gênero (FENNER et al., 1989; ESPOSITO & FENNER, 2001). A histopatologia é uma técnica auxiliar no diagnóstico de VACV, e permite identificar as alterações histológicas caracterizadas por vesículas e pústulas epidermais, com áreas de acantose, hiperqueratose ortoqueratótica, degeneração balonosa, necrose e corpúsculos de inclusão eosinofílicos intracitoplasmáticos em formato circular ou oval (LOBATO et al., 2005; TRINDADE et al., 2006; 2009; BRUM et al., 2010, SANT'ANA et al., 2013).

2.2 Epidemiologia, aspectos moleculares e infecções de outras espécies

No Brasil, casos de VACV em bovinos e humanos tem sido descritos desde a década de 90 (DAMASO et al., 2000). A origem dos isolados envolvidos nesses surtos permanece desconhecida (TRINDADE et al., 2007b). Especulava-se que a vacina utilizada no programa de erradicação da varíola humana, no final da década de 70, seria a origem desses surtos (DAMASO et al., 2000). Embora alguns isolados brasileiros compartilhem deleções no gene da HA, semelhante à uma cepa vacinal, outros isolados brasileiros não possuem essa assinatura molecular. Relatos sobre a origem do VACV na natureza sugerem que esse vírus tenha sido inicialmente isolado de equinos (TAYLOR, 1993; SYMONS et al., 2002),

derivado do vírus da varíola equina (***Horsepox***) (HUYGELEN, 1996), evoluído do vírus da varíola humana após diversas passagens em humanos ou bovinos, derivado do vírus da varíola bovina, uma recombinação entre o vírus da varíola bovina e humana, ou oriundo de uma espécie animal já extinta (BAXBY, 1977; BULLER & PALUMBO, 1991).

A caracterização molecular dos isolados de VACV no Brasil identificou uma deleção de 18 nucleotídeos (nt) no gene da HA, e a análise filogenética permite classificar os isolados brasileiros em dois grandes genogrupos: grupo 1 – cujas amostras (isolados) compartilham a deleção (como no caso dos isolados Araçatuba, Cantagalo, Guarani 2, P2V, Passatempo, Mariana e Serro), e grupo 2 – cujos isolados não possuem essa deleção no gene da HA (Guarani 1, P1V, BeAn 58058, SPAn232, Belo Horizonte) (TRINDADE et al., 2007b; KROON et al., 2011). Além da classificação dos isolados feita pela presença/ausência da deleção no gene da HA, também é possível agrupá-los pelo perfil de digestão enzimática do gene codificador do corpúsculo de inclusão do tipo A (gene A26L) (TRINDADE et al., 2004). Por este método, os isolados brasileiros da VACV estariam divididos em três grupos: grupo 1 – padrão de digestão semelhante à cepa padrão Western Reserve (WR); grupo 2 – perfil de digestão compatível, mas não idêntica às cepas WR e Lister, apresentadas pelas cepas Araçatuba, Passatempo e Guarani 2; e grupo 3 – completa ausência do gene A26L, exceto pelos últimos 112 nt, o que é apresentado pelas cepas Belo Horizonte, Guarani 1 e BeAn 58058 (TRINDADE et al., 2007b). Outras assinaturas moleculares de VACV brasileiros tem sido investigadas e, uma deleção de 10 nt no gene C23L (que codifica uma proteína ligante de citocina) foi proposta como um novo marcador molecular de isolados pertencentes ao genogrupo 1 (ASSIS et al., 2012).

Desde 1999 são descritos relatos de infecção e doença pelo VACV em bovinos e humanos no Brasil (DAMASO et al., 2000; TRINDADE et al., 2007b). O primeiro surto de infecção pelo VACV em equinos ocorreu em 2008, no Rio Grande do Sul, onde animais de diversas idades e categorias desenvolveram lesões crostosas e ulcerativas no focinho, gengivas e nos tetos (BRUM et al., 2010). Pelo diagnóstico laboratorial foi possível concluir que as lesões eram decorrentes de uma coinfecção por dois isolados de VACV, denominados Pelotas 1 (P1V) e Pelotas 2 (P2V) (CAMPOS et al., 2011). P1V e P2V apresentam características fenotípicas e genotípicas distintas, pertencendo a diferentes genogrupos (CAMPOS et al., 2011). As principais diferenças entre esses vírus incluem a presença (P2V) ou ausência (P1V) da deleção de 18 nt no gene da HA, o tamanho e a morfologia de placas em cultivo celular (P2V – placas pequenas; P1V – placas grandes) e virulência em camundongos (P1V – altamente virulento; P2V – avirulento) (CAMPOS et al., 2011).

Portanto, o surto em equinos em Pelotas foi causado por uma coinfecção de cepas marcadamente diferentes (CAMPOS et al., 2011). A fonte de infecção deste surto não foi determinada, uma vez que os animais não foram movimentados da propriedade, bem como não foram recebidos animais de outras localidades, no período que antecedeu a doença. Também não foram relatados casos de lesões vesiculares nas mãos das pessoas que manipulavam os animais, descartando a possibilidade de uma antropozoonose (BRUM et al., 2010).

A maioria dos casos de infecções por VACV tem sido descritos na região central e sudeste do Brasil, e a partir deste foco inicial, o vírus tem se difundido pelo território brasileiro (MEDAGLIA et al., 2009; KROON et al., 2011). Mesmo assim, a propriedade onde foi descrita a doença em equinos (Pelotas – RS) é distante, pelo menos, 1300 km da região mais próxima onde são descritos casos de VACV em animais e humanos (Estado de São Paulo), levantando uma hipótese de que os vírus envolvidos nesse surto possam ter se originado de reservatórios animais na região do surto. Após a descrição deste surto, alguns estudos sorológicos e epidemiológicos foram conduzidos em rebanhos equinos no Brasil, sendo que sorologia positiva para ***Orthopoxvirus*** foi detectada em equinos da região centro-oeste do Brasil (BORGES et al., 2013; PERES et al., 2013). Além da detecção de anticorpos anti-***Orthopoxvirus***, em 2013, um surto de VACV em equinos também foi relatado no Estado de Minas Gerais, com características clínicas, patológicas e epidemiológicas semelhantes ao surto de VACV em Pelotas (MATOS et al., 2013), demonstrando que a infecção de equinos pelo VACV deixou de ser um evento raro.

Estudos epidemiológicos da região dos surtos de VACV bovina sugerem que animais silvestres ou ratos e camundongos, possam ser a fonte de infecção (ABRAHÃO et al. 2009a; 2009b). Sorologia positiva para VACV e detecção de antígenos viral nas fezes de ratos capturados nas propriedades leiteiras em que ocorreram os surtos, são os principais indicativos que esses roedores possam ser a origem da infecção para bovinos (ABRAHÃO et al., 2009a). O isolamento concomitante de um mesmo VACV oriundo de amostras de camundongos peri-domésticos, de um ordenhador e de uma vaca infectada, indicam que roedores que vivem próximos à fazendas e propriedades leiteiras podem representar uma ligação entre o ciclo doméstico, e um possível ciclo silvestre de VACV (ABRAHÃO et al., 2009a). A identificação de VACV em amostras de sangue e tecidos de roedores urbanos, também fortalece a hipótese de que o VACV é mantido na natureza nessas espécies (MIRANDA et al., 2013). Por isso, potenciais reservatórios para o VACV na natureza tem sido investigados (ABRAHÃO et al., 2010b).

Infecção experimental de VACV em camundongos demonstra que esses animais desenvolvem a doença de forma sistêmica, sendo o vírus encontrado em diversos órgãos, secreções e excreções (FERREIRA et al., 2008a; 2008b). Nas fezes, há relatos de detecção de partículas viáveis de VACV por até 20 dias pós-exposição no ambiente, demonstrando a alta estabilidade desse vírus (ABRAHÃO et al., 2009b). Além disso, dependendo do isolado de VACV, as manifestações clínicas em camundongos podem diferir. Inoculação de camundongos com isolados de VACV do genogrupo 1 resultam em infecção inaparente, apesar do vírus ser excretado nas fezes e excreções (FERREIRA et al., 2008b). Essas observações fortalecem a hipótese de que, dependendo do isolado de VACV, os roedores podem ser reservatórios do vírus na natureza.

Embora a infecção pelo VACV já tenha sido relatada em camundongos peri-domésticos e silvestres (ABRAHÃO et al., 2009a), e a doença reproduzida nesses animais (FERREIRA et al., 2008a; 2008b), as informações sobre a patogenia e a transmissibilidade de VACV em outros modelos animais são escassas, principalmente utilizando isolados oriundos de equinos. Infecção experimental de coelhos com ***Rabbitpox*** e cepas de VACV isoladas de bovinos, demonstrou que essa espécie é susceptível à infecção por ***Orthopoxvirus***, desenvolvendo lesões cutâneas locais e doença respiratória, com alta taxa de letalidade (ADAMS et al., 2007).

2.3 Genes envolvidos na virulência, espectro de hospedeiro e fenotipia

Uma característica comum dos poxvírus é a presença de genes que codificam proteínas envolvidas na evasão do sistema imune, virulência e determinação do espectro de hospedeiros (SMITH, 2007). Esses genes estão localizados nas regiões terminais do genoma, e podem sofrer variações entre os diferentes gêneros da família ***Poxviridae*** (FENNER et al., 1989).

O genoma completo de cepas brasileiras de VACV ainda não foi sequenciado. Porém, a sequência de alguns genes envolvidos na virulência e utilizados na caracterização dos isolados já são conhecidos. Entre eles, o gene da HA, do *vgf*, da timidina quinase (TK), o gene de resistência ao interferon (E3L), o gene da glicoproteína de superfície EV (B5R) e da proteína de ligação ao interferon do tipo I (B18R) (KROON et al., 2011). Além desses, outros genes também são utilizados para estudos de evolução viral e filogenia. São genes conservados presentes nas regiões terminais do genoma: C7L (envolvido no espectro de hospedeiro), C6L (função desconhecida), N1L (fator de virulência intracelular), K2L (inibidor de proteinase serina), F2L (dUTPase), F4L (ribonucleotídeo redutase), F6L e F8L (desconhecida), B1R (proteína quinase) e B15R (desconhecida) (GUBSER et al., 2004).

O gene C7L é responsável pelo espectro de hospedeiros *in vitro* e tropismo celular dos ***Orthopoxvirus*** (PERKUS et al., 1990). Os genes C7L e K1L possuem função semelhante, e permitem ao vírus replicar em células de linhagem humana, mas a deleção do C7L limita a replicação do vírus em células de linhagem murina, suína e leporina (PERKUS et al., 1990). Além desses achados, o gene C7L também tem sido descrito em eventos de prevenção de apoptose em células infectadas com vetores vacinais de VACV (NAJERA et al., 2010).

O gene N1L do VACV codifica uma proteína de 14kDa que é detectada no sobrenadante celular (10%) e também no interior de células infectadas (90%) (BARTLETT et al., 2002). Estudos utilizando mutantes de VACV deletados no gene da N1L demonstram que essa proteína é essencial para a virulência *in vivo*, uma vez que as cepas deletadas são altamente atenuadas após a inoculação experimental em camundongos pela via intracranial, intraperitonial (KOTWAL et al., 1989), intranasal e intradérmica (BARTLETT et al., 2002), tanto pela manifestação clínica mais leve, quanto pela excreção viral diminuída em relação às cepas parentais.

A proteína codificada pelo gene K2L do VACV foi classificada como um inibidor de proteases serinas (“serpins”), importante na patogenia de determinados poxvírus e também pelo fenótipo de placa em cultivo celular (LAW & SMITH, 1992). A deleção do gene K2L demonstrou que células infectadas por esse mutante sofriam extensiva policariocitose, decorrente da fusão celular, e apresentavam menor efeito citopático em relação ao vírus parental. A fusão celular pode ser benéfica ou maléfica para o ciclo viral: pode ser vantajosa por facilitar a disseminação viral entre células, sem expor os antígenos aos anticorpos neutralizantes circulantes, mas pode ser maléfica para o vírus, pois a fusão pode limitar o egresso e a disseminação viral (LAW & SMITH, 1992).

O gene B1R codifica uma proteína quinase (LIN et al., 1992), essencial para replicação, sendo empacotada na partícula viral (TRAKTMAN et al., 1989). Mutantes deletados em B1R não são capazes de sintetizar o DNA adequadamente, o que pode influenciar na atenuação viral (LIN et al., 1992). Outra função da proteína quinase B1R é diminuir a expressão da proteína celular p53, que é responsável por controlar o ciclo celular e a apoptose (SANTOS et al., 2004). O resultado da fosforilação da p53 pela proteína B1R leva à diminuição da sua expressão, aumentando a replicação celular e inibindo a apoptose, o que é vantajoso para a replicação viral (SANTOS et al., 2004).

Esta tese apresenta estudos que objetivaram investigar aspectos da patogenia e biologia molecular de VACV isolados de equinos (P1V e P2V). O Capítulo1 relata o estudo da patogenia da infecção pelo VACV (P1V e P2V) em coelhos após inoculação intranasal. O

Capítulo 2 investiga a patogenia da infecção isolada ou mista dos isolados P1V e P2V, após a inoculação intradérmica em coelhos. O Capítulo 3 investiga a susceptibilidade de cobaias à infecção por P1V e P2V, e o seu potencial de transmissão viral para animais sentinelas. O Capítulo 4 relata o sequenciamento e a análise de quatro genes dos isolados P1V e P2V, potencialmente envolvidos no fenótipo viral.

3. CAPÍTULO 1

Vaccinia viruses isolated from cutaneous disease in horses are highly virulent for rabbits

Juliana Felipetto Cargnelutti[1]; Candice Schmidt[1]; Eduardo Kenji Masuda[2]; Lisiane Danusa Braum[1]; Rudi Weiblen[1]; Eduardo Furtado Flores[1*]

[1]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Camobi, Santa Maria, Rio Grande do Sul, Brazil, CEP 97105-900, phone/fax 55 (55) 3220 8034
[2]Laboratório de Patologia Veterinária, Departamento de Patologia, Av. Roraima, 1000, Camobi, Santa Maria, Rio Grande do Sul, Brazil, CEP 97105-900.*Corresponding author: eduardofurtadoflores@gmail.com

(Artigo publicado no periódico *Microbial Pathogenesis*, v.52, n.3, p.192-199, 2012).

ABSTRACT

Two genotypically distinct *Vaccinia viruses* (VACV), named P1V and P2V, were isolated from an outbreak of cutaneous disease in horses in Southern Brazil. We herein investigated the susceptibility of rabbits, a proposed animal model, to P1V and P2V infection. Groups of weanling rabbits were inoculated intranasally (IN) with P1V or P2V at low ($10^{2.5}$ $TCID_{50}$), medium ($10^{4.5}TCID_{50}$), or high titer ($10^{6.5}TCID_{50}$). Rabbits inoculated with medium and high titers shed virus in nasal secretions and developed serous to hemorrhagic nasal discharge and severe respiratory distress, followed by progressive apathy and high lethality. Clinical signs appeared around days 3 to 6 post-inoculation (pi) and lasted up to the day of death or euthanasia (around days 5 to 10). Virus shedding and clinical signs were less frequent in rabbits inoculated with low virus titers. Viremia was detected in all groups, with different frequencies. Viral DNA was detected in the feces of a few animals inoculated with P1V and P2V, low titer, and with P2V at high titer. Gross necropsy findings and histological examination showed diffuse interstitial fibrosing pneumonia with necrosuppurative bronchopneumonia and intestinal liquid content. Neutralizing antibodies were detected in all inoculated animals surviving beyond day 9pi. These results show that rabbits are highly susceptible to VACV isolated from horses, and develop severe respiratory and systemic disease upon IN inoculation. Thus, rabbits may be used to study selected aspects of VACV infection and disease.

Key-words: *Orthopoxvirus*, pathogenesis, virulence, animal model.

1.INTRODUCTION

Vaccinia virus (VACV) is an *Orthopoxvirus*, family *Poxviridae,* associated with exanthematic and vesicular cutaneous disease in cattle, buffaloes and humans [1]. The origin of VACV is still unknown and several hypotheses have been proposed. An evolution from the variola virus (VARV), evolution from cowpox virus (CPXV), recombination between VARV and CPXV and a possible origin from an extinct animal species or virus are among the speculated origins [2]. Due to its low virulence for humans and antigenic similarity with VARV, VACV strains were used during decades in the vaccine employed in the World Program of Eradication of Smallpox [3].

A number of outbreaks of exanthematic and vesicular disease affecting dairy cows and milkers has been reported in the last decade in Southeast Brazil [4-6]. The disease has assumed considerable importance in public and animal health in some rural communities [6-

8]. VACV strains have been repeatedly isolated from these outbreaks [7-9], yet their origin and epidemiology remain uncertain. Characterization of VACV strains involved in these outbreaks has led to the identification of two distinct groups of viruses (VACV groups 1 and 2), differing from each other in genetic and biological aspects [10]. Although genetically distinct and displaying different virulence for mice, viruses from both VACV groups cause a clinically indistinguishable disease in cattle [9, 11]. The surprising emergence of VACV in Brazil has suscited interest in elucidating their origin, ecology and epidemiology [11-13].

Our group reported an outbreak of cutaneous disease in horses in Southern Brazil (Pelotas county, Rio Grande do Sul state, Brazil), in which a mixed VACV infection was demonstrated [14, 15]. Genetic and biological analysis of equine isolates revealed a co-infection with two VACV strains belonging to distinct groups, named thereafter Pelotas 1 (P1V) and Pelotas 2 virus (P2V) [15]. These findings were somewhat unexpected since natural VACV infections in horses are extremely rare [16]. Likewise, equine VACV infections have never been reported in Brazil, even in areas and herds experiencing cattle and human disease. In addition, the location of this outbreak was far distant from the previously reported VACV cases in cattle and human.

Thus, we decided to investigate some aspects of the biology, epidemiology and pathogenesis of these horse VACV strains. We first sought to investigate the susceptibility of rabbits to P1V and P2V after IN inoculation.

2. MATERIAL AND METHODS

2.1 Cells and viruses

Vero cells (*African Green Monkey)* were used for virus amplification, quantitation and virus isolation. Cells were cultured in RPMI medium, containing ampicillin (1.6mg/L), streptomycin (0.4mg/L) and amphotericin B (2.25mg/L), supplemented with 10% bovine fetal serum. P1V and P2V strains were isolated concomitantly from sick horses in an outbreak of cutaneous disease in Southern Brazil [14]. Preliminary characterization of PV1 and PV2 has been carried out by Brum et al. [14] and by Campos et al. [15]. Cell cultures and virus growth were performed at 37°C with CO_2 at 5%.

2.2 Animals and virus inoculation

Thirty five weanling New Zealand white rabbits (30-40 days-old), weighing approximately 300-400g were randomly allocated in seven groups. Animals of each group were inoculated with a different VACV strain (P1V or P2V), at three different titers, as

follows: P1V - group 1 (low titer - $10^{2.5}$ $TCID_{50}$/animal); 2 (medium titer - $10^{4.5}$ $TCID_{50}$) and 3 (high titer - $10^{6.5}$ $TCID_{50}$); P2V – group 4 (low titer - $10^{2.5}$ $TCID_{50}$); 5 (medium titer - $10^{4.5}$ $TCID_{50}$) and 6 (high titer - $10^{6.5}$ $TCID_{50}$). Rabbits of group 7 were inoculated with RPMI and served as mock-infected controls. Prior to virus inoculation, rabbits were anesthetized by ketamine (50mg/Kg) and xylazine (5mg/Kg). The IN inoculation was performed into the paranasal sinuses [17]; each animal received 0.5mL of the viral suspension in each nostril. Rabbits from different groups were housed in separate cages to avoid cross-contamination and were given food and water *ad libitum*. All procedures of animal handling and experimentation were performed under veterinary supervision and according to the recommendations of the Brazilian Committee on Animal Experimentation (COBEA, law # 6.638 of May, 8th, 1979). The experiments were approved by an Institutional Committee on Ethics and Animal Welfare and Experimentation (UFSM, Comitê de Ética e Experimentação Animal: process 97/2010).

2.3 Animal monitoring, sample collection and testing

Rabbits were monitored for clinical signs on a daily basis during 30 days pi (dpi) and weighted every two days up to day 12pi. Nasal swabs, peripheral blood and feces were collected for virological examination (virus isolation and quantitation) and for PCR. Virus isolation from swabs was performed in monolayer of Vero cells. Samples were considered negative after three passages of five days each without cytopathic effect (*cpe*). Virus titers in nasal secretions were quantitated by limiting dilution, calculated according to Reed & Munch [18] and expressed as $Log_{10}TCID_{50}$. Lung and gut samples collected at necropsy were submitted to virus isolation and quantitation in Vero cells. Tissue fragments were homogenized in MEM (10% w/v) and submitted to virus isolation as described above. Infectivity in positive samples was quantitated by limiting dilution as described above, and virus titers were expressed as $Log_{10}TCID_{50}$/g.

Pools of peripheral blood and feces collected from each group were submitted to a semi-nested PCR to amplify a sequence of VACV growth factor gene (*vgf*) [19]. DNA extraction was performed with DNAzol reagent (Invitrogen®). The primers used were: forward 5′-CGCTGCTATGATAATCAGATCATT-3′ and reverse 5’-ACAATGGATATTTACGAC-3’. A second PCR was performed using the same forward primer with an internal reverse primer 5′- TAAAAATTATGGCACAACCATATC -3′ to amplify a product of 400bp [20]. PCR reactions were performed in a 25µl volume, using 2µl of template DNA (total DNA extracted from 80µl of total blood or 50-100mg of feces), 12.5µM of each primer, 2.5mM $MgCl_2$, 10mM of dNTPs, 1× reaction buffer and 0.75 units of Taq polymerase

(Invitrogen®). PCR conditions were: initial denaturation (95°C for 10 min), followed by 30 cycles of 95°C – 60s; 45°C – 60s for primer annealing and 72°C – 60s for primer extension; and a final extension of 7 min at 72°C. Products were visualized in an 1% agarose gel, stained with Gel Red (Life Technologies®) and visualized under UV light. In all reactions, DNA extracted from Vero cells infected with VACV and blood of a rabbit inoculated with VACV were used as positive controls; sterilized ultrapure water was used as negative control.

Serum samples collected at the day of virus inoculation and at day 30pi (or at the day of death) were submitted to a standard virus neutralization (VN) assay in 96-well plates, testing two-fold dilutions of sera against a fixed dose of virus (100 - 200 $TCID_{50}$/well). VN readings were performed after five days of incubation. VN titers were considered as the reciprocal of highest dilution of sera that prevented the production of *cpe* in indicator Vero cells. Serum from a rabbit infected with VACV and fetal bovine serum were used as positive and negative controls, respectively.

Sections of brain, heart, lung, liver, spleen, gut and kidney specimens were collected at necropsy. Tissue fragments were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5µm, stained with hematoxilin and eosin (H&E) and submitted to microscopic examination.

Titers of virus shedding and weight gain/loss by experimental and control groups were compared using the ANOVA and a *post hoc* Tukey test ($p<0.05$: statistical significance). Weight data were converted to percentage and analyzed for individual by group. Statistical analysis was performed using GraphPad Prism Software, version 5.0. (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

3.1 Clinical findings

Rabbits inoculated with P1V and P2V at different titers developed respiratory and systemic signs, yet with different severity and frequency (Figures 1 and 2). The disease was more frequent in animals of groups 2, 3, 5 and 6 (100% morbidity; 80-100% mortality), and less frequent in animals of groups 1 (40% morbidity/mortality) and 4 (80% morbidity/mortality), noticeably the animals inoculated with the lowest viral titers. The survival rate of inoculated animals is represented in Figure 3.

Clinical signs included serous to hemorrhagic nasal discharge (Figure 4A), serous ocular secretion (Figure 4B), breathing distress, dark diarrhea (Figure 4C) and progressive

apathy. The progression of clinical disease was similar in most animals, starting between days 3 to 5 pi and lasting until the death or euthanasia *in extremis*. Clinical signs were usually delayed and less frequent in rabbits inoculated with low virus titers (groups 1 and 4). Pock lesions (vesicles and pustules) were observed in the ears of two rabbits, from groups 1, 3 and 4 (between days 12 and 17 pi). In general, animals inoculated with the highest virus titers ($10^{4.5}$ and $10^{6.5}TCID_{50}$) presented an earlier onset and a more severe clinical disease. Two animals of group 4 (P2V low titer) presented delayed clinical signs (after days 15-18pi). No evident differences in the nature of clinical signs were observed between P1V and P2V groups, yet the disease was usually more severe in animals inoculated with medium and high virus titers.

Inoculated animals were weighed every two days up to day 12pi. As a number of animals died or were euthanized between days 6 and 9, we compared weight gain/loss only up to day 8 (Figure 5). Control rabbits showed a steady weight gain up to day 8pi (and thereafter up to day 12pi; not shown). Rabbits of groups 2, 3, 5 and 6 lost weight in relation to control group ($p<0.05$) at days 2 (group 5), 4 (groups 2, 3, 5 and 6) and 6 (groups 2, 3 and 5). Statistical analysis was not performed for group 6 at day 6pi, since only one animal survived by this day. Thus, VACV infection had a significative impact in weight gain in most experimental groups.

3.2 Virological and serological findings

Virus shedding in nasal secretions was first detected at days 1 to 4 pi in all animals of groups 2, 3, 5 and 6, usually progressing until the death or euthanasia (Figures 1 and 2). In groups 1 and 4, the onset of virus shedding was delayed (starting usually at days 4 - 5pi). In some animals surviving acute infection, virus shedding was detected up to days 19 and 21pi. The highest titers were observed between days 8 and 12 pi in groups 1, 4 and 5, between days 6 and 8 in group 3, at day 6pi in group 6 and 10pi in group 2. Virus shedding peaked earlier (at days 6 pi) in rabbits inoculated with high virus titers (Figure 6). The amount of virus shed among the groups for each day p.i. was not statistically different.

Table 1 shows the virus titers detected in lung and gut specimens collected at necropsy. Virus titers in the lungs were generally higher in animals of groups 2, 3, 5 and 6 than in the animals inoculated with lower titers. Nevertheless, some rabbits inoculated with low virus doses harbored high virus titers in the lungs (Table 1). Low to moderate viral titers were detected in gut samples from all groups, regardless the strain or dose, suggesting that viral replication took place in this organ.

Viral DNA was detected by PCR in the blood of animals inoculated with P1V up to day 9pi. In P2V groups, blood samples were positive for VACV DNA in alternate days.

PCR for VACV DNA in feces was positive in alternate days, only in samples of animals inoculated with P1V at low and high titers, and from animals inoculated with P2V at high dose. Virus shedding, viremia and viral DNA in feces were not detected in control animals.

Serology detected neutralizing antibodies to VACV in all animals that survived beyond day 9pi (Figures 1 and 2). VN titers ranged from 2 to 128, evidencing seroconversion to VACV regardless the viral dose or strain. Rabbits from control group remained seronegative to VACV (VN titers <2).

3.3 Pathological findings

At necropsy, inoculated animals presented the same nature of gross and histological changes, yet with variable intensity. The lungs presented edema and interstitial pneumonia with multiple areas of hemorrhage (Figure 4D). In many cases, subcutaneous edema, intestinal fluid content and diarrhea were also observed. One animal of group 4 (P2V low dose) that survived until day 21 presented *pock-like* lesions on the subcutaneous tissue (not shown). The severity of lesions varied among the groups inoculated with different VACV strains and titers. Rabbits inoculated with P2V at medium (group 5) and high titers (group 6) developed moderate to severe lesions in the lungs. In contrast, animals inoculated with same titer of P1V developed only mild to moderate lesions.

Histological examination of lungs showed lesions characterized as bronchial and alveolar hyperplasia (Figure 7A) with proliferation of type-2 pneumocytes. Multiple areas of alveolar and bronchial necrosis were observed (Figures 7A and D), with intra-alveolar fibrinous aggregates and formation of few hyaline membranes (Figures 7A and B). The alveoli were filled with edema, histiocytes (Figures 7C) and few heterophiles. Animals inoculated with P1V developed the following lesions: group 1 – no lesions in lungs; group 2 – proliferative interstitial pneumonia, multifocal, with mild fibrinonecrotic bronchopneumonia; group 3 – proliferative interstitial pneumonia, multifocal to coalescent, moderate, with fibrinonecrotic bronchopneumonia, multifocal and moderate. Rabbits inoculated with P2V presented the following lesions according to the groups: group 4 – proliferative interstitial pneumonia, multifocal, mild, with mild fibrinonecrotic bronchopneumonia; group 5 – proliferative interstitial pneumonia, multifocal to coalescent, moderate, with fibrinonecrotic bronchopneumonia, multifocal to coalescent, moderate; and group 6 – proliferative interstitial

pneumonia, diffuse, severe, with fibrinonecrotic bronchopneumonia, multifocal to coalescent, severe, with edema. Rabbits of control group did not present macro or microscopic changes when submitted to euthanasia at day 30pi.

4. DISCUSSION

We herein demonstrate that rabbits are highly susceptible to VACV strains isolated from cutaneous disease in horses [14]. Inoculated rabbits developed respiratory and systemic disease upon IN inoculation, with morbidity and mortality rates reaching 80 to 100%. Experimental infections of rabbits with VACV have been previously reported [2, 21, 22]. However, their susceptibility to VACV remains controversial since both susceptibility and relative resistance have been reported [2, 21, 22]. As VACV strains P1V and P2V present unique clinico-pathological and epidemiological features – their origin in severely affected horses – we sought to investigate their behavior in rabbits, a proposed animal model for VACV.

The two VACV strains isolated from horses (P1V and P2V) display distinct genetic and biological properties. P1V is highly virulent for mice: inoculated animals developed severe clinical and pathological changes, shedding virus in nasal discharges and feces [15]. In contrast, mice inoculated with P2V replicated and shed virus in feces and nasal discharge, but did not develop clinical disease [15]. In a previous experiment, intradermal (ID) inculation of weanling rabbits with P1V, P2V or mixed P1V+P2V infection produced cutaneous lesions, respiratory and systemic disease in most animals, regardless the viral strain. Hence, P1V and P2V apparently do not show differential virulence for weanling rabbits. Similar findings have been reported in cattle and horses infected with VACV strains belonging to different genogroups [5, 9, 14]. The lack of differential virulence for rabbits (group 1 and 2 VACV) contrasts with mice studies, suggesting that the expression of virulence is highly dependent on the host and, thus, may vary among species.

A delayed virus shedding was observed in rabbits inoculated with low P1V and P2V titers (detected after 4 or 5 days), comparing with animals inoculated with medium and high virus titers, in which virus shedding was detected as early as at day 1pi (Figures 1 and 2). These findings suggest the lower respiratory tract, especially the lungs, as the main site of virus replication upon IN inoculation. The detection of high virus titers (table 1) and marked histological changes in the lungs support this hypothesis. Regrettably, we did not perform immunohistochemistry to determine the cell types supporting virus replication in the lungs. Viral DNA was detected in the blood of animals from all groups during several days,

indicating sustained systemic spread after primary replication. A secondary viremic peak was observed in animals from all P2V groups (Table 1), similar to that reported in mice inoculated with VACV and humans infected with VARV [22]. The appearance of pock lesions on the ears and eyelids of two animals also indicates systemic virus dissemination. The detection of viral DNA in the feces (P1V low and high titer; P2V high titer), and the presence of virus in the gut also supports systemic spread. Indeed, following ID inoculation of rabbits, VACV produces sustained viremia accompanied by replication in secondary target organs (lungs and intestine) and shedding in respiratory secretions and feces (Cargnelutti et al. 2011, submitted).

The main clinico-pathological findings of our experiment are similar to those described upon ID inoculation of rabbits with VACV Western Reserve (VACV-WR) [21]. Systemic, respiratory signs and secondary lesions in the skin (pocks) were observed in animals inoculated with 10^3 PFU of VACV-WR, yet with lower lethality (60%) [21]. In addition, pathological changes observed in the lungs of rabbits inoculated with P1V and P2V were similar to those observed in VACV-WR inoculated rabbits [21]. Although these two studies reveal a common pattern of VACV pathogenesis in rabbits, differential pathogenesis and different degrees of rabbit susceptibility might be observed by using genotypically distinct VACV strains. In this sense, distinct virulence of VACV strains and/or different degrees of host susceptibility might help in explaining some failures in reproducing VACV disease in rabbits, as revised by Adams and col. [21].

Pathological changes in the lungs were characterized by proliferative interstitial pneumonia, with multiple areas of alveolar and bronchial necrosis and presence of inflammatory cells. The intensity of lesions ranged from moderate to severe in rabbits inoculated with P2V at medium and high titers, and from mild to moderate in rabbits inoculated P1V at medium and high titers. Similar lesions were observed in mice inoculated with VACV belonging to Brazilian genogroups 1 and 2 [10]. Pulmonary changes were focal and mild in mice inoculated with Brazilian VACV group 1 (P2V), and moderate to severe in mice inoculated with virus from group 2 (P1V) [10]. Rabbits inoculated ID with P1V, P2V or P1V + P2V developed similar pathological changes in the lungs. An interstitial pneumonia with multiple areas of edema and hemorrhage, with mild to moderate bronchiolar hyperplasia with mild multifocal bronchial and alveolar necrosis were observed (Cargnelutti et al. 2011, submitted). The high virus titers recovered from the lungs of rabbits inoculated with P1V and P2V by ID or IN routes suggest that pulmonary changes were a consequence of massive virus replication in lung tissue.

The crescent number of outbreaks of VACV infection in Southeast Brazil in the last decades has suscited a growing interest in the origin, ecology (natural hosts, maintenance and transmission) and pathogenic potential of these viruses [11-13, 23]. The outbreak of disease in horses, in a remote Brazilian location without evident epidemiological links with the previously reported outbreaks, added another piece to the complex and intriguing VACV epidemiology. In some Brazilian VACV outbreaks, *Orthopoxvirus* particles and antibodies were detected in peridomestic rodents, suggesting a role for these animals in the maintenance of the virus in the wild [13]. Serological surveys in a wide range of domestic and wild species living in the region where the horse outbreaks were reported [14] are currently underway to identify potential reservoirs for the virus.

The unique clinico-pathological and epidemiological features surrounding the outbreak associated with mixed P1V and P2V infection prompted us to investigate the susceptibility of rabbits to these viruses, as to provide insights into their biology and pathogenesis. In summary, our study demonstrated that rabbits are fully susceptible to VACV P1V and P2V and develop severe respiratory and systemic disease upon IN inoculation. In this sense, the susceptibility of rabbits may be used to study selected aspects of VACV pathogenesis.

REFERENCES

[1] Esposito, J.J., Fenner, F., 2001, Poxviruses, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.) Fields Virology, Philadelphia, Pa: Lippincott Williams & Wilkins; 2007, p. 2885-2981.

[2] Buller RM & Palumbo GJ. Poxvirus pathogenesis. Microbiol Rev 1991; 55:80-122.

[3] Baxby D. The origins of vaccinia virus. J Infect Dis 1977; 136:453-455.

[4] Damaso CR, Esposito JJ, Condit RC & Moussatche N. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. Virology 2000; 277:439-449.

[5] Leite JA, Drumond BP, Trindade GS, Lobato ZI, da Fonseca FG, dos SJ, Madureira MC, Guedes MI, Ferreira JM, Bonjardim CA, Ferreira PC & Kroon EG. Passatempo virus, a vaccinia virus strain, Brazil. Emerg Infect Dis 2005; 11:1935-1938.

[6] Trindade GS, Guedes MI, Drumond BP, Mota BE, Abrahao JS, Lobato ZI, Gomes JA, Correa-Oliveira R, Nogueira ML, Kroon EG & da Fonseca FG. Zoonotic vaccinia virus: clinical and immunological characteristics in a naturally infected patient. Clin Infect Dis 2009; 48:e37-40.

[7] Lobato ZIP, Trindade GS, Frois MCM, Ribeiro EBT, Teixeira BM, Lima FA, Almeida GMF & Kroon EG. Outbreak of exantemal disease caused by Vaccinia virus in human and cattle in Zona da Mata region, Minas Gerais. Arq Bras Med Vet Zootec 2005; 57:423-429.

[8] Abrahão JS, Oliveira TM, Campos RK, Madureira MC, Kroon EG & Lobato ZI. Bovine vaccinia outbreaks: detection and isolation of vaccinia virus in milk samples. Foodborne Pathog Dis 2009; 6:1141-1146.

[9] Trindade GS, Lobato ZI, Drumond BP, Leite JA, Trigueiro RC, Guedes MI, da Fonseca FG, dos Santos JR, Bonjardim CA, Ferreira PC & Kroon EG. Short report: Isolation of two vaccinia virus strains from a single bovine vaccinia outbreak in rural area from Brazil: Implications on the emergence of zoonotic orthopoxviruses. Am J Trop Med Hyg 2006; 75:486-490.

[10] Ferreira JM, Drumond BP, Guedes MI, Pascoal-Xavier MA, Almeida-Leite CM, Arantes RM, Mota BE, Abrahao JS, Alves PA, Oliveira FM, Ferreira PC, Bonjardim CA, Lobato ZI & Kroon EG. Virulence in murine model shows the existence of two distinct populations of Brazilian Vaccinia virus strains. PLoS One 2008; 3:e3043.

[11] Trindade GS, Emerson GL, Carroll DS, Kroon EG & Damon IK. Brazilian vaccinia viruses and their origins. Emerg Infect Dis 2007; 13:965-972.

[12] Abrahão JS, Trindade Gde S, Ferreira JM, Campos RK, Bonjardim CA, Ferreira PC & Kroon EG. Long-lasting stability of Vaccinia virus strains in murine feces: implications for virus circulation and environmental maintenance. Arch Virol 2009; 154:1551-1553.

[13] Abrahão JS, Guedes MI, Trindade GS, Fonseca FG, Campos RK, Mota BF, Lobato ZI, Silva-Fernandes AT, Rodrigues GO, Lima LS, Ferreira PC, Bonjardim CA & Kroon EG. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? PLoS One 2009; 4:e7428.

[14] Brum MC, Anjos BL, Nogueira CE, Amaral LA, Weiblen R & Flores EF. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. J Vet Diagn Invest 2010; 22:143-147.

[15] Campos RK, Brum MC, Nogueira CE, Drumond BP, Alves PA, Siqueira-Lima L, Assis FL, Trindade GS, Bonjardim CA, Ferreira PC, Weiblen R, Flores EF, Kroon EG & Abrahao JS. Assessing the variability of Brazilian Vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. Arch Virol 2011; 156:275-283.

[16] Kaminjolo JS, Jr., Johnson LW, Frank H & Gicho JN. Vaccinia-like Pox virus identified in a horse with a skin disease. Zentralbl Veterinarmed *B* 1974; 21:202-206.

[17] Flores EF, Weiblen R, Vogel FSF, Dezengrini R, Almeida SR, Spilki FR, Roehe PM. Experimental neuropathogenesis of bovine herpesvirus 5 infection in rabbits. Pesq Vet Bras 2009; 29:1-16.

[18] Reed LJ & Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27: 493-497.

[19] Fonseca FG, Lanna MC, Campos MA, Kitajima EW, Peres JN, Golgher RR, Ferreira PC & Kroon EG. Morphological and molecular characterization of the poxvirus BeAn 58058. Arch Virol 1998; 143:1171-1186.

[20] Abrahão JS, Drumond BP, Trindade Gde S, da Silva-Fernandes AT, Ferreira JM, Alves PA, Campos RK, Siqueira L, Bonjardim CA, Ferreira PC & Kroon EG. Rapid detection of Orthopoxvirus by semi-nested PCR directly from clinical specimens: a useful alternative for routine laboratories. J Med Virol 2010; 82:692-699.

[21] Adams MM, Rice AD & Moyer RW. Rabbitpox virus and vaccinia virus infection of rabbits as a model for human smallpox. J Virol 2007; 81:11084-11095.

[22] Chapman JL, Nichols DK, Martinez MJ & Raymond JW. Animal models of orthopoxvirus infection. Vet Pathol 2010; 47:852-870.

[23] Ferreira JM, Abrahao JS, Drumond BP, Oliveira FM, Alves PA, Pascoal-Xavier MA, Lobato ZI, Bonjardim CA, Ferreira PC & Kroon EG. Vaccinia virus: shedding and horizontal transmission in a murine model. J Gen Virol 2008; **89:**2986-2991.

Table 1 – Virus titers in lungs and gut, viral DNA in the blood and feces of rabbits inoculated with VACV P1V and P2V at three different doses.

Group	Animal	Virus titer in lung $TCID_{50}/g$ [c]	Virus titer in gut $TCID_{50}/g$ [c]	Viremia[a] (days)	DNA in feces[b] (days)
1 (P1V low dose)	11	7.1	+	2-9	6,11,13
	12	-*	-		
	13	-	-		
	14	5.3	-		
	15	4.9	-		
2 (P1V medium dose)	1	9.0	-	5-9	nd***
	2	+**	-		
	3	8.0	4.5		
	4	7.3	3.3		
	5	+	-		
3 (P1V high dose)	6	8.0	+	1-9	12
	7	+	-		
	8	+	-		
	9	8.7	2.8		
	10	7.9	-		
4 (P2V low dose)	21	7.9	3.8	3 - 9, 13, 17	nd
	22	8.1	5.8		
	23	5.8	-		
	24	6.9	+		
	25	-	-		
5 (P2V medium dose)	1	+	-	2 - 8, 12, 15	nd
	2	5.8	+		
	3	7.8	+		
	4	8.7	+		
	5	8.0	5.8		
6 (P2V high dose)	6	6.8	-	1 - 3, 7	1, 2,6
	7	5.7	-		
	8	8.3	2.8		
	9	8.7	2.8		
	10	8.7	3.6		

[a]Viremia was detected by PCR of pools of blood samples/group; results are presented by groups. [b]DNA in feces was detected by PCR of pools of samples/group; results are representative of the groups.[c]Virus titers are presented as $\log_{10}$. *Negative for virus; **Positive for virus, titer $<10^{2.8}$ $TCID_{50}/g$; ***not detected.

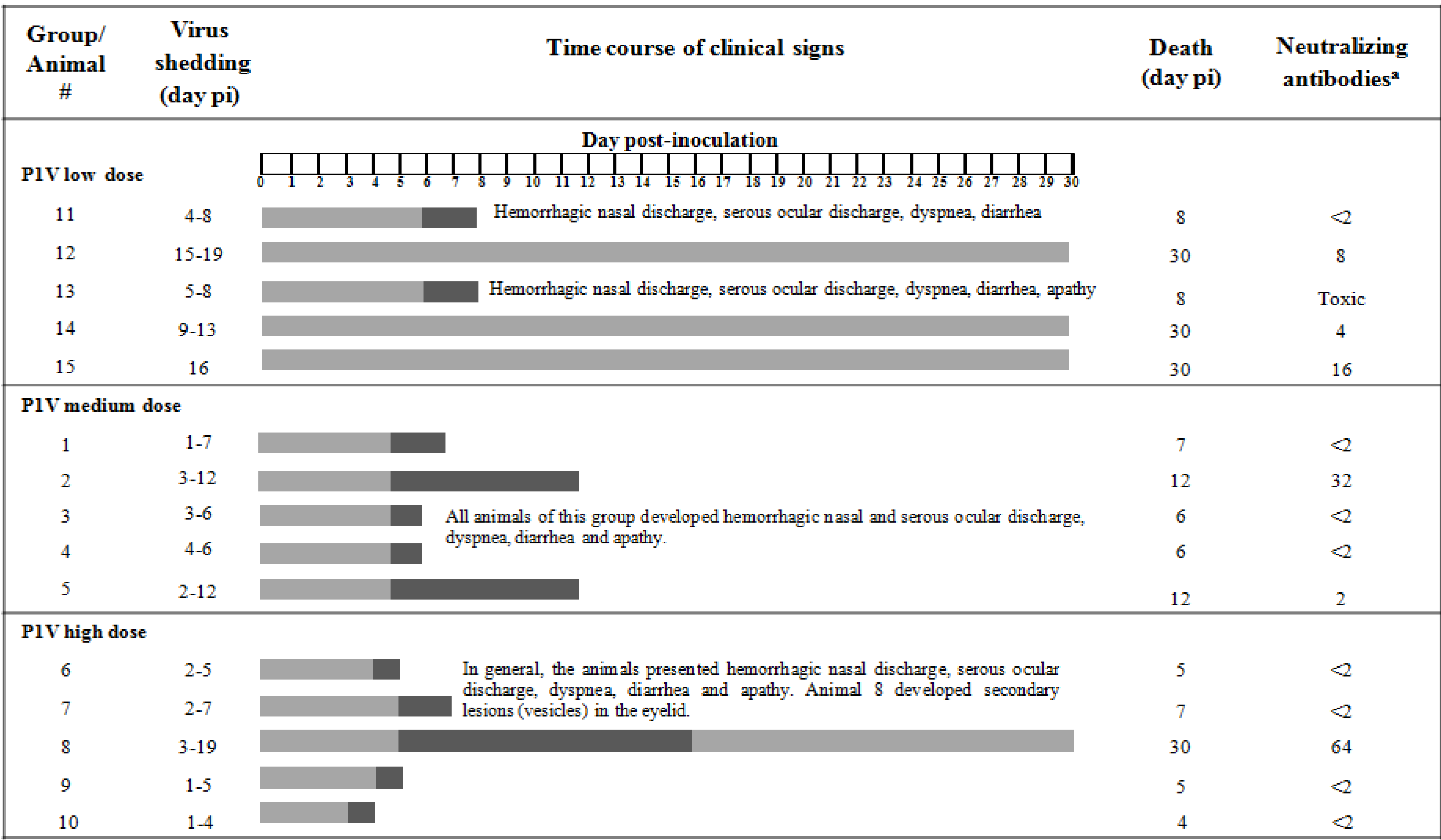

Figure 1 – Virus shedding, time course of the disease and serological response of rabbits inoculated intranasally with VACV P1V at three different doses. Dark bars show the duration of disease signs; light bars mean absence of clinical signs. [a]Neutralizing antibodies were detected in serum samples collected at the day of death.

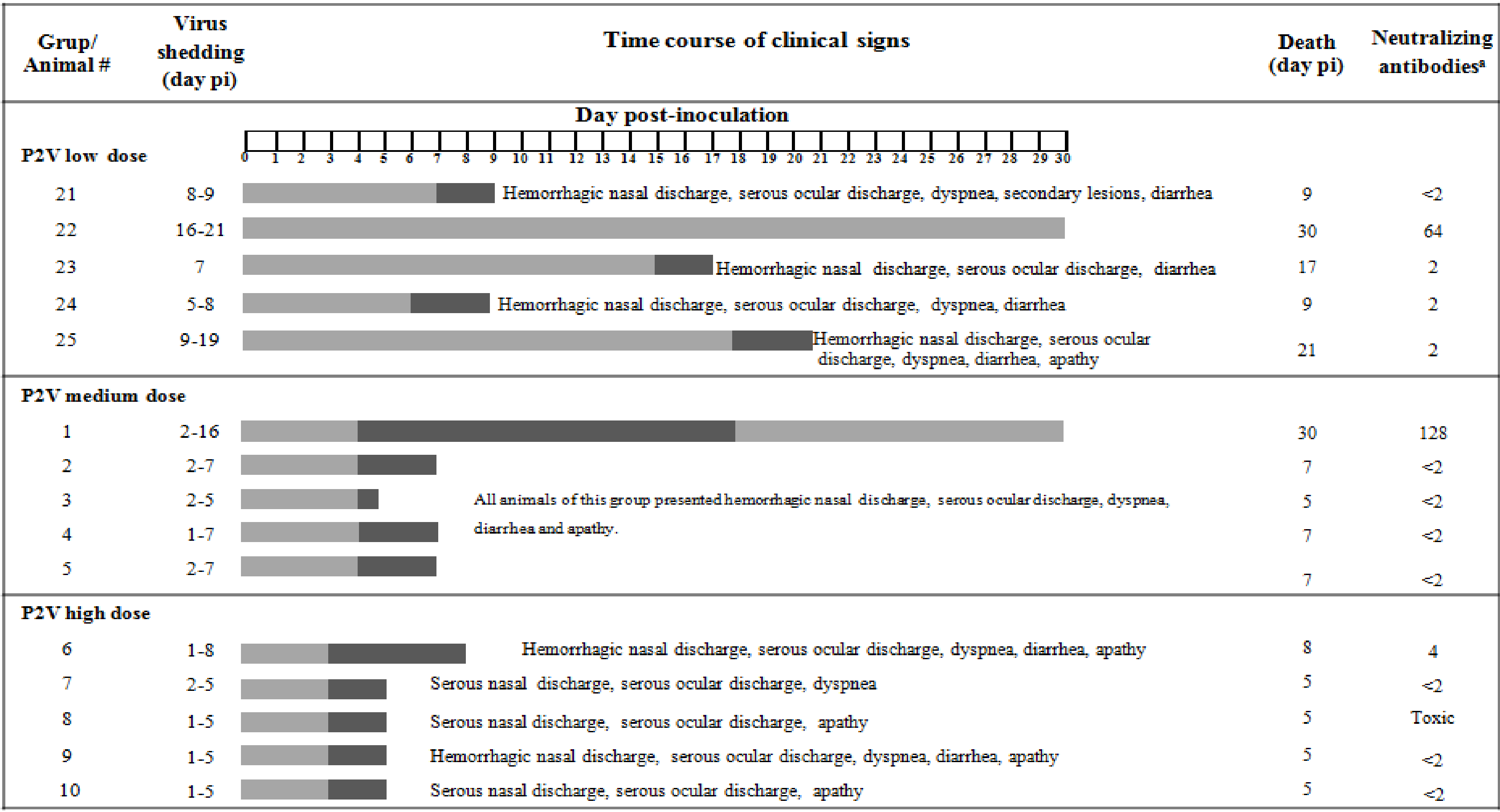

Figure 2 - Virus shedding, time course of the disease and serological response of rabbits inoculated intranasally with VACV P2V at three different doses. Dark bars show the duration of disease signs; light bars mean absence of clinical signs. [a]Neutralizing antibodies were detected in serum samples collected at the day of death.

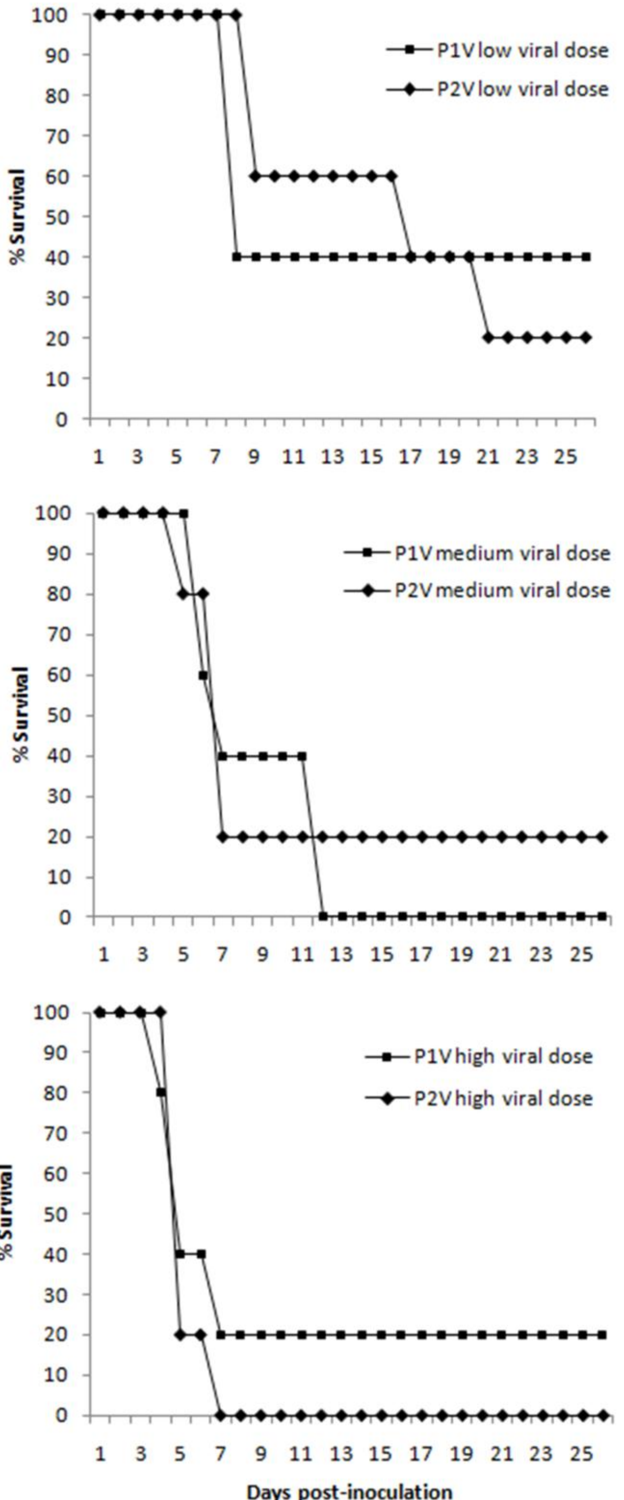

Figure 3 – Survival rate of rabbits inoculated with three different doses of VACV P1V and P2V.

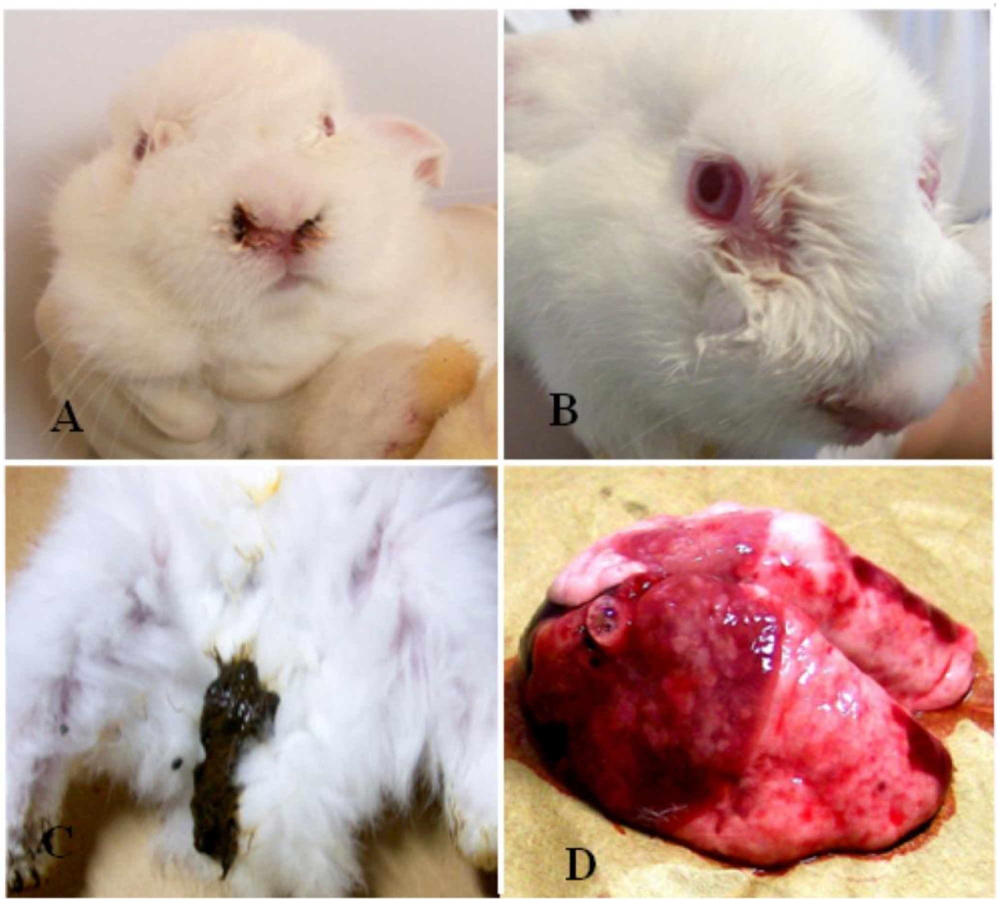

Figure 4 – Clinical signs and gross pathological changes observed in rabbits inoculated intranasally with three different doses of VACV P1V and P2V. A) Strain P1V, rabbit # 2, 9dpi. Hemorrhagic nasal discharge and serous ocular discharge. B) P2V, rabbit # 7, 4dpi. Serous ocular discharge. C) P2V, rabbit # 6, 7 dpi. Diarrhea. D) P2V, lung of rabbit # 6, 7 dpi. Bronchointerstitial pneumonia with extensive hemorrhagic areas.

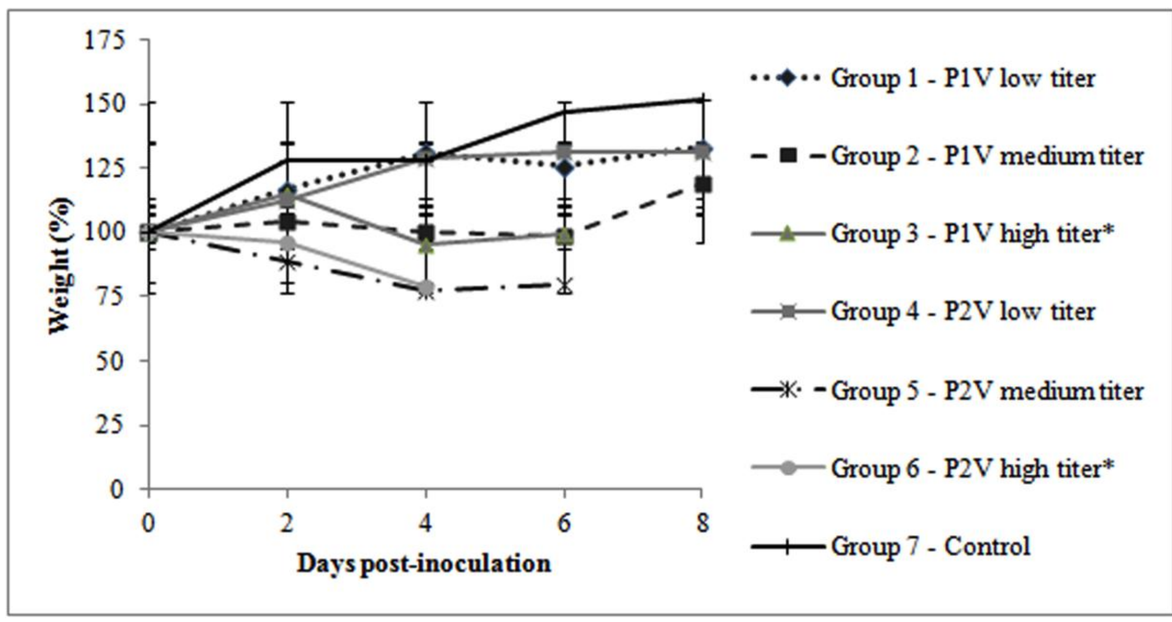

Figure 5 – Mean of body weights of rabbits inoculated with VACV P1V and P2V at three different doses. *The weights were statistically compared until day 6 post-inoculation.

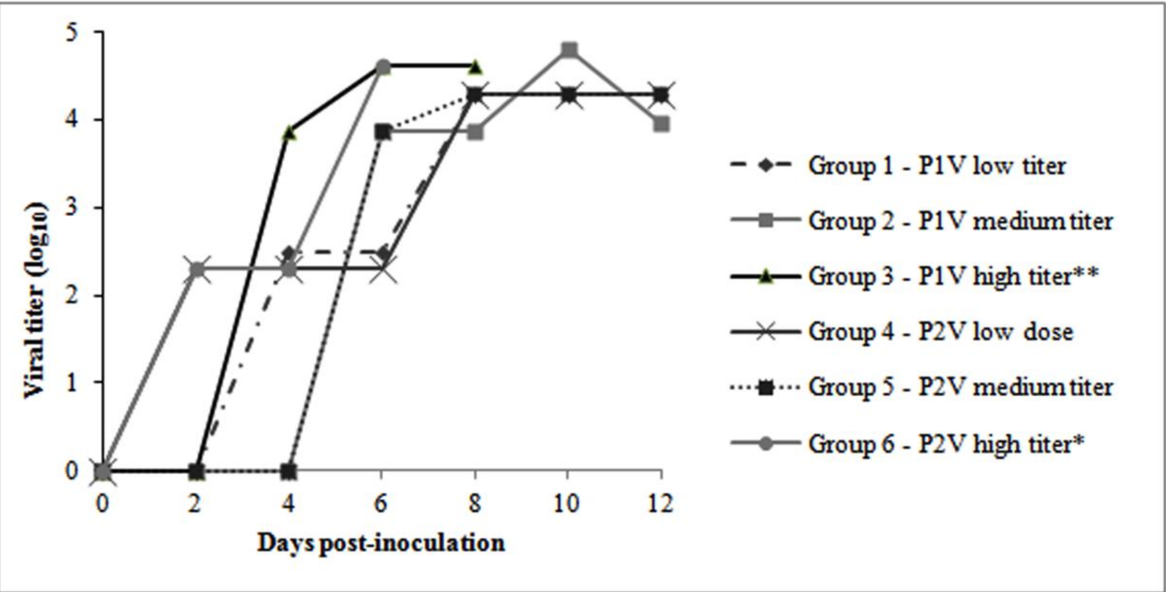

Figure 6 - Virus titers in nasal secretions of rabbits inoculated intranasally with two VACV strains (P1V and P2V) at three different doses. Infectivity in nasal secretions was quantified in a pool of samples of each group. **Viral shedding was measured until day 6 post-inoculation because after that, mostly animals died; *Viral shedding was quantitated until day 4 post-inoculation because many animals died after that.

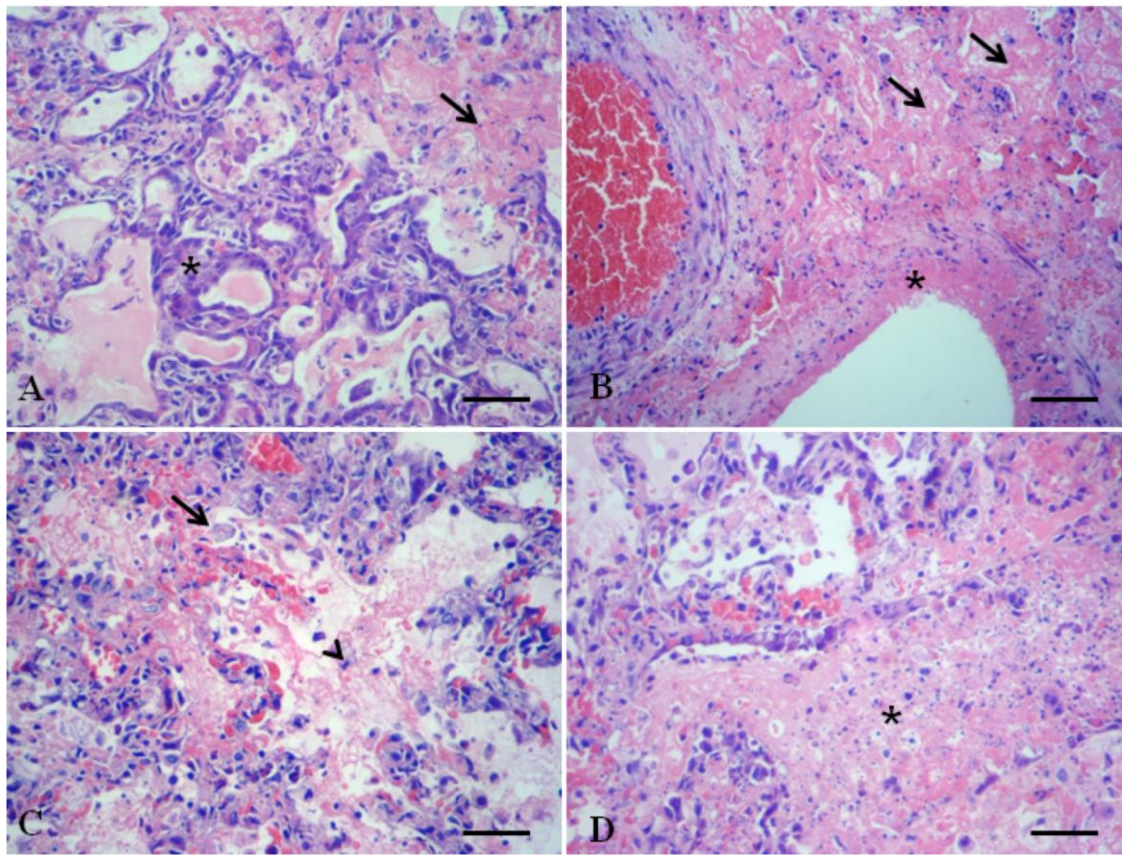

Figure 6 – Histological changes in the lungs of rabbits inoculated with three different doses of VACV P1V and P2V. A) Strain P1V, rabbit # 7, 7 dpi - alveolar hyperplasia with septal thickening (asterisk), alveolar edema and multiple areas of alveolar and bronchial necrosis (arrow) (H&E – bar 180µm). B) P2V, rabbit # 3, 5 dpi - severe intra-alveolar fibrinous aggregates (arrows) and severe bronchiolar necrosis (asterisk) (H&E – bar 360µm). C) P2V, rabbit # 7, 5 dpi - alveoli are filled with edema and fibrin, histiocytes (arrow) and few heterophiles (arrow head) (H&E – bar 180µm). D) P1V, rabbit # 9, 5 dpi - severe bronchio-alveolar necrosis (asterisk) (H&E – bar 180µm).

4. CAPÍTULO 2

Vaccinia viruses isolated from skin infection in horses produced cutaneous and systemic disease in experimentally infected rabbits

Juliana Felipetto Cargnelutti[a]; Candice Schmidt[a]; Eduardo Kenji Masuda[b]; Paula Rochelle Kurrle Nogueira[a]; Rudi Weiblen[a]; Eduardo Furtado Flores[a*]

[a]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Camobi, Santa Maria, Rio Grande do Sul, Brazil, CEP 97105-900. [b]Laboratório de Patologia Veterinária, Departamento de Patologia, Av. Roraima, 1000, Camobi, Santa Maria, Rio Grande do Sul, Brazil, CEP 97105-900, phone/fax 55 (55) 3220 8034 *Corresponding author: eduardofurtadoflores@gmail.com

(Artigo publicado no periódico *Research in Veterinary Science*, v.93, n.2, p.1070-1075, 2012).

Abstract

The susceptibility of rabbits to two isolates of Vaccinia virus (VACV) recovered from cutaneous disease in horses in Southern Brazil was investigated. Rabbits were inoculated in the ear skin with both VACV isolates, either in single or mixed infection. All inoculated animals presented local skin lesions characterized by hyperaemia, papules, vesicles, pustules and ulcers. Infectious virus was detected in the lungs and intestine of rabbits that died during acute disease. Histological examination of the skin revealed changes characteristic of those associated with members of the genus *Orthopoxvirus*. These results demonstrate that rabbits develop skin disease accompanied by systemic signs upon intradermal inoculation of these two equine VACV isolates, either alone or in combination, opening the way for using rabbits to study selected aspects of the biology and pathogenesis of VACV infection.

1. Introduction

Vaccinia virus (VACV) is the prototype of the genus *Orthopoxvirus*, family *Poxviridae* and has been associated with exanthematic and vesicular lesions in the skin of cattle, buffaloes and humans (Esposito and Fenner, 2001). In the past, VACV was used in the vaccine employed by the World Health Organization (WHO) in the Smallpox Eradication Programme due to its low virulence in humans and antigenic similarity to variola virus (VARV), the agent of smallpox (Baxby, 1977). The origin of VACV is still a matter of debate, and several hypotheses have been proposed, including evolution from VARV through multiple passages in human or cattle skin; evolution from cowpox virus (CPXV); recombination between VARV and CPXV; and from an extinct animal species (Buller and Palumbo, 1991).

During the past decade, several outbreaks of bovine and human skin vesicular disease associated with VACV infection have been reported in Southeast Brazil (Damaso et al., 2000; Leite et al., 2005; Trindade et al., 2006). The disease has since gained importance with respect to human and animal health in some rural communities (Lobato et al., 2005; Abrahão et al., 2009b). The origin of these Brazilian VACV isolates is still unknown. An initial hypothesis proposed that these viruses originated from escaped smallpox vaccine virus and was thereafter maintained in the wild for decades (Damaso et al., 2000). However, subsequent genetic analysis indicated that ancestral Brazilian VACV

isolates probably existed before the WHO global vaccination program, suggesting an autochthonous origin (Trindade et al., 2007).

The characterization of Brazilian VACV isolates has led to the identification of two distinct groups of viruses that differ from each other in genetic and biological aspects. Group 1 VACV are very similar to the Oswaldo Cruz Institute smallpox vaccine strain: they produce small plaques in cell monolayers, are avirulent in mice, share a common enzymatic digestion pattern and harbour the 18-nt deletion on the HA gene (Trindade et al., 2007). Group 2 VACV are similar to the prototypes Western Reserve VACV and vaccine strain Lister, produce large plaques in cell monolayers, are highly virulent in mice, lack the 18-nt deletion on the HA gene and share a common enzymatic digestion pattern (Trindade et al., 2007, Ferreira et al., 2008b). Both VACV groups cause indistinguishable diseases in cattle and, in at least one case, two viruses belonging to different genogroups were associated with the same outbreak of vesicular disease in dairy cows (Trindade et al., 2006).

The surprising emergence of VACVs in Brazil has generated interest concerning their origin, ecology and epidemiology (Trindade et al., 2007; Abrahão et al., 2009a; Abrahão et al., 2009c). Meanwhile, our group reported an outbreak of severe cutaneous disease in horses in Southern Brazil in which a mixed VACV infection was demonstrated (Brum et al., 2010; Campos et al., 2011). Genetic and biological analysis of the viruses recovered from affected horses revealed a co-infection with two VACV belonging to distinct groups (Campos et al., 2011). Pelotas 1 (P1V) and Pelotas 2 viruses (P2V) showed differences in plaque phenotype, virulence in mice and presence/absence of the 18-nt insertion in the HA gene (Campos et al., 2011). These findings were somewhat surprising since natural VACV infections in horses are very rare (Kaminjolo et al., 1974) and VACV infection in horses have not been reported in Brazil.

We herein investigated whether P1V and P2V might infect and cause disease in rabbits upon intradermal inoculation and describe the main aspects of their pathogenesis in rabbits.

2. Material and methods

2.1 *Cells and viruses*

Vero cells (*African Green Monkey)* (ATCC – CCL-81) were used for virus amplification, quantitation and isolation from cutaneous swabs and tissues. Cells were cultivated in RPMI medium containing ampicillin (1.6 mg/L), streptomycin (0.4 mg/L) and

amphotericin B (2.25 mg/L) and supplemented with 10% bovine foetal serum (Cultilab, Brazil). The P1V and P2V isolates were isolated concomitantly from sick horses in an outbreak of severe cutaneous disease in Southern Brazil (Brum et al., 2010; Campos et al., 2011). Cell cultures and virus growth were performed at 37°C and 5% CO_2.

2.2 *Animals and virus inoculation*

Twenty weanling New Zealand white rabbits (30-40 days old) weighing approximately 300-400 g each were randomly allocated into four groups and inoculated with one or both viruses as follows: group 1, P1V; group 2, P2V; group 3, a mixture of P1V and P2V (50% P1V: 50% P2V) (co-infection); and group 4 (control group), minimal essential medium (MEM). For virus inoculation, rabbits were anaesthetised with ketamine (50 mg/kg) and xylazine (5 mg/kg). Virus inoculation was performed with a cotton swab immersed in 150 µL of cell supernatant containing $10^{6.5}$ median tissue culture infectious dose ($TCID_{50}$)/mL after scarification of the skin of the ear with a hypodermic needle. The contralateral ear was scarified and inoculated with culture medium, serving as an internal control. Rabbits from different groups were housed in separate cages and provided food and water *ad libitum*. Animals that developed severe systemic disease, and presented severe apathy and anorexia, were euthanised. All animal handling producers and experiments were performed under veterinary supervision and according to the recommendations of the Brazilian Committee on Animal Experimentation (COBEA, law # 6.638 of May, 8th, 1979). The experiments were approved by an Institutional Committee on Ethics and Animal Welfare and Experimentation (UFSM, Comitê de Ética e Experimentação Animal: process 97/2010).

2.3 *Animal monitoring, sample collection and assays*

Animals were monitored daily for 30 days. Swabs collected from skin lesions were submitted for virus isolation and quantitation. Peripheral blood was collected for the detection of viral DNA by PCR. Virus isolation and quantitation from swabs was performed in Vero cell monolayers. Samples were considered negative after three passages of five days each without cytopathic effect (CPE). Virus titres were determined by limiting dilution, calculated according to Reed and Munch (1938) and expressed as Log_{10} $TCID_{50}$/mL. Tissue fragments from the lungs and intestine were homogenized in MEM (10% w/v) and submitted for virus isolation in Vero cells.

Peripheral blood was submitted to a semi-nested PCR to amplify a target sequence of the VACV vascular and growth factor gene (*vgf*) (Fonseca et al., 1998, Abrahão et al., 2010). Pools of three blood samples were tested to represent each group. DNA extraction from clinical samples was performed with DNAzol reagent according to the manufacturer's instructions (Invitrogen, CA, USA). The reaction was designed to amplify a target region of 1476 bp in the first reaction, followed by a second PCR to amplify a product of 400 bp (Fonseca et al., 1998; Abrahão et al., 2010). PCR products were visualized on a 1% agarose gel stained with Gel Red (Life Technologies, India) and visualized under UV light. In all reactions, DNA extracted from Vero cells infected with VACV and from the blood of a rabbit inoculated intranasally with VACV were used as positive controls. Sterile ultrapure water was used as a negative control.

Serum samples collected at days 0 and 30 pi were submitted to a standard VN assay for VACV antibodies. VN assays were performed in 96-well plates, testing two-fold dilutions of sera against a fixed dose of virus (100 - 200 $TCID_{50}$/well). Vero cells were used as indicators of virus replication. VN readings were performed after five days of incubation. The VN titer was considered the reciprocal of the highest dilution of serum that prevented the development of a CPE in indicator cells. Positive VACV bovine serum from infected cattle and foetal calf sera were used as positive and negative controls, respectively.

Biopsies of ear skin samples were collected between days 4 and 6 pi, according to the evolution of lesions, or at death. Samples of the lungs and intestine were collected at necropsy. Tissue fragments were fixed in 10% buffered formalin, routinely processed for histopathology, embedded in paraffin and stained with haematoxylin and eosin (H&E).

3. Results

3.1 *Clinical findings*

The local, primary signs developed by inoculated rabbits are illustrated in Figure 1. Animals in all three groups (P1V, P2V and P1V+P2V) developed cutaneous lesions following ID inoculation. In general, the nature of the lesions was similar, regardless of the group. The clinical course progressed through the stages of hyperaemia, macules, papules, vesicles, erupted vesicles, pustules and, finally, scabs. Typically, these lesions were confined to the sites of virus inoculation and the adjacent areas, though some animals developed macules and pustules outside of the lines of inoculation (Figure 1). The local signs usually appeared by days 1 or 3 pi and residual signs were still observed at 30 pi in

some animals. Animals in group 3 (mixed infection) developed large areas of oedema, haemorrhage, necrosis and hyperthermia in the inoculated ears (Figure 1C). Secondary lesions (hyperaemia, papules and crusts in the contralateral ears and abdominal skin) were observed in three animals from each group (Figure 2A) and typically appeared between days 7 and 8 pi and were short-lived, subsiding within three to four days. Pendular ears due to severe oedema were observed mainly in rabbits of group 3 (Figure 2B). Most animals recovered from the cutaneous lesions between days 20 and 30 pi.

Serous ocular discharge was observed in all VACV inoculated animals. Progressive apathy, anorexia, depression and respiratory signs (serous to sanguineous nasal discharge and respiratory distress) developed in animals in the three inoculated groups. Serosanguineous nasal discharge was observed in four animals in group 3 and in two rabbits in each of groups 1 and 2. Systemic signs were pronounced in animals from group 3 and also observed in animals of group 2 - and persisted until days 17 or 27 pi. Animals that developed respiratory or systemic disease died (3 animals) or were euthanized (2 animals) between days 8 and 9 pi (Table 1). On the other hand, a mild and short-term systemic course was observed mostly in rabbits (n=4) in group 1 (Table 1). Dark diarrhoea was present only in animals of group 3 (n=2).

None of the control animals developed clinical signs. The lesions from the scarifications healed within 2 to 3 days after the procedure.

3.2 *Virological findings*

Infectious virus was recovered from skin lesions between days 2-3 pi and 15-18 pi (Table 1). Virus titres in skin lesions peaked between days 3 and 4 in group 1, progressively subsiding thereafter (Figure 3). During acute infection, viral DNA was detected by PCR in the blood of animals in all groups, on alternate days, though the frequencies and duration of viraemia differed among groups (Table 2). Statistical analysis to compare the length of viraemia was not performed since we tested pooled blood samples. In any case, a considerable long-lasting viraemia was detected in all groups, especially in group 3.

Infectious virus was consistently detected in the lungs and intestines of rabbits that died or were euthanized during acute infection (animals 1a, 2a, 2d, 12b, 12c) indicating secondary virus replication in these organs (Table 2). Interestingly, infectious virus was also isolated from either organ in animals of group 2 that survived to acute infection and were euthanized at day 30pi (Table 2, animals 2b, 2c and 2e). Taken together, the detection

of virus in the blood, lungs and intestines, the development of secondary lesions in the skin and the pulmonary and systemic signs indicated a systemic spread and replication in target secondary tissues.

All inoculated animals that survived beyond day 9 pi seroconverted to VACV, presenting VN titres reaching 64 to 256 at day 30 pi. Control rabbits remained healthy and seronegative throughout the observation period; virus isolation attempts and PCR of blood DNA were negative.

3.3 *Pathological findings*

At necropsy, all inoculated animals that developed acute respiratory disease had interstitial pneumonia with multiple areas of oedema and haemorrhage in the lungs (Figure 4A). Similar macroscopic changes were observed in animals at 30 days pi (not shown). The histopathological lesions in the lungs were similar among all inoculated groups. However, animals that died from acute disease had more severe acute lesions than those that survived for a longer period. The latter had more severe interstitial pneumonia and bronchiolar epithelial hyperplasia and the former had more severe necrosis. The lungs had severe interstitial pneumonia, characterized by multifocal to coalescent areas of type-2 pneumocyte hyperplasia, severe proliferation of bronchiolar epithelium and multiple areas of necrosis with mild haemorrhage, and occasionally histiocytes (Figure 4B).

Animals from all VACV inoculated groups also presented a similar histological pattern in skin lesions examined during the clinical course. Multifocal to coalescent foci of acanthosis with severe ballooning degeneration were observed in the keratinocytes (Figure 4C). Multiple subcorneal vesicles and pustules and a few acantholytic cells were evident. Some disrupted pustules were accompanied by fibrin and degenerated neutrophils. Some oedematous keratinocytes around pustules had a few globoid to botryoid eosinophilic bodies, ranging from 1 to 2 µm, compatible with intracytoplasmic viral inclusion bodies (Figure 4D). The superficial and deep dermis had extensive areas of haemorrhage. Fibrinoid necrosis of the vascular wall accompanied by thrombosis was observed in several dermal blood vessels.

No pathological changes were observed in the intestinal sections of inoculated animals. Likewise, tissues obtained from control animals at day 30 pi appeared normal.

4. Discussion

The present article describes the virological and clinicopathological aspects of cutaneous, respiratory and systemic disease developed by rabbits inoculated with two VACV strains isolated from a mixed infection associated with cutaneous disease in horses in Southern Brazil (Brum et al., 2010). The two VACV isolates, in single or mixed infection, replicated efficiently in skin of the ear and produced typical poxvirus lesions, frequently followed by respiratory and systemic signs. Animals in all groups developed similar local, respiratory and systemic signs. Although VACV-Western Reserve (WR) and rabbitpox virus were shown to be transmitted by aerosol and penetrate through the nasal mucosa (Adams et al. 2007), the nature of muzzle lesions in the horse outbreak strongly suggested local virus penetration and replication (Brum et al., 2010). Thus, this finding prompted us to examine the pathogenesis of these VACV isolates following intradermal inoculation.

Experimental infection of rabbits has been performed to study VACV biology and epidemiology (Adams et al., 2007; Chapman et al., 2010). Rabbits have also been used to study the pathogenesis and immune response to several poxviruses, including VACV and Rabbitpox (Adams et al., 2007; Chapman et al., 2010) and orf virus (Cargnelutti et al., 2010). Our experiments demonstrate that rabbits are susceptible to VACV isolated from horses, developing the classical series of clinicopathological events that accompany poxvirus infections in several species, e.g., cutaneous infection followed by viraemia and secondary replication in target tissues (Buller and Palumbo, 1991). Inoculated rabbits developed skin lesions and shed virus through these lesions for several days. The appearance of systemic signs, pustules and vesicles in uninoculated skin regions followed an expected chronological order for poxvirus infections, and such signs were observed several days after the development of the skin lesions. In the meantime, VACV DNA was detected by PCR in pooled blood samples from all groups, demonstrating systemic spread. These findings suggest VACV replication at the inoculated sites (skin), followed by blood-systemic spread and secondary replication in target tissues (lungs, intestines). Systemic spread and secondary replication in target tissues were also accompanied by clinical signs in most animals. The detection of infectious virus in the lungs associated with marked histopathological changes indicates replication in lung tissues. The secondary lesions in the contralateral ears and in the body skin of many animals were also supportive of systemic viral dissemination. The long-term detection of viral DNA in the blood (Table 2) indicates either sustained viraemia or the occurrence of two viraemic peaks, following primary and secondary tissue replication, respectively. Intranasally inoculated mice developed primary

viraemia, followed by systemic dissemination and secondary viraemia, as in human infection with smallpox virus (Chapman et al., 2010).

The clinical course developed by rabbits infected with VACV isolates in the present study was similar to that observed in rabbits inoculated with VACV-WR or Rabbitpox virus (Adams et al., 2007). Rabbits inoculated intradermally with these viruses developed pustular, black and necrotic local lesions between 3 and 5 days pi. Respiratory signs first appeared between 5 and 7 days pi, initially as a mild, clear discharge from the nose, followed by respiratory distress (Adams et al., 2007). In the present experiment, rabbits inoculated with P1V and P2V, or both isolates, also developed poxvirus-related skin eruptions. The findings of the present study confirm the susceptibility of rabbits to VACV isolates from horses and, thus, open the way for the use of these animals to study different aspects of *Orthopoxvirus* biology.

Rabbits developed local and systemic signs regardless the equine VACV isolate used in the inoculation, against the phenotype presented by P1V and P2V in inoculated mice. Although the routes of inoculation were distinct between these species (rabbits – intradermal, mice – intranasal) mice did not display clinical signs (Campos et al., 2011). P1V inoculated mice showed weight loss, ruffling of fur and arching back within 3–7 days p.i., and mortality rates of 50%. In contrast, none of P2V inoculated mice died or lost weight, showing the distinct phenotype of P1V and P2V in mice (Campos et al., 2011). Therefore, mice inoculated with Brazilian VACV belonging to genogroup 1 (the same of P2V) also did not develop clinical signs, but shed virus in saliva and faeces (Ferreira et al., 2008a). The histopathological analyses of lungs from mice inoculated with both genogroups of Brazilian VACV revealed distinct degrees of pneumonia: in genogroup 1 inoculated mice, mild to moderate lesions were observed, contrasting with lesions developed by VACV genogroup 2, where mice developed moderate to severe lesions in lungs, characterized by interstitial pneumonia and bronchiolitis (Ferreira et al., 2008b). Although the pathological changes observed in rabbits are similar to those seen in mice, the magnitude of the pulmonary lesions was greater in rabbits, independent of VACV isolated. These results showed the variable signs developed by mice and rabbits inoculated with different VACV.

From an epidemiological point of view, the susceptibility of rabbits to VACV infection may be considered from two opposite perspectives. Susceptibility to infection would be a requisite for a possible participation of rabbits (or a related species) in the natural history of VACV. Virus detection beyond day 30pi in some animals, in spite of

high neutralizing antibody titres, would favor virus transmission and spread. On the other hand, the relatively high mortality rates might argue against a possible role of rabbits as natural reservoirs for VACV. In any case, the participation of a related species in the maintenance of VACV in nature should not be discarded. In summary, our study demonstrated that rabbits are susceptible to the Brazilian VACV isolates P1V and P2V and develop severe cutaneous and systemic disease upon ID inoculation. The susceptibility of rabbits to VACV may open the way for using these animals to study selected aspects of VACV biology and pathogenesis.

References

Abrahão, J.S., Drumond, B.P., Trindade Gde, S., da Silva-Fernandes, A.T., Ferreira, J.M., Alves, P.A., Campos, R.K., Siqueira, L., Bonjardim, C.A., Ferreira, P.C., Kroon, E.G., 2010. Rapid detection of Orthopoxvirus by semi-nested PCR directly from clinical specimens: a useful alternative for routine laboratories. Journal of Medical Virology 82, 692-699.

Abrahão, J.S., Guedes, M.I., Trindade, G.S., Fonseca, F.G., Campos, R.K., Mota, B.F., Lobato, Z.I., Silva-Fernandes, A.T., Rodrigues, G.O., Lima, L.S., Ferreira, P.C., Bonjardim, C.A., Kroon, E.G., 2009a. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? PLoS One 4, e7428.

Abrahão, J.S., Oliveira, T.M., Campos, R.K., Madureira, M.C., Kroon, E.G., Lobato, Z.I., 2009b. Bovine vaccinia outbreaks: detection and isolation of vaccinia virus in milk samples. Foodborne Pathogens Disease 6, 1141-1146.

Abrahão, J.S., Trindade Gde, S., Ferreira, J.M., Campos, R.K., Bonjardim, C.A., Ferreira, P.C., Kroon, E.G., 2009c. Long-lasting stability of Vaccinia virus strains in murine feces: implications for virus circulation and environmental maintenance. Archives of Virology 154, 1551-1553.

Adams, M.M., Rice, A.D., Moyer, R.W., 2007. Rabbitpox virus and vaccinia virus infection of rabbits as a model for human smallpox. Journal of Virology 81, 11084-11095.

Baxby, D., 1977. The origins of vaccinia virus. Journal of Infectious Disease 136, 453-455.

Brum, M.C., Anjos, B.L., Nogueira, C.E., Amaral, L.A., Weiblen, R., Flores, E.F., 2010. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. Journal of Veterinary Diagnostic Investigation 22, 143-147.

Buller, R.M., Palumbo, G.J., 1991. Poxvirus pathogenesis. Microbiological Reviews 55, 80-122.

Campos, R.K., Brum, M.C., Nogueira, C.E., Drumond, B.P., Alves, P.A., Siqueira-Lima, L., Assis, F.L., Trindade, G.S., Bonjardim, C.A., Ferreira, P.C., Weiblen, R., Flores, E.F., Kroon, E.G., Abrahão, J.S., 2011. Assessing the variability of Brazilian Vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. Archives of Virology 156, 275-283.

Cargnelutti, J.F., Masuda, E.K., Martins, M., Diel, D.G., Rock, D.L., Weiblen, R., Flores, E.F., 2010. Virological and clinico-pathological features of orf virus infection in experimentally infected rabbits and mice. Microbial Pathogenesis 50, 56-62.

Chapman, J.L., Nichols, D.K., Martinez, M.J., Raymond, J.W., 2010. Animal models of orthopoxvirus infection. Veterinary Pathology 47, 852-870.

Damaso, C.R., Esposito, J.J., Condit, R.C., Moussatche, N., 2000. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. Virology 277, 439-449.

Esposito, J.J., Fenner, F., 2001. Poxviruses, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.) Fields Virology. pp. 2885-2981.

Ferreira, J.M., Abrahão, J.S., Drumond, B.P., Oliveira, F.M., Alves, P.A., Pascoal-Xavier, M.A., Lobato, Z.I., Bonjardim, C.A., Ferreira, P.C., Kroon, E.G., 2008a. Vaccinia virus: shedding and horizontal transmission in a murine model. Journal of General Virology 89, 2986-2991.

Ferreira, J.M., Drumond, B.P., Guedes, M.I., Pascoal-Xavier, M.A., Almeida-Leite, C.M., Arantes, R.M., Mota, B.E., Abrahão, J.S., Alves, P.A., Oliveira, F.M., Ferreira, P.C., Bonjardim, C.A., Lobato, Z.I., Kroon, E.G., 2008b. Virulence in murine model shows the existence of two distinct populations of Brazilian Vaccinia virus strains. PLoS One 3, e3043.

Fonseca, F.G., Lanna, M.C., Campos, M.A., Kitajima, E.W., Peres, J.N., Golgher, R.R., Ferreira, P.C., Kroon, E.G., 1998. Morphological and molecular characterization of the poxvirus BeAn 58058. Archives of Virology 143, 1171-1186.

Kaminjolo, J.S., Jr., Johnson, L.W., Frank, H., Gicho, J.N., 1974. Vaccinia-like Pox virus identified in a horse with a skin disease. Zentralbl Veterinarmed B 21, 202-206.

Leite, J.A., Drumond, B.P., Trindade, G.S., Lobato, Z.I., da Fonseca, F.G., dos, S.J., Madureira, M.C., Guedes, M.I., Ferreira, J.M., Bonjardim, C.A., Ferreira, P.C., Kroon, E.G., 2005. Passatempo virus, a vaccinia virus strain, Brazil. Emerging Infectious Disease 11, 1935-1938.

Lobato, Z.I.P., Trindade, G.S., Frois, M.C.M., Ribeiro, E.B.T., Teixeira, B.M., Lima, F.A., Almeida, G.M.F., Kroon, E.G., 2005. Surto de varíola bovina causada pelo vírus Vaccinia na região da Zona da Mata Mineira. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 57, 423-429.

Reed, L.J., Munch, H.A., 1938. A simple method of estimating fifty percent endpoints. American Journal of Epidemiology 27, 493-497.

Trindade, G.S., Emerson, G.L., Carroll, D.S., Kroon, E.G., Damon, I.K., 2007. Brazilian vaccinia viruses and their origins. Emerging Infectious Disease 13, 965-972.

Trindade, G.S., Lobato, Z.I., Drumond, B.P., Leite, J.A., Trigueiro, R.C., Guedes, M.I., da Fonseca, F.G., dos Santos, J.R., Bonjardim, C.A., Ferreira, P.C., Kroon, E.G., 2006. Short report: Isolation of two vaccinia virus strains from a single bovine vaccinia outbreak in rural area from Brazil: Implications on the emergence of zoonotic orthopoxviruses. The American Journal of Tropical Medicine and Hygiene 75, 486-490.

Table 1 – Virological, clinical and serological findings in rabbits inoculated intradermally with two Brazilian Vaccinia virus isolates.

Group	# Animal	Virus shedding (dpi[a])	Time of clinical signs (dpi)		Mortality	Antibodies	
			Local (ears)	Systemic		0	††
1	1a	2-7	2-8	7-8†	20% (1/5)	<2	<2
	1b	2-15	1-20	6-11		<2	256
	1c	2-15	2-24	7-17		<2	128
	1d	2-14	2-17	9-10		<2	128
	1e	2-15	2-27	6-17		<2	256
2	2a	2-9	2-9	6-9†	40% (2/5)	<2	<2
	2b	3-15	3-27	7-23		<2	256
	2c	2-13	2-22	6-13		<2	256
	2d	3-8	2-9	7-9†		<2	<2
	2e	5-13	2-12	-		<2	256
3	12a	3-14	2-30	8-17	40% (2/5)	<2	256
	12b	5-8	1-9	7-9†		<2	<2
	12c	2-8	1-8	7-8†		<2	<2
	12d	2-12	1-30	7-18		<2	64
	12e	3-18	2-28	9-27		<2	256

†Death or euthanasia *in extremis*. ††Virus neutralization performed at death date (death, euthanasia *in extremis* or 30 days pi). [a]dpi: days post-inoculation.

Table 2 – Infectious virus and viral DNA in tissues and blood of rabbits inoculated intradermally with two Brazilian vaccinia virus isolates.

Group	Animal #	Infectious virus		Viral DNA in blood (days)[c]
		Lung	Gut	
1 (P1V)	1A	+[a]	+	3-9,13,19
	1B	-[b]	+	
	1C	-	-	
	1D	-	-	
	1E	-	-	
2 (P2V)	2A	+	+	3-8,12,17,20,23
	2B	+	-	
	2C	-	+	
	2D	+	+	
	2E	-	+	
3 (P1V + P2V)	12A	-	-	3-12,18,29
	12B	+	+	
	12C	+	+	
	12D	-	-	
	12E	-	-	

[a]Positive for virus; [b]Negative for virus; [c]Viremia was detected by PCR of pools of blood samples/group; results are presented by groups.

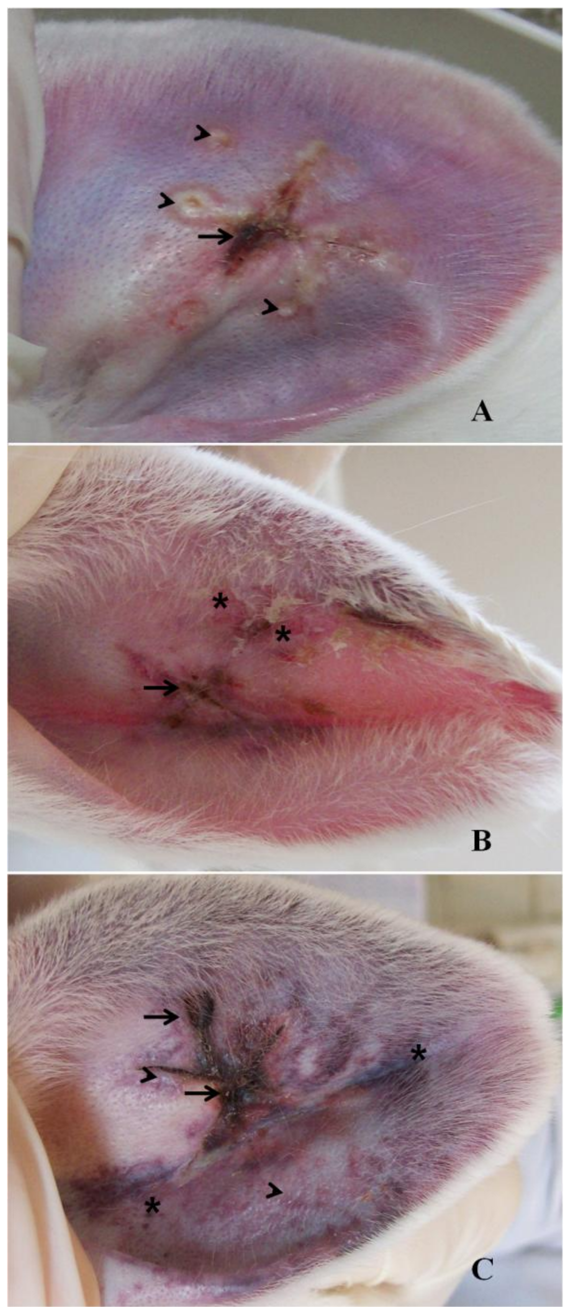

Figure 1 – Lesions in the ears in rabbits inoculated with Brazilian VACV isolates. A) Group 1 (P1V), rabbit 1B, 7 days pi. Pustules (arrowheads) and scabs (arrow). B) Group 2 (P2V), rabbit 2D, 7 days pi. Hyperemia, ulcerated pustules (arrowheads) and scabs (arrow). C) Group 3 (P1V+P2V), rabbit 12B, 7 days pi. Vesicles (arrowheads), hematomes (asterisk), congestion and necrosis (arrows).

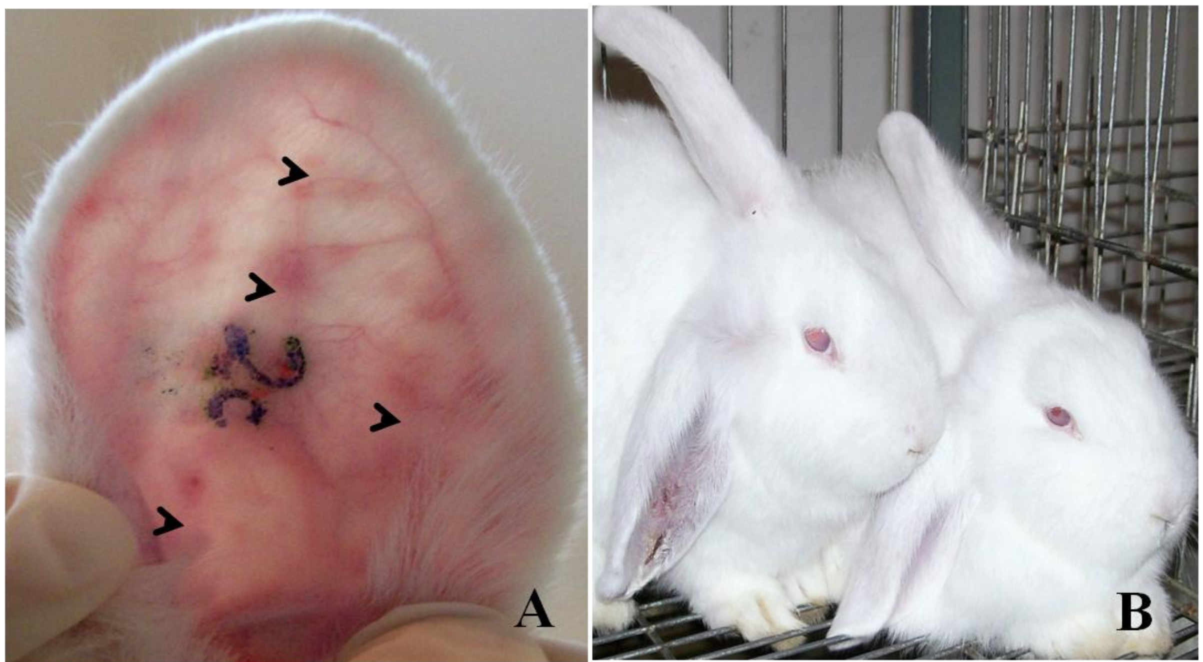

Figure 2 – Secondary signs developed by rabbits inoculated with two Brazilian VACV isolates. A) Group 2 (P2V), rabbit 2C, 7dpi. Secondary lesions (vesicles and pustules) in the contralateral ear (arrowhead). B) Group 3 (P1V + P2V), rabbits 12A and 12E. Animals presented pendulous ears due to severe edema.

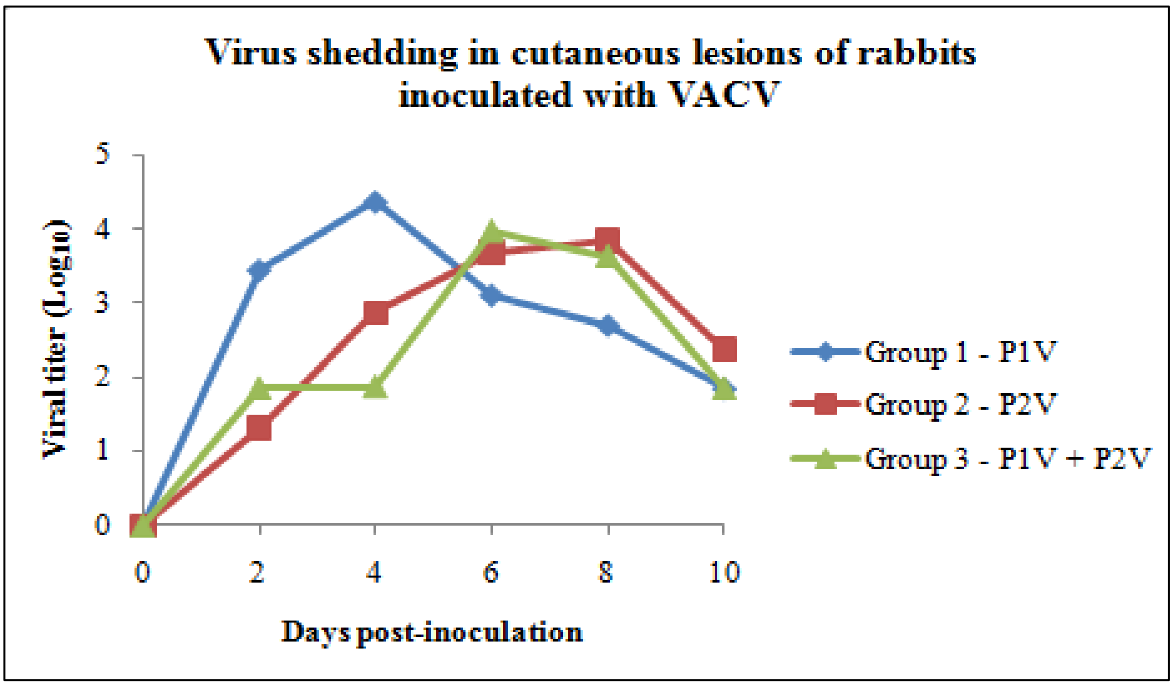

Figure 3 – Virus shedding from ear lesions of rabbits inoculated with two Brazilian VACV isolates. The figure shows virus titers up to day 10 post-inoculation. Some animals shed virus beyond this day.

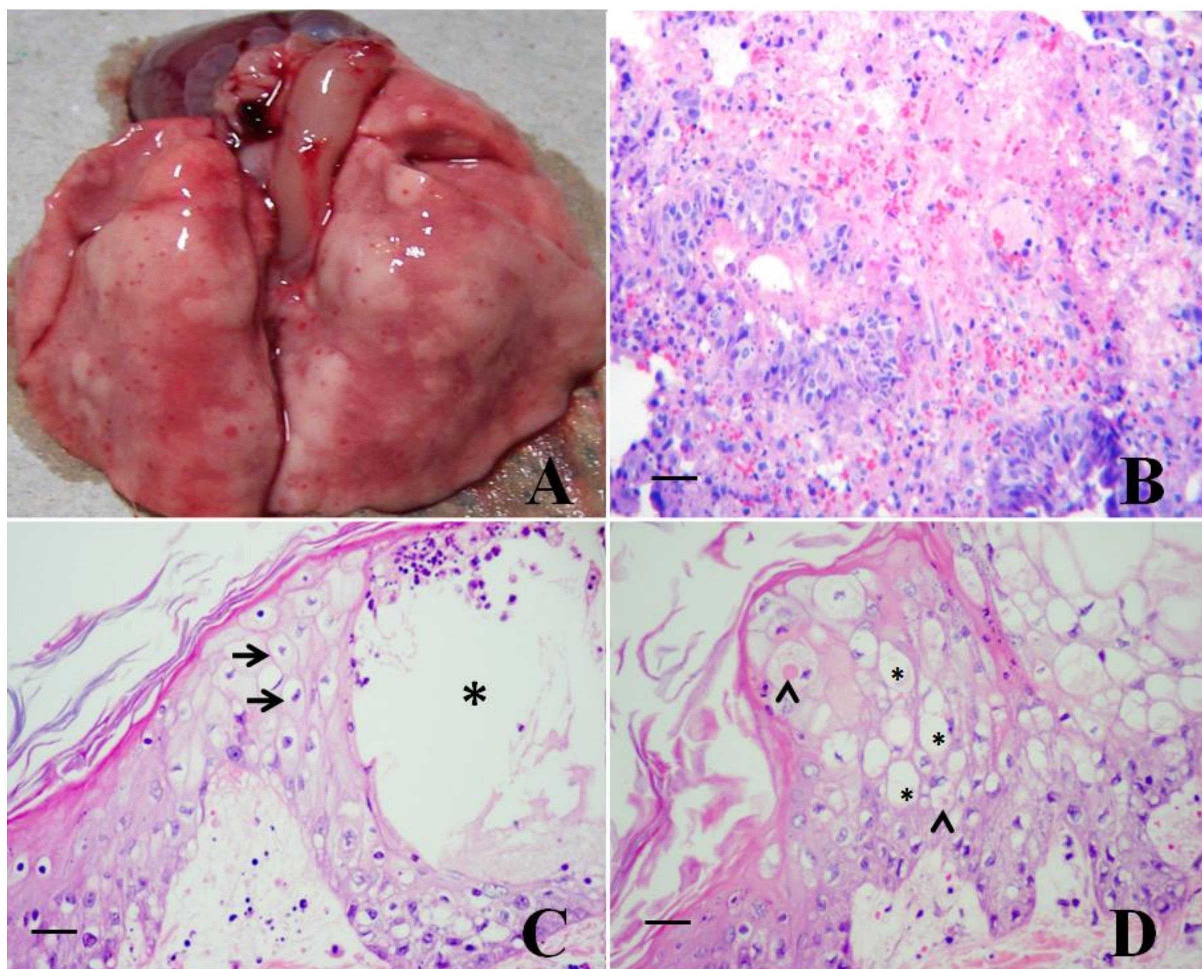

Figure 4 – Gross and microscopic changes in the lungs of rabbits inoculated with two Brazilian VACV isolates. A) Group 2 (P2V), rabbit 2A, 9dpi. Lungs, interstitial pneumonia, diffuse, severe, and hemorrhage (petechiae). B) Group 3 (P1V + P2V), rabbit 12C, 8dpi. Lung. The bronchiolar epithelium is severely hyperplastic, with an extensive area of necrosis, mild haemorrhage and severe multifocal type-2 pneumocyte hyperplasia (hematoxylin & eosin, bar 150μm). C) Group 1, (P1V), rabbit 1A, 8dpi. Skin, focally extended subcorneal pustule (asterisk), severe, surrounded by severe acanthosis and epidermal ballooning degeneration (arrow). Moderate to severe orthocerathotic hypercerathosis (hematoxylin & eosin, bar 150μm). D) Group 3 (P1V + P2V), rabbit 12B, 8dpi. Skin, focally extended severe acanthosis with balloning degeneration (asterisk). There are some viral eosinophilic intracytoplasmic inclusion bodies corpuscles (arrowhead) (hematoxylin & eosin, bar 150μm).

5. CAPÍTULO 3

Guinea pigs experimentally infected with vaccinia virus replicate and shed, but do not transmit the virus

Juliana Felipetto Cargnelutti[I], Adriéli Wendlant[I]; Rudi Weiblen[I]; Eduardo Furtado Flores[I*]

[I]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Camobi, Santa Maria, Rio Grande do Sul, Brazil, CEP 97105-900.

Fone/fax 55 (55) 3220 8034 *Corresponding author: eduardofurtadoflores@gmail.com

(Nota publicada no periódico *Ciência Rural*, v.42, n.6, p.1057-1060, 2012).

ABSTRACT

The origin of vaccinia viruses (VACV) associated with vesicular disease in cattle and humans in Southeast Brazil remains uncertain, yet the role of wild species in virus transmission has been suggested. This study investigated the susceptibility and transmission potential by guinea pigs (***Cavia porcellus***) – phylogenetically close to an abundant Brazilian rodent (***Cavia aperea***) – to two VACV strains (P1V and P2V) isolated from an outbreak of cutaneous disease in horses in Southern Brazil. Eight guinea pigs inoculated intranasally with P1V and P2V (10^6 $TCID_{50}.ml^{-1}$) did not develop clinical signs, but six animals shed virus in nasal secretions (day 1 to 9 post-inoculation – pi), developed viremia (between days 1 and 10 pi) and seroconverted to VACV. In spite of virus replication and shedding, the virus was not transmitted to sentinel animals by direct or indirect contact (aerosols) or through food and water contaminated with virus. These results demonstrate that, in spite of replicating and shedding the virus, guinea pigs do not transmit the virus upon experimental inoculation. This finding makes unlikely a possible participation of related species in VACV maintenance and transmission in nature.

Key words: ***Cavia porcellus***, ***Cavia aperea, Orthopoxvirus***, transmission, epidemiology.

RESUMO

A origem dos vírus vaccínia (VACV), envolvidos em surtos de doença vesicular em bovinos e humanos no Sudeste do Brasil, permanece desconhecida, e a participação de espécies silvestres na manutenção e transmissão do vírus tem sido sugerida. O objetivo deste trabalho foi investigar a susceptibilidade e o potencial de transmissão por cobaias (***Cavia porcellus***) - filogeneticamente relacionada a uma espécie de roedor, conhecido por preá (***Cavia aperea***), bastante abundante no país - a duas cepas de VACV (P1V e P2V) isoladas de um surto de doença cutânea em equinos no Rio Grande do Sul. Oito cobaias inoculadas pela via intranasal com uma mistura das amostras P1V e P2V (10^6 $DICC_{50}.ml^{-1}$) não apresentaram sinais clínicos, porém seis animais excretaram o vírus nas secreções nasais (1 a 9 dias pós-inoculação – pi), desenvolveram viremia (1 a 10 dias pi) e soroconverteram ao VACV. Apesar da replicação e excreção viral, o vírus não foi transmitido a sentinelas por contato direto, indireto (aerossóis) ou por água e alimentos contaminados com fezes deliberadamente contaminadas com o vírus. Esses resultados demonstram que, apesar de replicar e excretar o vírus, as cobaias não transmitem o VACV nas condições estudadas. Esses achados tornam pouco provável a participação de espécies relacionadas na manutenção e transmissão do VACV na natureza.

Palavras-chave: ***Cavia porcellus***, ***Cavia aperea***, ***Orthopoxvirus***, reservatórios, epidemiologia.

Vaccinia virus (VACV) is the prototype member of the family ***Poxviridae***, genus ***Orthopoxvirus,*** whose infection results in vesiculo-pustular and scab lesions, mainly in cattle and man (BULLER & PALUMBO, 1991). In Brazil, several outbreaks of VACV infection have been described since the 90's, especially in the Southeast region. These cases were characterized by vesicular and pustular lesions in the udder and teats of milking cows; in the lips, tongue and muzzle of suckling calves and, occasionally, on the hands and fingers of milkers (DAMASO et al., 2000; SILVA-FERNANDES et al., 2009). The origin of these viruses has been a matter of debate, but is still uncertain. During decades, VACV was used as the vaccine strain in the world programme of eradication of smallpox, due to its low virulence and antigenic similarity with the agent of smallpox, variola virus (VarV) (BAXBY, 1977). Thus, it has been suggested that the virus causing these outbreaks was originated from a vaccine escape virus. Later evidences, however, argue against this hypothesis and indicate an autochthonous origin of these VACV strains, possibly maintained in wild species and occasionally transmitted to cattle and man (KROON et al., 2011). Hence, potential reservoirs for VACV in nature have been investigated by serology and virus isolation attempts from wild rodents (da FONSECA et al., 2002; ABRAHÃO et al., 2009).

An outbreak of cutaneous disease in horses was described in Southern Brazil in 2008, in which a mixed VACV infection was demonstrated. Two VACV strains (P1V and P2V), belonging from different genogroups, were isolated from the same sick animal (BRUM et al., 2010, CAMPOS et al., 2011). The origin of this outbreak is still under investigation yet some hypotheses have been considered and, progressively discarded. First, the introduction of the virus into the herd through an infected horse is very unlikely. VACV infection in horses is very rare and this is the first report in Brazil. In addition, no animal had been introduced into the herd in the period preceding the outbreak. Second, the introduction through an infected bovine is also unlikely since the farm raises exclusively horses and there is no report of such disease in cattle in neighboring farms (BRUM et al., 2010). Third, the farm where the outbreak occurred is far distant from the cattle and human outbreaks in Southeast Brazil. As a part of the epidemiological investigation, a serological survey in cattle and horses of the region of the outbreak was conducted, with negative results. Then, a serological survey in some wild mammals of the region was performed, also yielding negative serology.

Concomitantly, we investigated the susceptibility of some species to VACV strains P1V and P2V.

Rabbits were shown to be highly susceptible to these strains and developed severe systemic disease upon nasal or cutaneous inoculation (not showed). Mice are also highly susceptible to VACV P1V e P2V (CAMPOS et al., 2011). Based on these studies, rabbits were proposed as a model for VACV pathogenesis (not showed) and mice have been used for preliminary phenotypic screening of VACV isolates (FERREIRA et al., 2008; CAMPOS et al., 2011).

The present experiment focused on an epidemiological aspect of VACV infection, investigating the susceptibility and transmission potential of P1V e P2V by guinea pigs (***Cavia porcellus***). Guinea pigs are phylogenetically related to a rodent species (***Cavia aperea*** or *"preás")*, very abundant in many Brazilian rural areas, including the region of the outbreak. In Rio Grande do Sul state, these rodents are particularly abundant, live in brushes nearby pastures and, thus, share grass pastures with livestock (SANTOS et al., 2008), Thus, our hypothesis was that a rodent species related to guinea pigs (in his case, ***Cavia aperea***) might serve as a reservoir for VACV in nature. As obtaining wild "preás" for the experiment would be difficult, we decided first to investigate the susceptibility and transmission potential of a related species, guinea pigs, to P1V and P2V.

The susceptibility of guinea pigs to VACV was investigated by intranasal (IN) inoculation of eight animals (400 to 500g, adults, both genders) with a mixed inoculum (P1V and P2V, virus titer of 10^6 $TCID_{50}$/animal), after anesthesia with ketamine (50mg Kg^{-1}) and xilazine (5mg Kg^{-1}). Animals were monitored daily for 30 days regarding clinical and virological aspects. Nasal swabs were submitted to for virus isolation in Vero cell monolayers, and monitored for cytopathic effect (CPE) during three passages of five days each. Pools of peripheral blood and faeces were submitted to PCR for viral DNA PCR reaction for *vgf* gene was performed according to ABRAHÃO et al. (2010). Serology was performed in serum samples collected at days 0 and 30 post-inoculation (pi), and submitted to a standard virus-neutralization (VN) assay in 96-well plates, testing two-fold dilutions of sera against a fixed dose of virus (100 - 200 TCID50/well). Vero cells were used as indicators of virus replication. VN readings were performed after five days of incubation. The VN titers were considered as the reciprocal of highest dilution of sera that prevented the production of CPE in the indicator cells. Serum from infected rabbit with VACV and fetal bovine serum were used as positive and negative controls, respectively.

Six out of eight inoculated animals shed virus in nasal secretions during an average of 5 days (days 1 to 9pi) and seroconverted to VACV (VN titers of 2 to 16 at day 30pi). Viral DNA was detected by PCR in pools of peripheral blood between days 1 and 10 pi, supporting systemic viral spread following IN inoculation. In spite of efficient virus replication and systemic spread, the animals remained healthy. No local (nasal) or systemic clinical signs (temperature, alertness, food and water consumption, weight gain) were observed. Likewise, no virus shedding was detected in faeces. These results showed that guinea pigs are susceptible to VACV infection but do not develop disease, under our experimental conditions.

Wild rodents have been investigated as possible reservoirs for VACV in Brazilian regions where the cattle and human cases have been reported. Positive serology and viral particles have been found in some mice species living in the affected farms (ABRAHÃO et al., 2009). The isolated virus was shown to be genetically and phenotypically similar to the virus infecting dairy cows and milkers in this outbreak, suggesting a possible epidemiological link (ABRAHÃO et al., 2009).

In the present study, VACV susceptibility and excretion by guinea pigs would be compatible with a possible participation of a related species (“preás”) in the epidemiology of VACV. Even though such extrapolation would appear excessive, we sought to investigate the potential virus transmission by inoculated animals.

The transmissibility of VACV by guinea pigs was investigated in three experiments: in Exp. #1, nine controls (non-inoculated) were housed together with two animals inoculated IN with 1mL of a mixture of P1V and P2V (10^6 $TCID_{50}.ml^{-1}$). In Exp. #2, four sentinel guinea pigs were housed in a cage 5 cm distant from another cage housing four animals inoculated with P1V + P2V (10^6 $TCID_{50}.ml^{-1}$). The only contact between the two groups would be by aerosols. In Exp. #3, four sentinel animals received water and food contaminated with faeces containing VACV. Animals were monitored during 60 (Exp. #1), 30 (Exp. #2) and 45 days (Exp. #3). VN performed after the observation period resulted negative for VACV antibodies. These results demonstrated that virus excretion by inoculated animals did not result in transmission to sentinel animals, regardless the proximity of the contact. The lack of transmission may be attributed to the low titers of virus shedding by inoculated animals (below $10^{1.8}$ $TCID_{50}.ml^{-1}$).

Summarizing, the results obtained herein demonstrate that guinea pigs are susceptible to infection with VACV strains isolated from horses since they replicate, shed the virus and seroconverted. The magnitude of virus shedding, however, seemed not to suffice to ensure transmission. Although using a related species (guinea pigs) rather than the target animal

species ("preás"), these results argue against the participation of "preás" in the maintenance and transmission of VACV in nature. Obviously, definitive evidence would be obtained after screening "preás" for VACV antibodies in the wild and definitively proving they are not susceptible to VACV infection.

COMMITEE FOR ETHICS AND ANIMAL WELFARE

All animal proceedings were approved by Committee for Ethics and Animal Welfare of Universidade Federal de Santa Maria (Protocol 23081.018003/2010-22; decision 97/2010).

REFERENCES

ABRAHÃO, J.S. et al. Rapid detection of orthopoxvirus by a semi-nested PCR directly from clinical specimes: a useful alternative for routine laboratories. **Journal of Medical Virology**, v.82, n.4, p.692-699, 2010. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20166167. Accessed: jan 31, 2012. doi: 10.1002/jmv.21617.

ABRAHÃO, J.S. et al. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? **PLoS One**, v.4, n.10, p.e7428, 2009. Available from: http://www.plosone.org/article/info:doi%2F10.1371%2Fjournal.pone.0007428. Accessed: jan 31, 2012. doi: 10.1371/journal.pone.0007428.

BAXBY, D. The origins of vaccinia virus. **Journal of Infectious Disease**, v.136, n.3, p.453-455, 1977. Available from: http://jid.oxfordjournals.org/content/136/3/453.long. Accessed: jan 31, 2012. doi: 10.1093/infdis/136.3.453.

BRUM, M.C.S. et al. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. **Journal of Veterinary Diagnostic Investigation**, v.22, n.1, p.143-147, 2010. Available from: http://vdi.sagepub.com/content/22/1/143.full. Accessed: jan 31, 2012. doi: 10.1177/104063871002200132.

BULLER, R.M.; PALUMBO, G.J. Poxvirus pathogenesis. **Microbiological Reviews**, v.55, n.1, p.80-122, 1991. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1851533. Accessed: jan 31, 2012. doi: 0146-0749/91/010080-43$02.00/0.

CAMPOS, R.K. et al. Assessing the variability of Brazilian vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. **Archives of Virology**, v.156, n.2, p.275-283, 2011. Available from: http://www.springerlink.com/content/252n3x54250131p1/. Accessed: jan 31, 2012. doi: 10.1007/s00705-010-0857-z.

da FONSECA, F.G. et al. Characterization of a vaccinia-like virus isolated in a Brazilian forest. **Journal of General Virology**, v.83, n.1, p.223-228, 2002. Available from: http://www.springerlink.com/content/252n3x54250131p1/. Accessed: jan 31, 2012.

DAMASO, C.R. et al. An emergent poxvirus from humans and cattle in Rio de Janeiro state: Cantagalo virus may derive from Brazilian smallpox vaccine. **Virology**, v.277, n.2, p.439-449, 2000. Available from: http://www.sciencedirect.com/science/article/pii/S0042682200906032. Accessed: jan 31, 2012. doi: http://dx.doi.org/10.1016/j.bbr.2011.03.031.

FERREIRA, J.M. et al. Virulence in murine model shows the existence of two distinct populations of Brazilian vaccinia virus strains. **PLoS One,** v.3, n.8, p.e3043, 2008. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2518622/. Accessed: jan 31, 2012. doi: 10.1371/journal.pone.

KROON, E.G. et al. Zoonotic Brazilian vaccinia virus: from field to therapy. **Antiviral Research**, v.92, n.2, p.150-163, 2011. Available from: http://www.sciencedirect.com/science/article/pii/S0166354211004190. Accessed: jan 31, 2012. doi: dx.doi.org/10.1016/j.antiviral.2011.08.018.

PFEFFER, M.; MEYER, H. Poxvirus diagnostics. In: MERCER, A.A. et al. **Poxviruses**. Berlin: Birkhauser Verlag, 2007. p.355-373.

SANTOS, T.G. et al. Mamíferos do campus da Universidade Federal de Santa Maria, Rio Grande do Sul, Brasil. **Biota Neutropica**, v.8, n.1, 2008. Available from: http://www.scielo.br/pdf/bn/v8n1/a15v8n1.pdf. Accessed: jan 31, 2012. doi: dx.doi.org/10.1590/S1676-06032008000100015.

SILVA-FERNANDES, A.T. et al. Natural human infections with vaccinia virus during bovine vaccinia outbreaks. **Journal of Clinical Virology**, v.44, n.4, p.308-313, 2009. Available from: http://www.journalofclinicalvirology.com/article/S1386-6532(09)00037-7 . Accessed: jan 31, 2012. doi: 10.1016/j.jcv.2009.01.007.

6. CAPÍTULO 4

Análise molecular de genes de virulência de amostras de vírus vaccínia isoladas de equinos

Molecular analysis of virulence genes of vaccinia virus isolated from horses

Juliana Felipetto Cargnelutti[I, II], Rudi Weiblen[I], Eduardo Furtado Flores[I*]

[I] Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria. Av. Roraima 1000, Santa Maria, Rio Grande do Sul, Brasil. 97105-900. [II] Programa de Pós-graduação em Medicina Veterinária, Universidade Federal de Santa Maria. Av. Roraima 1000, Santa Maria, Rio Grande do Sul, Brasil. *Autor para correspondência: eduardofurtadoflores@gmail.com

(Nota a ser submetida à revista *Ciência Rural*).

RESUMO

Duas amostras de vírus vaccínia (VACV) foram isoladas em infecção mista associada com doença vesicular em equinos. Além de diferenças genéticas no gene da hemaglutinina (HA), que as classificaram em genogrupos diferentes, as amostras P1V e P2V apresentaram fenótipo diferente em cultivo celular e em camundongos inoculados experimentalmente. O objetivo deste trabalho foi analisar a sequência de genes potencialmente envolvidos na virulência nesses isolados. Para isso, os genes C7L, N1L, B1R e K2L foram amplificados por PCR e os produtos foram submetidos à sequenciamento nucleotídico e análise das sequências. O alinhamento das sequências revelou que o isolado P2V (genogrupo 1) possui uma deleção de 15 nucleotídeos (nt) no gene K2L (posição 979-994), comparando-se com sequências de cepas de referência. Essa deleção também foi identificada em outros isolados brasileiros de VACV pertencentes ao mesmo genogrupo. Por outro lado, apenas mutações em ponto foram verificadas nos genes C7L e N1R do P2V quando comparados com o gene correspondente do P1V e da cepa padrão Western Reserve, e nenhuma mutação foi verificada no gene B1R dos vírus P1V e P2V quando comparadas com essa cepa padrão. Esses resultados demonstram que P1V e P2V possuem pequenas diferenças genéticas em genes de virulência, sendo que a deleção de 15 nt no gene K2L é a mais marcante. Além de uma possível associação com variação de fenótipo, essa deleção pode ser utilizada como marcador de isolados brasileiros de VACV do genogrupo 1.

Palavras-chave: ortopoxvírus, sequenciamento, VACV, marcadores moleculares.

ABSTRACT

Two distinct vaccinia viruses (VACV), named P1V and P2V, were isolated concomitantly from an outbreak of exanthemathous and vesicular disease in horses. P1V and P2V isolates display genetic differences in the hemagglutinin (HA) gene and differential phenotype in cell culture and in experimentally inoculated mice. These differences led to the classification of these isolates in different VACV genogroups. The purpose of this study was to analyze the nucleotide sequence of virulence and phenotype genes of these VACV isolates. C7L, N1L, B1R and K2L genes were amplified by PCR and the amplicons were submitted to nucleotide (nt) sequencing and molecular analysis. The sequence alignment demonstrated a 15 nt deletion in the K2L gene of P2V (genogroup 1, position 979-994) when compared with sequences of reference VACV strains. The same pattern of nt deletion was detected in other genogroup 1 Brazilian VACV isolates. Point mutations were observed in C7L and N1R genes from P2V isolates when compared to P1V and to a standard Western Reserve VACV strain.

No mutation was identified in B1R gene from P1V and P2V when compared to the standard strain. These results show that P1V and P2V have genetic divergences in virulence genes. The deletion of 15 nt in K2L gene might be associated with different phenotypes and could also be used as marker for genogroup 1 Brazilian VACV isolates.

Key words: ***Orthopoxvirus***, sequence analysis, VACV, molecular marker.

NOTA

O vírus vaccínia (VACV) pertence à família ***Poxviridae***, gênero ***Orthopoxvirus***, e está associado com uma importante doença zoonótica que acomete principalmente vacas leiteiras e ordenhadores (LOBATO et al., 2005). O VACV é um vírus envelopado, com genoma DNA dupla fita de aproximadamente 194kb, que codifica vários genes que, entre outras funções, estão envolvidos na determinação da virulência, espectro de hospedeiro e evasão do sistema imune do hospedeiro (SMITH, 2007).

Desde 1999, o VACV vem sendo identificado em casos de doença cutânea vesicular e crostosa em bovinos no Brasil, com frequente envolvimento de pessoas que possuem contato com animais afetados (DAMASO et al., 2000; LOBATO et al., 2005; DE SOUZA TRINDADE et al., 2007; TRINDADE et al., 2007; ABRAHÃO et al., 2009). A primeira ocorrência da infecção natural pelo VACV em equinos foi descrita em 2008 em Pelotas, no Rio Grande do Sul, envolvendo éguas e potros que apresentavam lesões crostosas e exudativas na face, gengivas e tetos (BRUM et al., 2010). A caracterização do agente isolado dos equinos afetados resultou na identificação concomitante de dois VACV, que diferiam geneticamente e fenotipicamente entre si, sendo denominados Pelotas 1 (P1V) e Pelotas 2 (P2V) (CAMPOS et al., 2011). Assim como os isolados brasileiros de VACV, o P1V e P2V apresentam características fenotípicas e genéticas que permitem a sua classificação em genogrupos diferentes. Dentre as características genéticas, os isolados de VACV do grupo 1, o qual pertence o P2V, apresentam uma deleção de 18 nucleotídeos (nt) no gene da hemaglutinina (HA), presente também em vários isolados do VACV, classificados como genogrupo 1. Por outro lado, essa deleção não está presente em outros isolados do VACV, como o P1V, o que os classifica no genogrupo 2 (CAMPOS et al., 2011). Outras características fenotípicas como morfologia de placa em cultivo celular e virulência em camundongos também diferem entre os isolados P1V e P2V (CAMPOS et al., 2011).

Animais inoculados concomitantemente com dois isolados de VACV pertencentes a genogrupos diferentes apresentaram diferentes apresentações clínicas, muitas vezes de

severidade maior do que a infecção simples (COTA et al., 2011; CARGNELUTTI et al., 2012). Essas diferenças fenotípicas podem estar relacionadas às diferenças genéticas nos genes codificadores de proteínas envolvidas na virulência. Uma característica comum dos poxvírus é a presença de genes que codificam proteínas envolvidas na evasão do sistema imune, no espectro de hospedeiro e na virulência (SMITH, 2007). Estes genes estão localizados nas regiões terminais do genoma (FENNER et al., 1989) e possuem grande divergência entre as espécies virais. Dentre eles, alguns genes merecem destaque, como o C7L (envolvido no espectro de hospedeiro *in vitro*, essencial para a replicação do VACV em alguns tipos celulares), o N1L (uma viroquina codificante de um fator de virulência *in vivo*), o K2L (envolvido na inibição de proteinase serina e na fusão célula-célula) e o gene B1R (codificante de proteína quinase essencial para a síntese de DNA viral e que também atua diminuindo a expressão da p53 celular) (PERKUS et al., 1990; LIN et al., 1992; BARTLETT et al., 2002; GUBSER et al., 2004; SANTOS et al., 2004).

Vários genes de VACV brasileiros já foram sequenciados para esclarecer as divergências fenotípicas observadas, e em alguns casos, assinaturas genéticas que permitem diferenciar os genogrupos foram identificadas (MARQUES et al., 2001; DAMASO et al., 2007; DRUMOND et al., 2008). Porém, a análise molecular de todos os genes de virulência dos VACV isolados de equinos ainda não foi realizada. Assim, o objetivo deste trabalho foi analisar e comparar a sequência nucleotídica de quatro genes envolvidos no espectro de hospedeiro, fenótipo e virulência do VACV, dos isolados P1V e P2V.

Quatro genes conservados entre os ortopoxvírus: B1R, C7L, K2L e N1L foram analisados (Tabela 1). Para isso, DNA total extraído de células Vero inoculadas com cada isolado foi submetido a amplificação por PCR com oligonucleotídeos iniciadores descritos na tabela 2. Cada reação de PCR foi realizada com, aproximadamente, 8 µl do DNA total adicionado a 92 µl de uma solução contendo 2µM dos iniciadores, 10 mM de dNTPs, 10 µl de tampão de reação 10x, 0,4 mM de $MgCl_2$ e 1 U da enzima Taq polimerase (Invitrogen, Carlsbad, CA, USA), sob as seguintes condições: um ciclo de 95°C por 9 minutos (min), seguidos por 30 ciclos de denaturação (95°C, 1 min), anelamento dos iniciadores (1 min, temperatura dependente de cada gene) e extensão (72°C, 1 min), com uma extensão final de 10 min (72°C). A temperatura de anelamento dos iniciadores foi de 47°C para o gene C7L, 44°C para o B1R, 46°C para o K2L e 42°C para o N1L. Os produtos de PCR (5 µl) foram analisados em gel de agarose a 1%, coradas com o reagente Gel Red® (Life Technologies, Carlsbad, CA, USA) sob luz ultravioleta. Para o sequenciamento, 90 µl do produto de PCR de cada amostra foi purificado utilizando o kit Ilustra GFX PCR DNA® (GE HealthCare, Uppsala, Sweden)

conforme instruções do fabricante. As amostras foram sequenciadas em quadruplicatas em sequenciador MEGABACE (Amersham Biosciences). As sequências obtidas foram analisadas pelo programa Staden (STADEN, 1996), para obtenção da sequência consenso. O alinhamento e a matriz de identidade foram realizados utilizando o programa BioEdit Sequence Alignment Editor Software suíte, versão 7.0.5.3. As sequências foram comparadas entre elas e com as depositadas no Genbank.

Utilizando as condições de PCR descritas, foi possível amplificar todos os genes pesquisados para cada um dos dois isolados. A análise da sequência consenso no Genbank confirmou que as sequências geradas correspondiam aos genes B1R, C7L, K2L e N1L do VACV, com homologia variando de 97-100%. A identidade de nucleotídeos entre P1V e P2V foi elevada, variando de 98,3% na sequência do C7L a 99,6% nas sequências do B1R, N1L e K2L. Embora as sequências analisadas se localizem nas regiões terminais do genoma, onde a variação genética entre isolados é geralmente grande, a identidade de nucleotídeos observada entre o P1V e P2V foi bastante elevada e diverge de outros genes do VACV, como o gene da hemaglutinina (HA). Neste gene, que tem sido utilizado para a caracterização do isolados de VACV, P1V e P2V apresentam identidade de nucleotídeos de 95,7%, e menor de aminoácidos (92,9%) (CAMPOS et al., 2011).

O alinhamento das sequências consenso de cada gene foi realizada utilizando cepas de referência de VACV, cepas vacinais e isolados brasileiros de VACV. A cepa Western-Reserve (VACV-WR) foi utilizada como padrão para comparação com todas as sequências. A análise das sequências do gene K2L do P2V revelou uma deleção de 15 nucleotídeos (posição 979-994) quando comparado com as sequências de K2L do P1V e da cepa padrão VACV-WR (Figura 1). Essa deleção no gene K2L do P2V já foi observada em outros isolados brasileiros de VACV, como Araçatuba, Cantagalo, Guarani 2 e Passatempo (DRUMOND et al., 2008) que são isolados de bovinos pertencentes ao genogrupo 1. Por outro lado, essa deleção está ausente no P1V e em vários isolados brasileiros de VACV, demonstrando a heterogeneidade desses isolados em relação ao gene K2L.

A proteína codificada pelo gene K2L é homóloga ao inibidor de protease serina presente no vírus cowpox, a SPI-3 (TURNER & MOYER, 2006). A SPI-3 é um componente do envelope viral nas partículas extracelulares envelopadas e é requerida para inibir a fusão entre células nas infecções por ortopoxvírus, sendo que pode estar associada à HA na superfície de células infectadas (LAW & SMITH, 1992; TURNER & MOYER, 2006). Além disso, a proteína SERP1 presente no poxvírus do fibroma maligno dos coelhos, que tem a sequência homóloga ao K2L do VACV, está envolvida na síndrome imunossupressiva, sistêmica e de

evolução fatal em coelhos, sendo que a sua deleção torna o vírus atenuado (UPTON et al., 1990). Alguns autores sugerem que a deleção presente no gene K2L de isolados brasileiros de VACV possa alterar a associação da proteína codificada pelo K2L com a HA na superfície das partículas extracelulares envelopadas (DRUMOND et al., 2008). Mesmo assim, a deleção de 15 nt no gene K2L dos VACV pertencentes ao genogrupo 1 parece não ter implicações fenotípicas, pois mutantes de VACV com deleção total do gene K2L não são atenuados em camundongos (LAW & SMITH, 1992). Além disso, a deleção do gene K2L gera um mutante que produz extensa policariocitose em cultivo celular (LAW & SMITH, 1992), o que não é observado no isolado P2V, que possui deleções nesse gene. Assim, embora a deleção de 15 nt no gene K2L pareça não afetar a fenotipia do P2V, a ausência dessas sequências em algumas amostras brasileiras de VACV pode ser usada como um marcador adicional para a classificação desses vírus em genogrupos.

O gene da HA tem sido utilizado como marcador molecular para a classificação de ortopoxvírus. A deleção de 18 nt neste gene em alguns isolados de VACV do Brasil, associado à morfologia de placa dos vírus e ao seu fenótipo em camundongos, são critérios que vem sendo utilizados para classificar os isolados de VACV em dois genogrupos (LEITE et al., 2005; TRINDADE et al., 2007; FERREIRA et al., 2008; KROON et al., 2011). Essa assinatura molecular (deleção de 18 nt) está presente em diversos isolados brasileiros de VACV, e também foi identificada em uma cepa vacinal utilizada no Brasil durante a campanha de erradicação da varíola humana na década de 70 (DAMASO et al., 2000). A análise filogenética da sequência da HA demonstra uma clara segregação dos isolados brasileiros de VACV em dois grupos, de acordo com o padrão de deleção visualizado nesse gene (KROON et al., 2011). Associado à análise do gene HA, o sequenciamento do gene K2L poderia auxiliar na classificação dos isolados de VACV brasileiros.

Mutações pontuais também foram verificadas na sequência do K2L do P2V, sendo que três destas alteram a sequência de aminoácidos: nt 338 (serina por leucina), nt 515 (isoleucina por treonina) e nt 578 (treonina por metionina). Essas mutações também estavam presentes em alguns isolados de VACV brasileiros pertencentes ao genogrupo 1, mas não parecem ser mutações consistentes para serem utilizadas como marcadores de VACV brasileiros. Além disso, uma alteração de nucleotídeo (posição 767) que origina uma mudança de aminoácido foi identificada no P2V e também no P1V (fenilalanina por serina).

Embora o P1V e P2V tenham sido isolados de equinos, uma espécie raramente afetada pelo VACV, a análise da sequência do gene K2L não identificou um padrão diferente dos VACV isolados de lesões em bovinos e roedores peri-domésticos. Semelhante ao que ocorre

na sequência do gene HA (CAMPOS et al., 2011), P1V também diverge de uma das cepas vacinais quando se analisa o gene K2L.

Nas sequências dos genes C7L e N1L do P2V também foram observadas substituições de nt que geraram alterações de aminoácidos. No gene C7L foi observada que a mutação de adenina para citosina na posição 121 promove a alteração de lisina por cisteína. No gene N1L, o aminoácido glicina é substituído por serina decorrente de uma mutação no nucleotídeo 109. Essas alterações não estavam presentes nas sequências do P1V quando comparadas com a cepa VACV-WR. A análise molecular do gene B1R não identificou alterações importantes entre os isolados analisados. As mutações presentes nos genes C7L e N1L do P2V são caracterizadas por polimorfismos únicos de nucleotídeos (SNPs) que resultam em diferenças na sequência de aminoácidos, e estão associadas com seleção positiva, indicando que os vírus estão em constante evolução. Embora os genes analisados pareçam estar envolvidos no fenótipo do VACV, é difícil afirmar se essas mutações estão envolvidas nas diferenças fenotípicas observadas entre P1V e P2V.

Os resultados obtidos neste trabalho demonstram que os vírus P1V e P2V isolados de equinos possuem algumas diferenças em genes de virulência, que incluem desde mutações pontuais até deleção de sequências de nucleotídeos. Além disso, se propõe que a deleção de 15 nt no gene K2L possa ser utilizada como um marcador adicional para a classificação de isolados brasileiros de VACV em genogrupos.

REFERÊNCIAS

ABRAHÃO, J. S. et al. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? **PLoS One**, v. 4, n. 10, p. e7428, 2009. Disponível em: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0007428. Acessado em: 02 de outubro de 2013. doi: 10.1371/journal.pone.0007428

BARTLETT, N.et al. The vaccinia virus N1L protein is an intracellular homodimer that promotes virulence. **Journal of General Virology**, v. 83, n. Pt 8, p. 1965-1976, 2002. Disponível em: http://vir.sgmjournals.org/content/83/8/1965.full.pdf+html. Acessado em: 02 de outubro de 2013.

BRUM, M. C. S. et al. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. **Journal of Veterinary Diagnostic Investigation**, v. 22, n. 1, p. 143-147, 2010. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/20093706. Acessado em: 02 de outubro de 2013. doi: 10.1177/104063871002200132

CAMPOS, R. K. et al. Assessing the variability of Brazilian Vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. **Archives of Virology**, v. 156, n. 2, p. 275-283, 2011. Disponível em: http://link.springer.com/article/10.1007%2Fs00705-010-0857-z. Acessado em: 02 de outubro de 2013. doi: 0.1007/s00705-010-0857-z

CARGNELUTTI, J. F. et al. Vaccinia viruses isolated from skin infection in horses produced cutaneous and systemic disease in experimentally infected rabbits. **Research in Veterinary Science**, v. 93, n. 2, p. 1070-1075, 2012. Disponível em: http://www.sciencedirect.com/science/article/pii/S0034528811005054. Acessado em: 02 de outubro de 2013. doi: 10.1016/j.rvsc.2011.12.016

COTA, M. et al. Estudo de patogênese de co-infecção de amostras brasileiras de vaccinia virus em modelo murino. In: VII Fórum de Microbiologia, 2011, Belo Horizonte. Caderno de Resumos, 2011.

DAMASO, C. R. et al. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. **Virology**, v. 277, n. 2, p. 439-449, 2000. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/11080491. Acessado em: 02 de outubro de 2013. doi: 10.1006/viro.2000.0603

DAMASO, C. R. et al. A PCR-based assay for detection of emerging vaccinia-like viruses isolated in Brazil. **Diagnostic Microbiology and Infectious Disease**, v. 57, n. 1, p. 39-46, 2007. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/16949244. Acessado em: 02 de outubro de 2013. doi: 10.1016/j.diagmicrobio.2006.07.012

DE SOUZA TRINDADE, G. et al. Zoonotic vaccinia virus infection in Brazil: clinical description and implications for health professionals. **Journal of Clinical Microbiology**, v. 45, n. 4, p. 1370-1372, 2007. Disponível em: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1865848/. Acessado em: 02 de outubro de 2013. doi: 0.1128/JCM.00920-06

DRUMOND, B. P. et al. Brazilian Vaccinia virus strains are genetically divergent and differ from the Lister vaccine strain. **Microbes and Infection**, v. 10, n. 2, p. 185-197, 2008. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/18248758. Acessado em: 02 de outubro de 2013. doi: 10.1016/j.micinf.2007.11.005

FENNER, F. & WITTEK, R. et al. **The Orthopoxviruses**, Academic Press Inc: London. 1989, 432p.

FERREIRA, J. M. et al. Virulence in murine model shows the existence of two distinct populations of Brazilian Vaccinia virus strains. **PLoS One**, v. 3, n. 8, p. e3043, 2008. Disponível em: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0003043. Acessado em: 02 de outubro de 2013. doi: 1 10.1371/journal.pone.0003043

GUBSER, C. et al. Poxvirus genomes: a phylogenetic analysis. **Journal of General Virology**, v. 85, n. 1, p. 105-117, 2004. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/14718625. Acessado em: 02 de outubro de 2013. doi: 10.1099/vir.0.19565-0

KROON, E. G. et al. Zoonotic Brazilian Vaccinia virus: from field to therapy. **Antiviral Research**, v. 92, n. 2, p. 150-163, 2011. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/21896287. Acessado em: 02 de outubro de 2013. doi: 0.1016/j.antiviral.2011.08.018

LAW, K. M. & SMITH, G. L. A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. **Journal of General Virology**, v. 73, n. 3, p. 549-557, 1992. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/1545218. Acessado em: 02 de outubro de 2013. doi: 10.1099/0022-1317-73-3-549

LEITE, J. A. et al. Passatempo virus, a vaccinia virus strain, Brazil. **Emerging Infectious Disease**, v. 11, n. 12, p. 1935-1938, 2005. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/16485483. Acessado em: 02 de outubro de 2013. doi: 10.3201/eid1112.050773

LIN, S. et al. The vaccinia virus B1R gene product is a serine/threonine protein kinase. **Journal of Virology**, v. 66, n. 5, p. 2717-2723, 1992. Disponível em: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC241026/. Acessado em: 02 de outubro de 2013. doi: 0022-538X/92/052717-07$02.00/0

LOBATO, Z. I. P. et al. Outbreak of exantemal disease caused by Vaccinia virus in human and cattle in Zona da Mata region, Minas Gerais. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 57, n. 4, p. 423-429, 2005. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-09352005000400001. Acessado em: 02 de outubro de 2013. doi: 10.1590/S0102-09352005000400001

MARQUES, J. T. et al. Characterization of ATI, TK and IFN-alpha/betaR genes in the genome of the BeAn 58058 virus, a naturally attenuated wild Orthopoxvirus. **Virus Genes**, v. 23, n. 3, p. 291-301, 2001. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/11778697. Acessado em: 02 de outubro de 2013.

PERKUS, M. E. et al. Vaccinia virus host range genes. **Virology**, v. 179, n. 1, p. 276-286, 1990. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/2171207. Acessado em: 02 de outubro de 2013. doi: 10.1016/0042-6822(90)90296-4

SANTOS, C. R. et al. The vaccinia virus B1R kinase induces p53 downregulation by an Mdm2-dependent mechanism. **Virology**, v. 328, n. 2, p. 254-265, 2004. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/15464845. Acessado em: 02 de outubro de 2013. doi: 0.1016/j.virol.2004.08.013

STADEN, R. The Staden sequence analysis package. **Molecular Biotechnology**, v. 5, n. 3, p. 233-241, 1996. Disponível em: http://link.springer.com/article/10.1007%2FBF02900361#page-1. Acessado em: 02 de outubro de 2013.

TRINDADE, G. S. et al. Brazilian vaccinia viruses and their origins. **Emerging Infectious Disease**, v. 13, n. 7, p. 965-972, 2007. Disponível em: http://wwwnc.cdc.gov/eid/article/13/7/pdfs/06-1404.pdf. Acessado em: 02 de outubro de 2013. doi: 0.3201/eid1307.061404

TURNER, P. C. & MOYER, R. W. The cowpox virus fusion regulator proteins SPI-3 and hemagglutinin interact in infected and uninfected cells. **Virology**, v. 347, n. 1, p. 88-99, 2006. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/16378629. Acessado em: 02 de outubro de 2013. doi: 10.1016/j.virol.2005.11.012

UPTON, C. et al. Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. **Virology**, v. 179, n. 2, p. 618-631, 1990. Disponível em: http://www.sciencedirect.com/science/article/pii/004268229090129F. Acessado em: 02 de outubro de 2013. doi: 10.1016/0042-6822(90)90129-F

Tabela 1 – Funções dos genes analisados.

Gene	Funções	Referências
B1R	Proteína quinase que fosforila resíduos de treonina e serina; essencial para a replicação do DNA viral; diminui a expressão da proteína p53 celular.	(LIN et al., 1992, SANTOS et al., 2004).
C7L	Espectro de hospedeiro *in vitro*.	(PERKUS et al., 1990).
K2L	Inibidor de proteinase serina; inibe fusão celular; envolvido no fenótipo *in vitro*.	(LAW and SMITH, 1992).
N1L	Codifica proteína intracelular não- essencial para replicação *in vitro*, envolvida na virulência *in vivo*.	(BARTLETT et al., 2002).

Tabela 2 – Sequência dos iniciadores utilizados na amplificação dos genes N1L, K2L, B1R e C7L.

Gene	Sequência dos iniciadores	ORF	Posição no genoma	Produto (pb)
N1L	N1LFor 5'-3' TTATTTTTCACCATATAGATCAATC N1l Rev 5'-3' ATGAGGACTCTACTTATTAGATA	028	21819 22172	354
K2L	K2L For 5'-3' TTAAGGAGATTCCACCTTACCC K2L Rev 5'-3' ATGATTGCGTTATTGATACTATCG	033	26147 27256	1110
B1R	B1R For 5'-3' ATGAACTTTCAAGGACTTGTGTTAAC B1R Rev 5'-3' TAATAATATACACCCTGCATTAATA	183	163878 164780	902
C7L	C7L For 5'-3' TTAATCCATGGACTCATAATCTCTA C7L Rev 5'-3' ATGGGTATACAGCACGAATTCGACA	021	15716 16168	453

```
                  ....|....| ....|....# ###############|....| ....|....| ....|....| ....|....| ....|....|
                       965        975        985        995       1005       1015       1025       1035
VACV - WR         CAAGGAACTG TAGCAGAGGC ATCTACTATT ATGGTAGCTA CGGCGAGATC ATCTCCTGAA AAACTGGAAT TTAATACACC
*VACV-P1V         .......... .......... .......... .......... .......... .......... .......... ..........
VACV - BAV        .......... .......... .......... .......... .......... .......... .......... ..........
VACV - VBH        .......... .......... .......... .......... .......... .......... .......... ..........
VACV - GP1V       .......... .......... .......... .......... .......... .......... .......... ..........
VACV - SAV        .......... .......... .........C .......... .......... .......... .......... ..........
VACV - IHD-W      .......... .......... .......... .......... .......... .......... .......... ..........
VACV - Acambis    .......... .......... .........C .......... .......... .......... .......... ..........
VACV - Copenhagen .......... .......... .........C .......... .......... .......... .......... ..........
VACV - Ankara     .......... .......... .......... .......... .......... .......... .......... ..........
VACV - Lister     .......... .......... .........C .......... .......... .......... .......... ..........
VACV - Dryvax     .......... .......... .........C .......... .......... .......... ---------- ----------
VACV - IOC        .......... .........- ---------- ----...... .......... .......... .......... ..........
*VACV - P2V       .......... .........- ---------- ----...... .......... .......... ......---- ----------
VACV - Araçatuba  .......... .........- ---------- ----...... .......... .......... .......... ..........
VACV - Cantagalo  .......... .........- ---------- ----...... .......... .......... .......... ..........
VACV - GP2V       .......... .........- ---------- ----...... .......... .......... .......... ..........
VACV - PSTV       .......... .........- ---------- ----...... .......... .......... .......... ..........
```

Figura 1 – Alinhamento parcial das sequências do gene K2L do P1V, P2V, isolados e cepas vacinais de VACV. Os isolados P1V e P2V estão identificados com asterisco (*). A deleção de 15 nucleotídeos em alguns isolados de VACV está identificada por sustenido (#) e negrito. Os códigos de acesso das sequências de VACV obtidas do Genbank são: WR (AY243312), BAV (EF175990), VBH (EF175993), GP1V (EF175991), SAV (EF175992), IDH-W (KC201194), Acambis (AY313848), Copenhagen (M35027), Ankara (U94848), Lister (EF175994), Dryvax (JN654986), IOC (EU528618), Araçatuba (EF175987), Cantagalo (HQ336401), GP2V (EF175988) e PSTV (EF175989). As sequências de P1V e P2V foram depositadas no Genbank (acesso X e y, respectivamente).

7. CONCLUSÕES

O VACV é um agente importante de doença vesicular e exantemática em bovinos, com repercussão em saúde humana. No Brasil, desde 1999, vários surtos de enfermidade vesicular tem sido descritos em rebanhos leiteiros, com frequente envolvimento de ordenhadores e outras pessoas que mantém contato com animais afetados. Caracterização genética e fenotípica de isolados de VACV recuperados desses casos demonstra uma marcada dicotomia entre as amostras brasileiras. Deleções de nucleotídeos em determinados genes, marcada divergência no padrão de virulência em camundongos, diferenças em morfologia de placa em cultivo celular e em perfis de restrição enzimática, tem permitido agrupar os isolados brasileiros de VACV em dois genogrupos (1 e 2), de acordo com as semelhanças apresentadas.

A investigação da etiologia de doença cutânea em equinos no município de Pelotas, no Rio Grande do Sul (no ano de 2008) indicou uma infecção mista por dois isolados de VACV, divergentes genética e fenotipicamente. Esses surto ganhou destaque por ser o primeiro relato de infecção por VACV em equinos no Brasil, e também pela coinfecção por vírus de dois genogrupos. Essas observações justificaram a investigação da patogenia desses vírus em diferentes modelos animais, bem como a realização de análise de genes potencialmente envolvidos na virulência e no espectro de hospedeiros.

Desta forma, os resultados obtidos nesta tese permitem concluir que:

1. O P1V e P2V são virulentos para coelhos Nova Zelândia e não apresentaram diferenças fenotípicas após inoculação pelas vias intranasal ou intradérmica nessa espécie;

2. O P1V e P2V produzem doença respiratória e sistêmica, geralmente fatal, em coelhos inoculados pelas vias intranasal e intradérmica;

3. A infecção intradérmica mista (P1V + P2V) resulta em lesões cutâneas aparentemente mais graves do que as resultantes de inoculação com cada isolado individualmente;

4. Cobaias são susceptíveis à infecção experimental com P1V + P2V, replicam e excretam o vírus, porém não desenvolvem sinais clínicos;

5. A análise molecular de quatro genes dos isolados P1V e P2V envolvidos na virulência e espectro de hospedeiro revelou mutações pontuais, provavelmente não relevantes

em termos de fenótipo; e uma deleção de 15 nt no gene K2L do P2V, que pode servir de marcador molecular de isolados de VACV do genogrupo 1.

8. REFERÊNCIAS

ABRAHÃO, J. S. et al. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? **Plos One**, San Francisco, v. 4, n. 10, p. e7428, Oct. 2009a.

ABRAHÃO, J. S. et al. Long-lasting stability of vaccinia virus strains in murine feces: implications for virus circulation and environmental maintenance. **Archives of Virology**, New York, v. 154, n. 9, p. 1551-1553, May 2009b.

ABRAHÃO, J. S. et al. Human vaccinia virus and pseudocowpox virus co-infection: clinical description and phylogenetic characterization. **Journal of Clinical Virology**, Amsterdam, v. 48, n. 1, p. 69-72, May 2010a.

ABRAHÃO, J. S. et al. Vaccinia virus infection in monkeys, Brazilian Amazon. **Emerging Infetious Disease**, Atlanta, v. 16, n. 6, p. 976-979, June 2010b.

ABRAHÃO, J. S. et al. Rapid detection of orthopoxvirus by semi-nested PCR directly from clinical specimens: a useful alternative for routine laboratories. **Journal of Medical Virology**, New York, v. 82, n. 4, p. 692-699, Apr. 2010b.

ADAMS, M. M. et al. Rabbitpox virus and vaccinia virus infection of rabbits as a model for human smallpox. **Journal of Virology**, Washington, v. 81, n. 20, p. 11084-11095, Oct. 2007.

ASSIS, F. L. et al. Characterization of a new vaccinia virus isolate reveals the C23L gene as a putative genetic marker for autochthonous group 1 Brazilian vaccinia virus. **Plos One**, San Francisco, v. 7, n. 11, p. e50413, Nov. 2012.

BARTLETT, N. et al. The vaccinia virus N1L protein is an intracellular homodimer that promotes virulence. **The Journal of General Virology**, London, v. 83, n.8, p. 1965-1976, Aug. 2002.

BAXBY, D. The origins of vaccinia virus. **The Journal of Infectious Disease**, Oxford, v. 136, n. 3, p. 453-455, Sep.1977.

BORGES, I. A. et al. Evaluation of Orthopoxvirus circulation among equids from Minas Gerais State, Brazil: Serological and historical implications. IN: XXIV BRAZILIAN CONGRESS OF VIROLOGY & VIII MERCOSUR MEETING OF VIROLOGY, 2013, Porto Seguro. **Anais eletrônicos**... Virus Reviews and Research, v.18, supl. 1, 2013. Disponível em: <http://www.sbv.org.br/vrr/ISSUE18.pdf>. Acesso em: 02 out. 2013.

BRUM, M. C. S. et al. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. **Journal of Veterinary Diagnostic Investigation**, Thousand Oaks, v. 22, n. 1, 143-147, Jan. 2010.

BULLER, R. M. & PALUMBO, G. J. Poxvirus pathogenesis. **Microbiological Reviews**, Washington, v. 55, n. 1, p. 80-122, Mar. 1991.

CAMPOS, R. K. et al. Assessing the variability of Brazilian vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. **Archives of Virology**, New York, v. 156, n .2, p. 275-283, Nov. 2011.

CARGNELUTTI, J.F. et al. An outbreak of pseudocowpox in fattening calves in southern Brazil. **Journal of Veterinary Diagnostic Investigation**, Thousand Oaks, v. 24, n. 2, 437-441, Mar. 2012.

CARGNELUTTI, J.F. et al. Pseudovaríola e estomatite papular em bovinos no Estado de Rondônia, Brasil. **Ciência Rural**, Santa Maria, 2013. Aceito para publicação.

DAMASO, C. R. et al. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. **Virology**, New York, v. 277, n. 2, p. 439-449, Nov. 2000.

DAMASO, C. R. et al. A PCR-based assay for detection of emerging vaccinia-like viruses isolated in Brazil. **Diagnostic Microbiology and Infectious Disease**, New York, v. 57, n. 1, p. 39-46, Jan. 2007.

ESPOSITO, J. J. & FENNER, F. Poxviruses. In: KNIPE, D. M.; HOWLEY, P. M.; GRIFFINET, D. E. **Fields Virology**. 5. ed. Lippincott Williams & Wilkins: Philadelphia, 2001. p. 2885-2981.

FENNER, F. & WITTEK, R. et al. **The Orthopoxviruses**, Academic Press Inc: London. 1989, 432p.

FERREIRA, J. M. et al. Vaccinia virus: shedding and horizontal transmission in a murine model. **The Journal of General Virology**, London, v. 89, n. 12, p. 2986-2991, Dec. 2008a.

FERREIRA, J. M. et al. Virulence in murine model shows the existence of two distinct populations of Brazilian vaccinia virus strains. **PLoS One**, San Francisco, v. 3, n. 8, p. e3043, Aug. 2008b.

GUBSER, C. et al. Poxvirus genomes: a phylogenetic analysis. **The Journal of General Virology**, London, v. 85, n. 1, p. 105-117, Jan. 2004.

HUYGELEN, C. Jenner's cowpox vaccine in light of current vaccinology. **Verhandelingen Koninklijke Academie voor Geneeskunde van België**, Brussels, v. 58, n. 5, p. 479-536, 1996.

ICTV. **Virus Taxonomy**. San Diego, 2012. Disponível em: <http://www.ictvonline.org/virusTaxonomy.asp>. Acesso em: 02 out. 2013.

KOTWAL, G. J. et al. Mapping and insertional mutagenesis of a vaccinia virus gene encoding a 13,800-Da secreted protein. **Virology**, New York, v. 171, n. 2, p. 579-587, Aug. 1989.

KROON, E. G. et al. Zoonotic Brazilian vaccinia virus: from field to therapy. **Antiviral Research**, Amsterdam, v.92, n.2, p.150-163, Nov. 2011.

LAW, K. M. & SMITH, G. L. A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. **The Journal of General Virology**, London, v. 73, n. 3, p. 549-557, Mar. 1992.

LEITE, J. A. et al. Passatempo virus, a vaccinia virus strain, Brazil. **Emerging Infectious Disease**, Atlanta, v. 11, n. 12, p. 1935-1938, Dec. 2005.

LEWIS-JONES, S. Zoonotic poxvirus infections in humans. **Current Opinion in Infectious Disease**, Hagerstown, v. 17, n. 2, p. 81-89, Apr. 2004.

LIN, S. et al. The vaccinia virus B1R gene product is a serine/threonine protein kinase. **Journal of Virology**, Washington, v. 66, n. 5, p. 2717-2723, May 1992.

LOBATO, Z. I. P. et al. Outbreak of exantemal disease caused by vaccinia virus in human and cattle in Zona da Mata region, Minas Gerais. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, Belo Horizonte, v. 57, n. 4, p. 423-429, Fev. 2005.

MATOS, A.C.D. et al. Outbreak of vaccinia virus infections in horses in Minas Gerais State, Brazil. In: XXIV BRAZILIAN CONGRESS OF VIROLOGY & VIII MERCOSUR

MEETING OF VIROLOGY, 2013, Porto Seguro. **Anais eletrônicos**... Virus Reviews and Research, v.18, supl. 1, 2013. Disponível em: <http://www.sbv.org.br/vrr/ISSUE18.pdf>. Acesso em: 02 out. 2013.

MEDAGLIA, M.L.G. et al. Spread of Cantagalo virus to Northern Brazil. **Emerging Infectious Disease**, Atlanta, v.15, n.7, p.1142-1143, July 2009.

MIRANDA, J.D. et al. Changing the paradigm: detection of vaccinia virus in urban areas in the State of Minas Gerais, Brazil. In: XXIV BRAZILIAN CONGRESS OF VIROLOGY & VIII MERCOSUR MEETING OF VIROLOGY, 2013, Porto Seguro. **Anais eletrônicos**... Virus Reviews and Research, v.18, supl. 1, 2013. Disponível em: <http://www.sbv.org.br/vrr/ISSUE18.pdf>. Acesso em: 02 out. 2013.

MOSS, B. Poxvirus DNA Replication. **Cold Spring Harbor Perspectives in Biology**, Woodbury, 2013. Disponível em: <http://cshperspectives.cshlp.org/content/early/2013/07/08/cshperspect.a010199.abstract> Acesso em: 02 out. 2013.

NAJERA, J. L. et al. Insertion of vaccinia virus C7L host range gene into NYVAC-B genome potentiates immune responses against HIV-1 antigens. **PLoS One**, San Francisco, v. 5, n. 6, p. e11406, June 2010.

PERES, M.G. Serological study of vaccinia virus reservoirs in areaswith and without official reports of outbreaks in cattle and humans in São Paulo, Brazil. **Archives of Virology**, New York, 2013. In press.

PERKUS, M. E. et al. Vaccinia virus host range genes. **Virology**, New York, v. 179, n. 1, p. 276-286, Nov. 1990.

ROPP, S. L. et al. PCR strategy for identification and differentiation of small pox and other orthopoxviruses. **Journal of Clinical Microbiology**, Washington, v. 33, n. 8, p. 2069-2076, Aug. 1996.

SANT'ANA, F.J.F et al. Bovine papular stomatitis affecting dairy cows and milkers in midwestern Brazil. **Journal of Veterinary Diagnostic Investigation**, Thousand Oaks, v. 24, n. 2, 442-445, Feb. 2012.

SANT'ANA, F.J.F. et al. Outbreaks of vesicular disease caused by *Vaccinia virus* in dairy cattle from Goiás State, Brazil (2010-2012). **Pesquisa Veterinária Brasileira**, Rio de Janeiro, v. 33, n. 7, p. 860-866, July 2013.

SANTOS, C. R. et al. The vaccinia virus B1R kinase induces p53 downregulation by an Mdm2-dependent mechanism. **Virology**, New York, v. 328, n. 2, p. 254-265, Oct. 2004.

SCHATZMAYR, H. G. et al. Animal infections by vaccinia-like viruses in the state of Rio de Janeiro: an expanding disease. **Pesquisa Veterinária Brasileira**, Rio de Janeiro, v. 29, n. 7, p. 509-514, July 2009.

SMITH, G. L. Genus orthopoxvirus: vaccinia virus. In: MERCER, A. A.; SCHMIDT, A.; WEBER, O. **Poxviruses**. Birkhauser Verlag Basel: Berlin, 2007. p. 1-33.

SYMONS, J. A. et al. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. **The Journal of General Virology**, London, v. 83, n. 8, p. 1953-1964, Aug. 2002.

TAYLOR, C. E. Did vaccinia virus come from a horse? **Equine Veterinary Journal**, Hobokken, v. 25, n. 1, p. 8-10, Jan. 1993.

TORRES, F. D. et al. Prevalence of antibodies to bovine herpes mammillitis virus in cattle of Rio Grande do Sul, Brazil. **Ciência Rural**, Santa Maria, v. 39, n. 6, p. 1901-1904, Jun. 2009.

TRAKTMAN, P. et al. Vaccinia virus encodes an essential gene with strong homology to protein kinases. **The Journal of Biological Chemistry**, Baltimore, v. 264, n. 36, p. 21458-21461, Dec.1989.

TRINDADE, G. S. et al. Belo Horizonte virus: a vaccinia-like virus lacking the A-type inclusion body gene isolated from infected mice. **The Journal of General Virology**, London, v. 85, n. 7, p. 2015-2021, July 2004.

TRINDADE, G. S. et al. Short report: Isolation of two vaccinia virus strains from a single bovine vaccinia outbreak in rural area from Brazil: Implications on the emergence of zoonotic orthopoxviruses. **The American Journal of Tropical Medicine and Hygiene**, Baltimore, v. 75, n. 3, p. 486-490, Sep. 2006.

TRINDADE, G. S. et al. Brazilian vaccinia viruses and their origins. **Emerging Infectious Disease**, Atlanta, v. 13, n. 7, p. 965-972, July 2007a.

TRINDADE et al. Zoonotic vaccinia virus infection in Brazil: clinical description and implications for health professionals. **Journal of Clinical Microbiology**, Washington, v. 45, n. 4, p. 1370-1372, Apr. 2007b.

TRINDADE, G. S. et al. Zoonotic vaccinia virus: clinical and immunological characteristics in a naturally infected patient. **Clinical infectious diseases : an official publication of the Infectious Diseases Society of America**, Chicago, v. 48, n. 3, p. e37-40, Feb. 2009.