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INFECÇÃO EXPERIMENTAL DE COELHOS E CAMUNDONGOS COM O VÍRUS DO ECTIMA CONTAGIOSO

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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INFECÇÃO EXPERIMENTAL DE COELHOS E CAMUNDONGOS COM O VÍRUS DO ECTIMA CONTAGIOSO

por

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INFECÇÃO EXPERIMENTAL DE COELHOS E CAMUNDONGOS COM O VÍRUS DO ECTIMA CONTAGIOSO

elaborada por
Juliana Felipetto Cargnelutti

Como requisito parcial para obtenção do título de
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RESUMO

Dissertação de Mestrado
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INFECÇÃO EXPERIMENTAL DE COELHOS E CAMUNDONGOS COM O VÍRUS DO ECTIMA CONTAGIOSO

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Santa Maria, 25 de fevereiro de 2010.

O ectima contagioso (ou *orf*) é uma doença infecto-contagiosa de pele que afeta principalmente ovinos e caprinos, e que ocasionalmente pode acometer o homem. O seu agente etiológico é o vírus da orf (ORFV). O ORFV produz lesões proliferativas, geralmente na comissura labial e no plano naso-labial de cordeiros, e também na pele do úbere, nos tetos e no rodete coronário dos cascos de animais adultos. A patogenia da infecção pelo ORFV é pouco conhecida, embora a doença já tenha sido reproduzida em ovinos, caprinos e coelhos. Essa dissertação relata os achados clínicos, virológicos e histopatológicos da infecção experimental de coelhos e camundongos pelo ORFV. Para isso, coelhos, camundongos e cordeiros foram inoculados pela via intradérmica (ID), após escarificação da pele com agulha hipodérmica. A inoculação dos cordeiros serviu como controle positivo. Uma suspensão viral da cepa IA-82 do ORFV ($10^{8,5}DICC_{50}$/mL) foi inoculada na face interna da orelha, na pele do dorso e na comissura labial dos coelhos; na face interna da orelha dos camundongos; e na comissura labial e face interna do membro pélvico dos cordeiros. Os animais foram monitorados por 21 dias nos aspectos clínicos, virológicos e patológicos. Todos os coelhos inoculados apresentaram lesões semelhantes nos locais de inoculação, iniciando com hiperemia, evoluindo para máculas, pápulas, vesículas, pústulas e crostas. Os sinais surgiram 3 a 4 dias pós inoculação (pi) e duraram por 3 a 10 dias. Excreção viral foi detectada entre os dias 2 e 14pi. A análise histológica das lesões revelou dermatite focal proliferativa, com degeneração balonosa e corpúsculos de inclusão intracitoplasmáticos eosinofílicos nos queratinócitos, semelhante às alterações histológicas observadas nos cordeiros. Lesões similares, mas de menor intensidade foram observadas em 5 de 10 camundongos. Os cordeiros, utilizados como controles positivos, apresentaram lesões clínicas e histopatológicas características de ectima contagioso entre os dias 3 e 18pi, sendo que o vírus foi recuperado das lesões entre os dias 2 e 19dpi. No dia 28pi, pelo teste de soroneutralização (SN), não foram detectados anticorpos neutralizantes no soro dos animais inoculados. Esses resultados demonstram que a inoculação de ORFV resulta em replicação viral e produção de lesões em coelhos e camundongos, porém a doença é reproduzida de forma mais consistente em coelhos. Portanto, sugere-se que coelhos possam ser utilizados como modelos para estudos *in vivo* com o ORFV.

Palavras-chave: ORFV; vírus da orf; patogenia; modelos experimentais.

ABSTRACT

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

EXPERIMENTAL INFECTION OF RABBITS AND MICE WITH CONTAGIOUS ECTHYMA VIRUS

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Santa Maria, February 25, 2010.

Contagious ecthyma (orf) is a cutaneous disease that affects sheep and goats, and may be occasionally transmitted to humans. The disease is caused by orf virus (ORFV). ORFV infection produces croustous and proliferative lesions, usually on the nostrils and labial commissures of lambs, and also in the udder, teat skin and coronary bands of adults animals. The pathogenesis of ORFV infection is poorly understood and a search for an adequate animal model is required, yet the disease has been already reproduced in sheep, goats and rabbits. This dissertation relates the clinical, virological and pathological aspects of ORFV infection in rabbits and mice experimental inoculated. Ten rabbits, ten mice and two lambs were inoculated intradermally after skin scarification with an hypodermic needle. A viral suspension of ORFV IA-82 strain ($10^{8.5}TCID_{50}$/mL) was inoculated in the internal face of the ear, back skin and labial commissure of rabbits; internal face of the ear of mice. Lambs were inoculated in the labial commissures and in the internal face of hind limbs. All animals were monitored clinically, virologically, and pathologically for 21 days. All rabbits developed clinical signs in the inoculation sites, begining with mild hyperemia that evolved to macules, papules, vesicle, pustules and scabs. Lesions appeared at days 3 and 4 post-inoculation (pi) and lasted to 3 to 10 days. Viral shedding was detected from days 2 to 14pi. Histological examination of lesions revealed focal proliferative dermatitis with ballooning degeneration and eosinophilic intracytoplasmic inclusions in keratinocytes, histological hallmarks of contagious ecthyma in sheep. A similar, albeit much milder clinical course was observed in 5 out of 10 inoculated mice. All lambs presented characteristic contagious ecthyma clinical and histopathologycal lesions from days 3 to 18pi, and the virus was recovered from lesions between days 2 and 19pi. At day 28pi, seroneutralization test (SN) was unable to detect neutralizing antibodies in all inoculated animals. These findings show that ORFV replicates and produce local lesions in rabbits and mice. However, rabbits are more susceptible to infection and disease, and may be used as an animal model to study some aspects of ORFV pathogenesis.

Keywords: ORFV; orf virus; pathogenesis; experimental models.

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LISTA DE ABREVIATURAS E SIGLAS

°C – grau centígrado

µL – microlitro

µm – micrômetro

C – citosina

CO_2 – dióxido de carbono

COBEA – Colégio Brasileiro de Experimentação Animal

CPE – efeito citopático

DNA – ácido desoxirribonucléico

g – grama

G – guanina

GIF – fator inibidor do fator estimulatório de colônia macrocítica e granulocítica

H&E – hematoxilina e eosina

IA-82 – cepa isolada de um surto de ectima contagioso em ovinos em Iowa no ano de 1982

Kb – quilopares de bases

L – litro

MEM – meio essencial mínimo

min – minutos

mg – miligrama

mL – mililitro

ORFV – vírus da orf

OTu – células de corneto etmoidal ovino

OVIFNR – proteína de resistência ao interferon

pi – pós-inoculação

$TCID_{50}$ – dose viral infectante para 50% dos cultivos celulares

UFSM – Universidade Federal de Santa Maria

VEGF-E – fator de crescimento endotelial e vascular

vIL-10 – proteína ortóloga à interleucina-10 de mamíferos

VN – teste de soroneutralização

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1. INTRODUÇÃO

O ectima contagioso, também conhecido como *orf*, dermatite pustular contagiosa e estomatite pustular contagiosa, é uma enfermidade viral que cursa com lesões crostosas e proliferativas na pele e nas junções mucocutâneas de ovinos, caprinos e, ocasionalmente, do homem (ROBINSON & BALASSU, 1981; HAIG & MERCER, 1998). A doença é causada pelo vírus da *orf* (ORFV), que pertence à família ***Poxviridae***, subfamília ***Chordopoxvirinae,*** gênero ***Parapoxvírus*** (BUTTNER & RZIHA, 2002). O ORFV está estreitamente relacionado aos vírus da estomatite papular bovina, pseudocowpox e parapoxvírus, que infectam cervídeos (HAIG & MERCER, 1998). Em ovinos e caprinos, as lesões produzidas pelo ORFV geralmente se localizam nas comissuras labiais, plano naso-labial, mas também podem ser encontradas na região genital, no rodete coronário dos cascos e nos tetos (ROBINSON & BALASSU, 1981). Em humanos, a doença é de caráter ocupacional, sendo os ordenhadores e Médicos Veterinários as classes mais propensas à infecção, pelo contato próximo com animais doentes. As lesões em pessoas são dolorosas, mas de pequena extensão e intensidade, restringindo-se geralmente aos dedos das mãos (AL-SALAM et al., 2008).

A infecção pelo ORFV possui distribuição mundial, sendo endêmica na maioria dos países onde existem criações comerciais de ovinos e caprinos (ROBINSON & BALASSU, 1981). A estimativa da soroprevalência da infecção pelo ORFV nos rebanhos é prejudicada pelo fato do vírus induzir baixos níveis de anticorpos neutralizantes, não sendo de fácil detecção em testes de soroneutralização (HAIG & MERCER, 1998). Por esse motivo, a prevalência têm sido estimada com base em relatos da ocorrência da doença em rebanhos (ROBINSON, 1983), ou por meio de testes imunoenzimáticos, como na Turquia, onde os valores de soroprevalência giram em torno de 53% (GOKCE et al., 2005).

No Brasil já foram descritos casos de ectima em ovinos e caprinos. Um surto da doença envolvendo ovinos no Rio Grande do Sul esteve relacionado ao modo de alimentação desses animais. Neste estudo, a fonte de infecção não foi determinada, mas a infecção e a disseminação da doença parecem ter sido facilitadas pelo hábito dos animais de mastigar a planta caraguatá (*Tillandsia usneoides*) que, devido sua morfologia, teria promovido abrasões e escarificações ao redor da boca e narinas dos ovinos (SALLES et al., 1992). Em caprinos, a ocorrência da doença também já foi descrita (MAZUR & MACHADO, 1989; NÓBREGA Jr et al., 2008). Morbidade de 100% e alta mortalidade de caprinos jovens ocorreram em

rebanhos fechados sem histórico de contaminação prévia pelo ORFV (MAZUR & MACHADO, 1989). Na região do semi-árido da Paraíba foram acompanhados diversos surtos de ectima contagioso em ovinos e caprinos, com casos de lesões em humanos. Os animais jovens foram os mais afetados, apresentando lesões crostosas principalmente na comissura labial e plano naso-labial. Animais adultos apresentaram sinais especialmente na região do úbere. Em humanos, as lesões foram restritas aos dedos das mãos do tratador dos animais (MACÊDO et al., 2008; NÓBREGA Jr et al., 2008). Nos Estados de Minas Gerais e Rio de Janeiro, o ORFV foi isolado a partir de crostas de caprinos naturalmente infectados (MAZUR & MACHADO, 1990). Estudos brasileiros mais recentes relatam a presença do vírus em ovelhas, no estado do Mato Grosso, onde também foi realizada a análise filogenética, demonstrando a diversidade das cepas que circulam no Brasil (ABRAHÃO et al., 2009).

Infecções pelo ORFV levam a importantes perdas econômicas nos rebanhos afetados, devido sua alta morbidade. Os cordeiros são os animais mais afetados, justamente pela ausência de imunidade ao vírus. Nesses animais, as lesões se localizam ao redor da boca e narinas que, além de predispor a infecções oportunistas, limitam a ingestão de alimentos, reduzindo o ganho de peso (HAIG & MERCER, 1998). Animais jovens são os mais acometidos e, embora a morbidade seja elevada, a mortalidade é baixa. Quando isso ocorre, pode-se atribuir à incapacidade de amamentação (devido às lesões) ou pelas infecções secundárias, por bactérias e parasitas (GUMBRELL & McGREGOR, 1997). Em animais adultos, as lesões são encontradas principalmente ao redor dos tetos, o que pode favorecer os quadros de mastite, aumentando as perdas (MAVROGIANNI et al., 2006). Assim como nos ovinos, as lesões em humanos são auto-limitantes e tendem a se resolver dentro de poucas semanas (HAIG & MERCER, 1998).

O ORFV penetra através de abrasões ou escarificações da pele, uma vez que a sua replicação ocorre nos queratinócitos basais. Por isso, o requerimento para a instalação da infecção viral não é a destruição da epiderme em si, mas a replicação nos queratinócitos associados ao processo de reparação da derme lesada (McKEEVER et al., 1988). Esse mecanismo de infecção explica o fato do ORFV não causar lesões na pele íntegra, ou após injeção intradérmica, intravenosa ou subcutânea (BOUGHTON & HARDY, 1934). O período de incubação da doença varia de 24 a 72 horas, sendo que inicialmente são observadas hiperemia leve e máculas. Dentro de 3 a 4 dias, as lesões evoluem a pápulas que, com o passar dos dias, transformam-se em vesículas, pústulas e crostas (ROBINSON & BALASSU, 1981). Na maioria dos casos, as lesões são agudas, restringindo-se à comissura labial, narinas e tetos dos animais (BOUGHTON & HARDY, 1934; TÓRTORA, 1987; HAIG & MERCER, 1998;

NÓBREGA Jr et al., 2008). Apesar dessas lesões serem geralmente auto-limitantes, já foram descritos casos de infecção crônica em caprinos (de la CONCHA-BERMEJILLO et al., 2003), lesões verrucosas atípicas na face e na pele dos membros de ovinos (YERUHAM et al., 2000), vesiculares na mucosa oral (McELROY & BASSETT, 2007) e proliferativas e crostosas na pele da cabeça, pescoço e flanco de caprinos (COATES & HOFF, 1990).

Até o presente não existe evidência da disseminação sistêmica do ORFV (HAIG, 2006). As lesões desenvolvidas na primeira infecção são severas, de caráter proliferativo e que tendem a se resolver espontaneamente dentro de seis semanas (HAIG & MERCER, 1998). Em casos de reinfecções, as lesões são evidentes, mas menos severas do que na infecção primária, e regridem em um período mais curto. Após a primeira infecção, a doença parece ocorrer periodicamente nesses rebanhos, provavelmente devido ao longo tempo de sobrevivência do vírus em crostas e nas pastagens (LIVINGSTON & HARDY, 1960), e à imunidade, que é de curta duração (FLEMING et al., 2007).

As alterações histológicas encontradas em surtos de ectima contagioso em ovinos e caprinos são caracterizadas por dermatite proliferativa, degeneração balonosa, acantose, vesículas e pústulas subcorneais, além de inclusões intracitoplasmáticas eosinofílicas em queratinócitos (TÓRTORA, 1987; COATES & HOFF, 1990; YERUHAM et al., 2000; CHAN et al., 2007; NÓBREGA Jr et al., 2008). Lesões semelhantes são encontradas em humanos, bovinos, caninos, felinos e animais silvestres infectados acidentalmente pelo ORFV (WILKINSON et al., 1970; TÓRTORA, 1987; GUO et al., 2004; AL-SALAM et al., 2008; FAIRLEY et al., 2008).

Quando a doença é endêmica nos rebanhos, opta-se pela vacinação anual dos animais jovens (ROBINSON & BALASSU, 1981). A imunização dos animais é realizada pela utilização de vacinas vivas, sendo obtida a partir de crostas de ovinos inoculados. Por essa razão, a vacinação coincide com a introdução do vírus no rebanho, o que pode ocasionar surtos da doença (TÓRTORA, 1987; HAIG & MERCER, 1998). O tratamento das lesões é feito a base de anti-sépticos, principalmente para combater infecções secundárias, embora existam fármacos capazes de controlar a replicação deste vírus, como os ésteres derivados de fosfatos de nucleosídeos acíclicos (DAL POZZO et al., 2007).

A resposta imune do hospedeiro à infecção pelo ORFV é principalmente celular, composta por linfócitos T $CD4^+$ e $CD8^+$, células dendríticas e neutrófilos adjacentes (HAIG & FLEMING, 1999; LLOYD et al., 2000). Anticorpos parecem ter menor importância no combate à infecção pelo ORFV em ovinos (HAIG et al., 1996). Da mesma forma, anticorpos

neutralizantes são indetectáveis ou presentes em baixos níveis em animais infectados (HAIG & MERCER, 1998).

O ORFV possui envelope, contém uma fita dupla de DNA linear como genoma, com aproximadamente 140kb, conteúdo médio de G + C de 64% e codifica aproximadamente 132 produtos (DELHON et al., 2004). Diversos genes codificam proteínas envolvidas no mecanismo de evasão do sistema imune do hospedeiro, como a proteína de resistência ao interféron (OVIFNR), o fator inibidor do fator estimulatório de colônia macrocítica e granulocítica (GIF), o fator de crescimento endotelial e vascular (VEGF-E), a proteína de ligação à heparina e citocinas, e a proteína ortóloga à interleucina-10 de mamíferos (vIL-10) (FLEMING et al., 1997; HAIG et al., 2002; DELHON et al., 2004; HAIG, 2006). Uma característica importante do ORFV é a sua capacidade de reinfectar e replicar repetidas vezes no seu hospedeiro (HAIG & FLEMING, 1999). Alguns fatores podem estar envolvidos neste evento, como o fato da infecção ser aguda e aparentemente restrita aos queratinócitos da epiderme, permitindo ao vírus uma replicação inicial anterior ao recrutamento dos mecanismos efetores da imunidade antiviral. A infecção viral pode não estimular uma resposta protetora; e o vírus pode ter desenvolvido mecanismos para subverter ou interferir com componentes do sistema imune. Dessa forma, a capacidade do ORFV de reinfectar o mesmo hospedeiro, embora produzindo lesões menos intensas quando comparadas à infecção primária, pode estar relacionada a esses mecanismos (HAIG et al., 1996).

Diversos aspectos da biologia complexa do ORFV ainda não são conhecidos, principalmente os mecanismos utilizados pelo vírus para evadir e modular a resposta imune à sua infecção (DELHON et al., 2004). Os mecanismos envolvidos na patogenia do ectima contagioso têm sido estudados por diferentes abordagens, incluindo a produção de cepas mutantes e recombinantes, deleção gênica e atenuação de cepas virais (FLEMING et al., 2007). No entanto, a aplicação prática de muitas pesquisas tem sido prejudicada pela falta de modelos animais para posterior reprodução da doença e estudos *in vivo*. Além disso, a utilização de ovinos e caprinos para estudos de patogenia é dificultada pela ampla distribuição do ORFV nos rebanhos, e pela conseqüente dificuldade de obter animais soronegativos (BARROS, 2007).

A doença já foi reproduzida em coelhos, mas com resultados controversos. A maioria dos pesquisadores falhou ao tentar infectar coelhos com o ORFV (AYNAUD, 1923, GLOVER, 1928, HOWARTH, 1929, BOUGHTON & HARDY, 1935 apud ABDUSSALAM, 1957; GREIG, 1956). Lesões leves foram observadas em coelhos inoculados com uma cepa de ORFV adicionada de hialuronidase, utilizada para aumentar a permeabilidade tecidual ao

vírus. Porém, neste trabalho não foram realizadas análises histológicas das lesões e não se observou a excreção viral (GREIG, 1956). Em outro estudo, coelhos inoculados com macerado de crostas de ovinos infectados pelo ORFV, após escarificação, apresentaram lesões eritematosas em um período de 20 dias. A análise histopatológica dessas lesões revelou hiperplasia da epiderme e infiltrado inflamatório, mas sem detecção de corpúsculos de inclusão, característicos da infecção por esse vírus (SELBIE, 1944). Resultados semelhantes foram observados em outros experimentos (WHEELER & CAWLEY, 1956; ABDUSSALAM, 1957) em que também não foram observadas lesões histológicas clássicas de ectima contagioso, nem mensurado o período de excreção viral das lesões.

Wheeler e Cawley (1956) observaram lesões máculo-papulares que surgiam 7 a 9 dias após a inoculação, e que duravam de 3 a 5 dias. Microscopicamente, apenas foram observados edema, dilatação vascular, infiltrado inflamatório leve e proliferação da derme. Abdussalam (1957) utilizou diversas diluições de uma suspensão de ORFV para inocular coelhos. Os animais foram inoculados após escarificação da pele, córnea, lábios, gengiva e língua. Somente a inoculação da pele resultou em lesões típicas de ectima contagioso, como hiperemia, máculas, pápulas, vesículas e crostas. No entanto, histologicamente não foram observados corpúsculos de inclusão e degeneração balonosa, que são lesões comumente encontradas em ovinos com ectima contagioso (McKEEVER et al., 1988).

Tentativas de infectar outras espécies animais também têm sido relatadas. Camundongos inoculados pela via intranasal e após escarificação da pele não apresentaram lesões (ABDUSSALAM, 1957). O mesmo ocorreu em camundongos e cobaias após inoculação de uma suspensão viral patogênica para coelhos (GREIG, 1956). Outros resultados negativos da inoculação nessas espécies foram observados por Blanc et al. (1923), Aynaud (1923), Jacotot (1926), Glover (1928), Blanc & Martin (1933) e Blaizot (1937) apud Abdussalam (1957).

Dessa forma, os objetivos do presente trabalho foram reproduzir a infecção e doença causada pelo ORFV em coelhos e camundongos, e caracterizar a infecção nos aspectos clínicos, virológicos e histopatológicos. Assim, abre-se a perspectiva de utilizar esses animais como modelos para estudos de patogenia com o ORFV.

2. CAPÍTULO 1

Virological and clinico-pathological features of orf virus infection in experimentally infected rabbits and mice

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Virological and clinico-pathological features of orf virus infection in experimentally infected rabbits and mice

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Running title: ORFV infection in animal models

Abstract

The biology of orf virus (ORFV) is complex and many aspects remain poorly understood. Experimental studies in the natural hosts have been partially impaired by the difficulty to obtain naïve animals. Attempts to establish animal models for ORFV infection have yielded conflicting and non-reproducible results. We herein describe the virological and clinico-pathological characterization of ORFV infection in rabbits and mice. A protocol of intradermal inoculation after skin scarification was employed to inoculate $10^{8.5}TCID_{50}$/mL of

ORFV strain IA-82 in the internal face of the ears, dorsal skin and labial commissures. All inoculated rabbits presented a similar clinical course, characterized by development of hyperemia or macules, papules/vesicles or pustules that eventually dried out originating scabs. Local signs started around days 3 and 4 post-inoculation (pi) and lasted 3 to 10 days. Infectious virus was recovered from lesions between days 2 and 14pi. Histological examination of lesions revealed focal proliferative dermatitis with ballooning degeneration and eosinophilic intracytoplasmic inclusion bodies in keratinocytes, histological hallmarks of contagious ecthyma in sheep. A similar, albeit much milder clinical course occurred in 5/10 inoculated mice; virus was recovered from lesions from three animals. Inoculated lambs – used as controls – developed severe lesions of contagious ecthyma from day 3 to 18pi; virus was isolated from lesions between days 2 to 19pi. VN tests performed at day 28pi failed to detect neutralizing antibodies in inoculated animals. Taken together, these results showed that rabbits are susceptible to ORFV infection and thus may be used to study to address selected aspects of ORFV biology.

Key words: contagious ecthyma, rabbits, mice, animal models, pathogenesis.

1. Introduction

Contagious ecthyma – also known as *orf*, contagious pustular stomatitis and contagious pustular dermatitis – is a cutaneous and debilitating disease naturally affecting sheep and goats (Haig and Mercer, 1998). A mild form of the disease has been occasionally described in wild ruminants (Vikoren et al., 2008) and in humans, in which is characterized by self-limiting, painful pustular lesions on the hands and fingers (Al-Salam et al., 2008). The disease is caused by orf virus (ORFV), a member of the family ***Poxviridae***, genus

Parapoxvirus (Haig and Mercer, 1998). ORFV infection in sheep and goats is associated with maculopapular, vesicular, pustular and proliferative lesions in the muco-cutaneous transition of lips and nose and affects mainly suckling lambs (McElroy and Bassett, 2007). The udder and teats of milking ewes can also be affected (Robinson and Balassu, 1981).

ORFV infection has a worldwide distribution and is endemic in most sheep and/or goat-raising countries (Robinson and Balassu, 1981). Severely affected animals – noticeably young lambs – dramatically reduce their food intake leading to transient growth impairment and, thus, to important economic losses to affected flocks (Haig and Mercer, 1998). In affected flocks, morbidity may reach nearly 100% of the young animals, yet the mortality is usually low and generally results from opportunistic parasitic or bacterial infections (Haig and Mercer, 1998; Robinson and Balassu, 1981). Udder lesions may also predispose lactating ewes to mastitis (Mavrogianni et al., 2006).

The infection usually starts through lesions or abrasions in the skin of muco-cutaneous junction of the lips, nose and oral mucosa (McElroy and Bassett, 2007). The infection evolves through multifocal erythema, papules, vesicles, pustules and scabs (Haig and Mercer, 1998). Proliferative and crostous lesions often dry out and bleed, predisposing to secondary infections and miiasis. The virus is shed to the environment through desquamated scabs, in which it may persist viable for months or even years (Robinson and Balassu, 1981). Virus transmission occurs through direct or indirect contact with fomites or contaminated grass/scabs. Grazing in abrasive grass facilitate viral penetration and infection in lambs (Munz and Dumbell, 1994).

The host immune response to ORFV has been extensively studied, yet many aspects of the complex host-virus interactions remain unclear. The short-term duration of the immunity and the resulting ability of the virus to reinfect its hosts are well documented whereas the immune mechanisms underlying these phenomena remain unclear (Fleming et al., 2007). As

demonstrated for a number of viruses, cell mediated immune response through specific CD4+ and CD8+ lymphocytes are believed to a play a pivotal role in protection against ORFV infection (Jenkinson et al. 1991, 1992 cited by Haig and Mercer, 1998; Lloyd et al., 2000). The role of the humoral response in preventing or reducing the severity of lesions upon reinfection is controversial (Fleming and Mercer, 2007). The ability of the virus to counteract specific host immune mechanisms has also been documented and may contribute to the evasion of the immune response (Haig, 2006).

The ORFV genome consists of a linear double stranded DNA molecule of approximately 140kb encoding around 132 gene products (Delhon et al., 2004). Most genes (around 80%) located in the central part of the genome are well conserved and are homologous to other poxvirus genes. These genes encode products involved in basic mechanisms of virus replication, structure and morphogenesis (Delhon et al., 2004; Mercer et al., 2006). In contrast, the terminal regions encode genes with no homology with other poxvirus sequences, whose products are presumably involved in determination of virulence, host range and evasion of the immune response (Delhon et al., 2004; Fleming et al., 1997; Mercer et al., 2006). Nevertheless, the biological function of many products of ORFV-unique genes remains unknown and, thus, awaits investigation. In particular, gene products involved in modulation of the immune response and evasion of host defenses are of special interest as they may provide new insights into the host-virus interactions (Delhon et al., 2004).

In depth studies on the function of specific gene products involved of the biology of ORFV have been somewhat impaired by the widespread distribution of the infection and, consequently, by the difficulty to find seronegative animals for experimentation. On the other hand, attempts to establish animal models for ORFV infection (e.g. guinea pigs, mice, rabbits) have yielded conflicting and poorly reproducible results reviewed by Boughton and Hardy (1934), Greig (1956) and Tórtora (1987). A few old articles and meeting abstracts reported

the reproduction of disease in rabbits upon ORFV inoculation, yet the protocols of virus inoculation, virological and clinico-pathological findings were only superficially described (Boughton and Hardy, 1934; Greig, 1956; Wheeler and Cawley, 1956). Other reports described the failure to reproduce the disease in rabbits upon experimental inoculation (Boughton and Hardy, 1934). In addition to studies on the biology of ORFV and pathogenesis of contagious ecthyma suitable animal models would also benefit vaccine and antiviral drug development and testing (Dal Pozzo et al., 2007; Pye, 1990).

Thus, the experiments described herein were designed to establish a protocol for ORFV inoculation in rabbits that would consistently reproduce the infection and disease; to provide a detailed description of the virological and clinico-pathological findings during acute infection and, thus, to evaluate the suitability of this species as animal model for ORFV. The susceptibility of mice to ORFV was also investigated.

2. Material and Methods

Rabbits and mice were inoculated with ORFV after scarification of the skin at different sites. Two lambs were inoculated in the labial commissure and in the internal face of the hind limbs and served as positive controls. Acute infection was monitored through daily clinical examination, by virus isolation and histological examination of skin fragments collected from lesions.

2.1 Cells and virus

Primary cultures of ovine turbinate cells (OTu) were used for virus amplification, quantification and isolation from lesions. Cells were cultivated in minimum essential medium (MEM), containing ampicillin (1.6mg/L), streptomycin (0.4mg/L) and nistatin (0.02mg/L)

supplemented with 10% fetal bovine serum. The ORFV samples used for animal inoculation were Iowa-82 strain (IA-82), kindly provided by Dr. Daniel Rock (College of Veterinary Medicine, University of Illinois at Urbana-Champaign, USA). Cell cultures and virus growth were performed at 37ºC with CO_2 at 5%.

2.2 Animals and virus inoculation

Ten weanling New Zealand rabbits (30 – 40 days-old), weighing approximately 300 – 400g; ten 30-days-old mice (*Mus musculus*) and two three-months-old Pollwarth lambs from a flock not experiencing cases of contagious ecthyma in the last 10 years were used for virus inoculation. Two animals of each species were inoculated with sterile MEM and used as controls. All the inoculations were performed after scarification of the skin with a hypodermic, sharp needle. In each site, the scarification consisted of three crossed lines, forming a six-pointed star or parallel lines. Following scarification, each site received 150µL of a virus suspension containing $10^{8.5}$ median tissue culture infectious doses ($TCID_{50}$/mL) of ORFV strain IA-82 (GenBank AY386263.1), passage 8 in culture cells, followed by a mild friction with a cotton swab to distribute the inoculum over the scarified line. The rabbits were inoculated in the internal face of the ears, in the dorsal skin (two sites per animal) and in the labial commissures. Mice were inoculated in the internal face of the ear and the lambs were inoculated in the labial commissures and in the internal face of hind limbs. In addition to control animals (inoculated with MEM), at least one site in each virus-inoculated animal (ear, dorsal skin, labial commissures) was inoculated with MEM to serve as an internal control.

The antibody response was evaluated by virus-neutralization assay (VN). Serum samples collected on the day of inoculation and at day 28 pi were submitted to standard VN assays, testing two fold dilutions of sera against a fixed dose of virus (100–200 $TCID_{50}$/well). OTu cells were used as indicators of virus replication.

Rabbits and mice were housed in cages with food and water *ad libitum;* lambs were kept in native grass with water *ad libitum*. Virus inoculated and control animals were kept in separate cages/pastures to avoid possible cross-contamination. All procedures of animal handling and experimentation were performed under veterinary supervision and according to recommendations by the Brazilian Committee on Animal Experimentation (COBEA, law # 6.638 of May, 8th, 1979). The experiments were approved by an institutional committee on Ethics on Animal Welfare and Experimentation (UFSM – Comitê de Ética e Experimentação Animal: process # 23081.002171/2009-62 and assent 11/2009).

2.3 Virological and clinico-pathological monitoring

The inoculated animals were monitored daily during three weeks following virus inoculation. Clinical monitoring was performed by a detailed and criterious examination of each inoculation site. Any perceptible visual change in the inoculation sites and vicinities was recorded in individual forms. After each daily clinical examination, swabs were collected from inoculation sites regardless the presence of lesions. The swabs were drained, vortexed, submitted to low speed centrifugation (300 x g for 5 min) and the supernatants were inoculated onto preformed monolayers of OTu cells. Inoculated cells were monitored for CPE during three passages of five days each. Skin biopsies of inoculation sites were performed in selected animals at determined intervals following virus inoculation (Table 1). Tissue fragments were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxilin and eosin (H&E) and submitted to microscopic examination according to routine protocols.

3. Results

3.1 ORFV infection in rabbits

Inoculation of ORFV strain IA-82 after scarification of the skin resulted in local lesions in all inoculated rabbits, regardless the site of inoculation (Table 1, Figures 1 and 2). In general, local diffuse hyperemia or macules at or nearby the inoculation sites were first observed between days 3 and 5pi. In a few animals, these signs appeared later (6 or 7pi). The peak of clinical signs occurred around day 6 to 8pi (Figure 1A), from which the signs gradually subsided up to day 12 - 13pi. The progression of the clinical course was similar in most animals and was characterized by progressive and consecutive appearance of diffuse or focal hyperemia at or around the inoculation sites, macules, papules, small vesicles, pustules and scabs (Figures 1A and B; 2 A-D). The time of appearance and the severity of most signs were roughly similar in the three inoculation sites, yet the development of pustules was manly observed in the labial commissures (Figure 2C). The overall duration of signs in the dorsal skin (x: 5 days) was slightly shorter than in the ears (x: 7.3) and labial commissures (7.0 days). In general, the vesiculo-pustular progression of the lesions was more evident and pronounced in the labial commissures than in the other sites (Figure 2A-D). The crusts and scabs in these sites were also more pronounced (Figure 2D). In the sites inoculated with MEM after scarification (control animals and contra-lateral sites of inoculated ones), a mild hyperemia along the scarified lines was observed for 1 to 2 days pi, followed by formation of dry scabs along the lines (not shown).

Infectious virus was recovered from lesions between days 2 and 14pi, with most lesions yielding virus from day 3 to 11pi (Table 1). The mean duration of virus shedding ranged between 7.8 days (labial commissures), 8.0 days (ears) to 8.6 days (dorsal skin).

Biopsies of skin lesions for histological examination were performed at day 5pi (papule, dorsal skin), day 6pi (papule and pustule from dorsal skin, vesicle from ear and labial commissure) at day 7pi (vesicle from ear skin, pustule and scab from labial commissure) and

at day 8pi (scab from labial commissure) (Table 1). Histological examination revealed the typical progression of cutaneous ORFV lesions (Figure 3A-C). Papules were observed at days 5 and 6pi and were microscopically characterized by focal acanthosis with a mild dermal lymphoplasmacytic inflammatory infiltrate. Vesicles (days 6 and 7pi) and scab (days 7 and 8pi) were histologically composed by moderate acanthosis with severe ballooning degeneration and moderate heterophylic, lymphoplasmacytic and histiocytic dermatitis (Figure 3A). Pustules were also found at day 6 and 7pi and were microscopically characterized as a focal hyperplasia with severe heterophylic and lymphoplasmocytic dermatitis. Several eosinophilic intracytoplasmic inclusion bodies were observed in keratinocytes of some lesions, mostly of the labial commissures (Figure 3C). The VN was unable to detect neutralizing antibodies in any animal at 28 days pi.

3.2 ORFV infection in mice

The skin signs developed by inoculated mice were less consistent (5 out of 10 animals) and were noticeably milder and of shorter duration than those produced in rabbits. Focal hyperemia, macules, papules, a few vesicles and small scabs were being progressively observed between days 5 and 12pi (Table 1, Figure 4). Virus isolation was achieved from three animals, in one swab collection (between days 8 and 10pi). Histological examination of papules (biopsy performed at day 6pi) and vesicles (day 8pi) from the inoculated ear revealed multifocal epidermal erosions, mild neutrophilic and lymphoplasmacytic inflammatory infiltrate and moderate dermal hemorrhage (not shown). Control animals and sites inoculated with MEM developed a transient hyperemia and a prompt development of thin dry scabs over the scarified lines. VN assays performed with sera collected at day 28pi yielded negative results.

3.3 ORFV infection in lambs

The two inoculated lambs developed the classical sequence of ORFV-induced lesions: hyperemic foci, macules, papules, vesicles, pustules that dried out and gave place to firmly attached scabs. The signs first appeared around day 3pi, peaked in severity between days 10 and 14dpi and spontaneously regressed by day 19pi. Virus isolation from the lesions was achieved between days 2 to 19pi (Table 1). Histological examination performed in a tissue fragment collected at day 12pi of labial commissure revealed severe acanthosis and hyperkeratosis, accompanied by ballooning degeneration, dyskeratosis, subcorneal pustules and several eosinophilic intracytoplasmic inclusion bodies. A marked dermal lymphoplasmacytic and histiocytic inflammatory infiltrate was also observed (not shown).

None of the control animals developed similar clinical signs. The scarifications regressed promptly within two to three days after the procedure. Likewise, no infectious virus was recovered from the sites of scarification in the control animals. VN assays performed with sera collected at day 28pi yielded negative results.

4. Discussion

We herein describe the consistent reproduction of acute maculopapular, vesicular and pustular lesions in the skin and labial commissure of rabbits through intradermal inoculation of ORFV strain IA-82. The local signs lasted several days and were accompanied by virus shedding during most of the clinical course. Histological examination of lesions revealed chronic inflammatory reaction and mild proliferative dermatitis, resembling some features of ORFV-induced histopathology in sheep. Ballooning degeneration and intracytoplasmic eosinophilic inclusion bodies in keratinocytes – which are histological hallmarks of contagious ecthyma in sheep and goats – are being described for the first time in rabbits.

Previous attempts to reproduce ORFV infection and disease in rabbits have yielded conflicting and barely reproducible results (reviewed in Robinson and Balassu, 1981 and Tórtora, 1987). Thus, the present report provides a detailed description of the virological and clinico-pathological features of ORFV infection in experimentally infected rabbits.

We also describe the reproduction of mild erythematous and vesicular in mice, accompanied by virus isolation from lesions, supporting the concept that mice can also harbor ORFV replication and eventually develop skin lesions. To our knowledge, this appears to be the first successful reproduction of ORFV infection and lesions in mice.

The motivation behind these experiments was a search for an animal model in which to study selected aspects of ORFV biology. Although the disease herein described in inoculated rabbits was less severe and of shorter duration than in sheep – and the histological changes were less pronounced (Abdussalam, 1957; Wheeler and Cawley, 1956) –, these results keep the way open for further studies on ORFV in rabbits. Unfortunately, the low susceptibility of mice is detrimental to their use as animal model for ORFV.

The varied ability among ORFV strains to replicate and produce lesions in rabbits has been well illustrated in several articles reporting either the success or failure in reproducing the infection and disease. Most early attempts to reproduce ORFV infection in rabbits failed (Aynaud, 1923, Glover, 1928 and Howarth, 1929 cited by Abdussalam, 1957; Jacotot, 1926 and Lanfranchi, 1925 cited by Selbie, 1944), while a few others were successful in producing virus replication and mild local signs (Abdussalam, 1957; Greig, 1956). In these later cases, the rabbit infection was reported as a mild skin disorder, lacking some important clinico-pathological features of the disease in the natural hosts. Upon intradermal inoculation of an ORFV strain amplified in tissue culture (Selbie, 1944), described the appearance of small hyperemic spots (maculopapules) subsequently covered by an yellowish-white scab. The author claimed they successfully performed 19 passages of the virus in rabbits. Greig (1956)

was able to reproduce mild and transient skin signs (tiny hyperemic spots, small macules) by inoculating rabbits with two field viruses obtained directly from sheep scabs (strains II and III), but not with one field virus (strain I). The three strains, however, showed a similar virulence in lambs. Previously, other authors (Bennet et al., 1944 cited by Greig, 1956) were also able to produce mild to moderate lesions in the skin of inoculated rabbits (hyperemic spots, skin eruptions, pustules, crusts) and, in one occasion, it was possible to recover the virus from lesions and transmit it to other animals (Blanc, 1933 cited by Greig, 1956). Wheeler and Cawley (1956) observed tiny maculopapules in the skin of inoculated rabbits, with an incubation period of 7 to 9 days and lasting 3 to 5 days. The authors were able to recover the virus and to reproduce lesions upon inoculation in lambs. Abdussalam (1957) also reported a successful reproduction of mild lesions in rabbits and the ability to perform multiple passages of the virus in this host.

Taken together, these conflicting results and our findings allow us to speculate that the choice of the viral strain, the virus titer in the inoculums, the procedure/site of inoculation – in addition to the genetic background and age of experimental animals – are critical determinants of the outcome of the inoculation of rabbits (and perhaps in mice) (Abdussalam, 1957; Tórtora, 1987). The inadequate combination of some of these factors would probably explain a number of conflicting and barely reproducible results of experimental inoculations of ORFV in rabbits and the consistent failure to reproduce the infection and pathology in mice (Tórtora, 1987). Likewise, particular combinations of some of these factors would explain the variations in morbidity, incubation period, severity and duration of local signs, gross and microscopic appearance of lesions described upon ORFV inoculation in rabbits (Abdussalam, 1957; Tórtora, 1987).

The right choice of the virus strain and the ability to grow the virus to high titers in tissue culture – in spite of possible, undesirable attenuation – would increase the chance of

achieving positive results (Abdussalam, 1957). In a pilot experiment, we were unable to produce lesions in mice and produced very mild signs in rabbits upon inoculation of a suspension of scabs obtained from an outbreak of contagious ecthyma. It is possible that the virus strain and/or the low virus titer in the scab suspension contributed for this partial failure in reproducing the infection. In a subsequent attempt, very mild and transient maculo-papular and vesicular lesions were produced in rabbits upon inoculation of a vaccine ORFV strain (Cargnelutti, J.F., unpublished). Taken together, these results suggest that the combination of an adequate virus strain (IA-82), inoculated at a high titer through the right procedure and route was probably determinant in producing efficient virus replication and pathology.

Inoculation of ORFV IA-82 in the labial commissures – an inoculation site not described in previous reports – was particularly efficient in producing marked local lesions which resulted in the formation of large, unique pustules that eventually ruptured and dried out giving place to scabs (Table 1, Figure 2). The consistent development of pustular, severe lesions in this site likely reflects the predilection of the virus for the muco-cutaneous transition of the lips observed in natural infections of sheep and goats (Haig and Mercer, 1998; Nóbrega Jr et al., 2008; Tórtora, 1987). Thus, the labial commissure should be especially targeted when aiming at reproducing lesions upon ORFV inoculation in rabbits.

Neutralizing antibodies were not detected by VN using serum samples collected on days 0 and 28 pi. ORFV-infected animals could have produced low or undetectable neutralizing antibody titers in this study (Haig and Mercer, 1998).

An interesting and unique finding in our experiments was the reproduction of some typical microscopic features of ORFV pathology in sheep, e.g. proliferative dermatitis, ballooning degeneration and cytoplasmic, eosinophilic inclusion bodies in keratinocytes. The few histological descriptions of the microscopic appearance of ORFV pathology in rabbits

usually referred to mild chronic inflammatory changes in the dermis. These descriptions emphasized the absence of ballooning degeneration, proliferative aspect or inclusion bodies (Abdussalam, 1957; Selbie, 1944; Wheeler and Cawley, 1956), which are typical microscopic findings accompanying natural and experimental ORFV disease in sheep (de la Concha-Bermejillo et al., 2003; Nóbrega Jr et al., 2008; Robinson and Balassu, 1981; Tórtora, 1987; Wheeler and Cawley, 1956). The development of these histological changes in the inoculated rabbits likely reflects the degree of virus replication, the extent and severity of tissue pathology derived thereof.

In summary, intradermal inoculation of rabbits with a high titer of ORFV strain IA-82 resulted in virus replication and the development of a clinical course somewhat resembling the appearance and progression of lesions presented by sheep naturally or experimentally infected with ORFV. Some microscopic changes resembling the histological features of contagious ecthyma, yet less prominent, were also observed in rabbit tissues. Although the clinico-pathological changes were noticeably less pronounced than in sheep, these results are promising towards the use of rabbits to address selected aspects of ORFV biology.

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Table 1 – Clinical and virological findings following intradermal inoculation of rabbits, mice and lambs with ORFV strain IA-82.

Animal	Clinical signs[a] (days pi)			Virus shedding (days pi)		
	Ear	Labial commissure	Dorsal skin (rabbits) Limb skin (lambs)	Ear	Labial commissure	Dorsal skin (rabbits) Limb skin (lambs)
Rabbit						
1	h, m, p, v, s (4-12)	h,m,p,v,pu***,s (4-11)	h,m,p,s (5-10)	2-9	4-11	5-13
2	m, v, s (5-10)	h,m,p,v,s*** (4-12)	h,p,s (7-10)	3-7	2-11	7-11
3	m, v, s (4-12)	h,p,pu,s**** (6-9)	h,s (6-11)	4-11	6-10	6-11
4	m,p (5-8)	h,v,s (4-11)	m,pu**,s (4-10)	7-12	8-11	3-11
5	h,m,p,v,s (5-13)	h,m,p,v,pu,s (3-11)	h,m,p,s (3-8)	2-14	2-11	3-14
6	h,m,p,v**,s (5-11)	m,v,s (5-11)	h,m,p*,v,s (3-11)	3-10	2-14	3-11
7	h,m,p,s (3-11)	h,p,v**,s (3-11)	m,p,v,s (3-10)	3-13	3-9	3-12
8	h,m,p,v,s (3-10)	h,p,v,pu,s (5-13)	m,p (3-6)	3-13	2-12	3-14
9	h,m,p,v***,s (3-13)	h,m,p,v,pu,s (3-12)	h,m (5-8)	3-11	2-11	2-14
10	h,m,p,s (3-13)	h,m,p,s (3-13)	h,m,p**,s (3-7)	4-14	2-10	3-12
Mouse						
1	h,m (5-9)	ni	ni	8	ni	ni
2	h,m (5-9)	ni	ni	10	ni	ni
3	m,p**,v,s (6-8)	ni	ni	9	ni	ni
4	-[b]	ni	ni	-	ni	ni
5	-	ni	ni	-	ni	ni
6	h,m,s (5-12)	ni	ni	-	ni	ni
7	-	ni	ni	-	ni	ni
8	-	ni	ni	-	ni	ni
9	h,p,v**** (5-6)	ni	ni	-	ni	ni
10	-	ni	ni	-	ni	ni
Lamb						
23	ni[c]	m, p,v,pu,s***** (3-18)	m,p (5-7)	ni	2-16	10
24	ni	m, p,v,pu,s***** (3-18)	m, p,v,pu,s (4-16)	ni	2-19	5-18

[a] Clinical signs: h- hyperemia, m-macule, p-papule, v-vesicle, pu-pustule, s-scab,; [b] –: negative, [c]ni: not infected
*Biopsy 5dpi; **Biopsy 6dpi; ***Biopsy 7dpi; ****Biopsy 8dpi; *****Biopsy 12dpi.

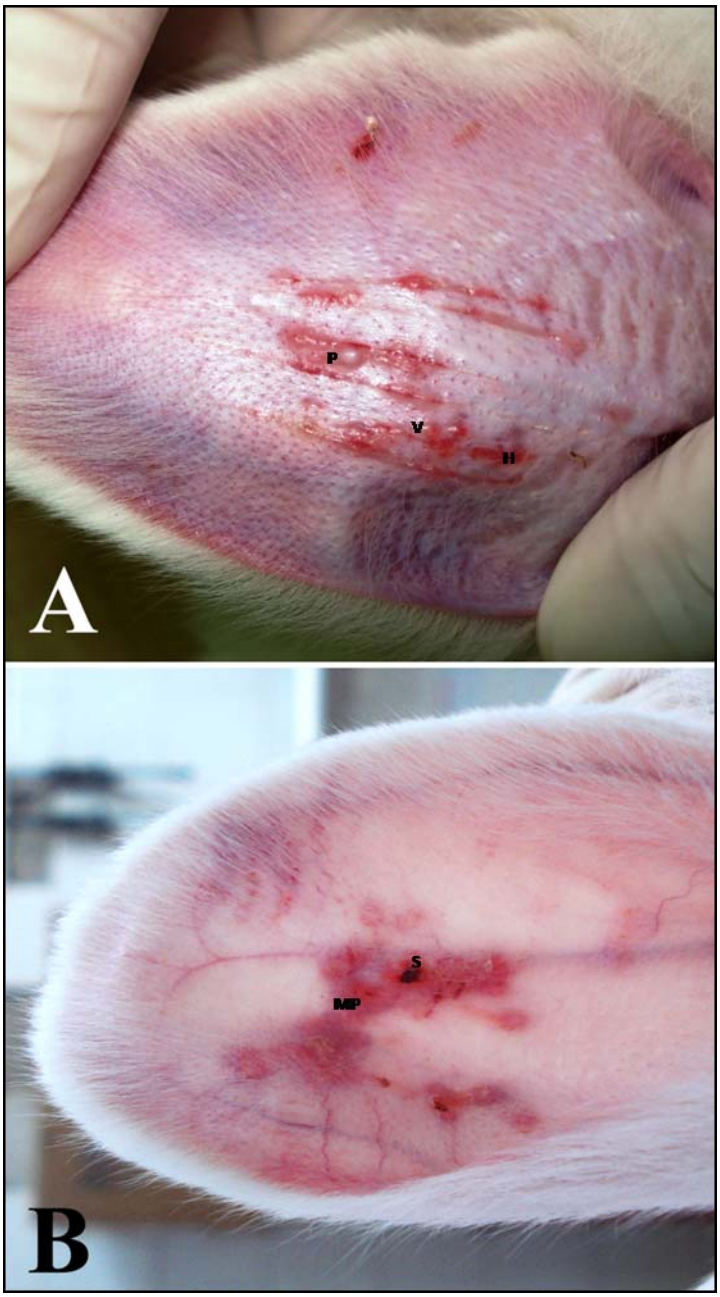

Figure 1. Lesions developed by rabbits inoculated with ORFV IA-82 in the ears. A. Rabbit 6, 7dpi: Hyperemia (H), vesicles (V) and pustules (P); B. Rabbit 8, 9dpi: macule-papular region (MP) and scabs (S).

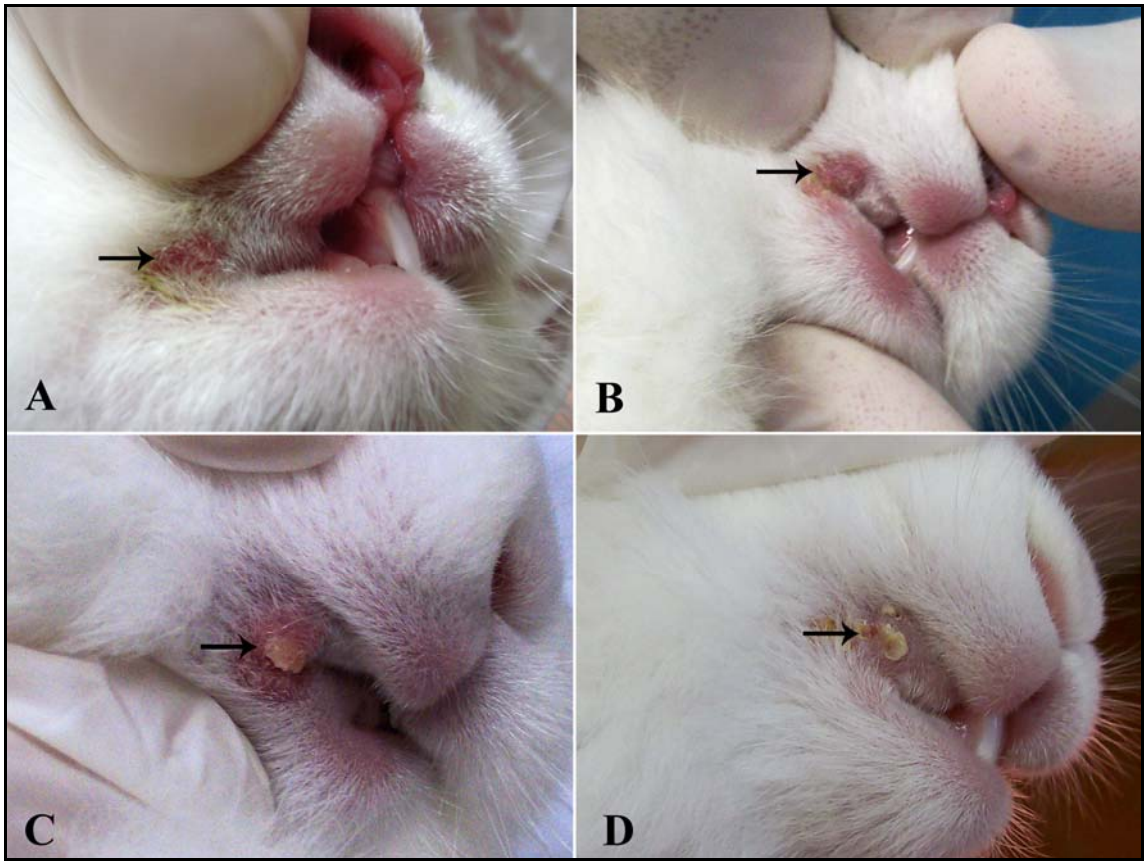

Figure 2. Progression of lesions in rabbits inoculated with ORFV IA-82 in the labial commissure. A. Rabbit 1, 3dpi: macule (arrow); B. Rabbit 2, 5dpi: papule (arrow); C. Rabbit 5, 7dpi: ulcerated pustule (arrow) surrounded by hyperemia; D. Rabbit 3, 8dpi: detaching scab (arrow).

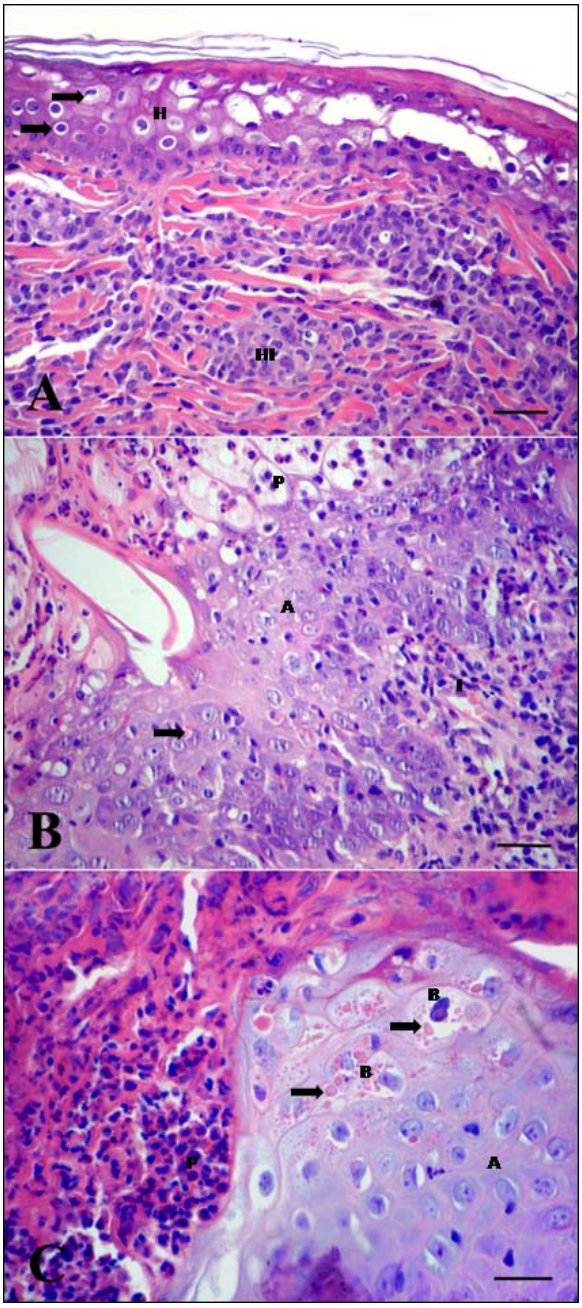

Figure 3. Muco-cutaneous section of the labial commissure of rabbits inoculated with ORFV IA-82. A. Rabbit 7, 6dpi. Moderate epidermal hyperplasia (H) with severe hydropic and ballooning degeneration (arrow). The dermis is infiltrated by a large number of histiocytes and a few neutrophils (N). B. Rabbit 2, 7dpi. Severe acanthosis (A) with subcorneal pustule formation (P), neutrophilic exocytosis and a few intracytoplasmic eosinophilic corpuscules (arrow). The dermis is severily infiltrate (I) by neutrophils, histiocytes and a few lymphocytes and plasmocytes. C. Rabbit 3, 8dpi. Severe acanthosis (A) with an extensive subcorneal pustule (P) and severe ballooning degeneration of keratinocytes (B). There are myriads of intracytoplasmic eosinophilic corpuscules inside degenerated keratinocytes (arrow) (Haematoxylin and eosin, bar 50µm).

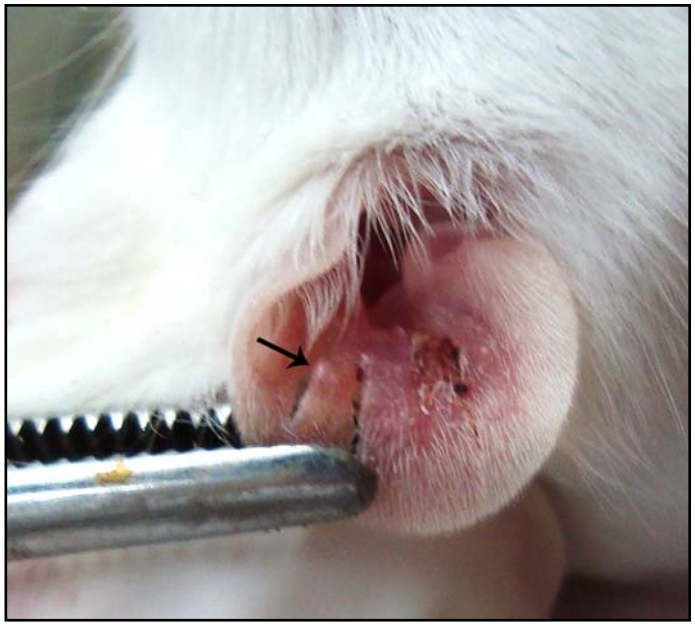

Figure 4. Lesions developed by a mouse inoculated with ORFV IA-82 in the ears. Mouse 3, 6dpi: vesicle (arrow) and scabs.

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