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Bovine vaccinia, a systemic infection: Evidence of fecal shedding, viremia and detection in lymphoid organs

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ABSTRACT

Bovine vaccinia (BV) is a zoonosis caused by Vaccinia virus (VACV) that affects dairy cattle and milkers, causing economic losses and impacting animal and human health. Based on the clinical presentation, BV appears to be a localized disease, with lesions restricted to the skin of affected individuals. However, there are no studies on the pathogenesis of the disease in cows to determine if there is a systemic spread of the virus and if there are different ways of VACV shedding. The objective of this work was to study if there is a systemic spread of VACV in experimentally infected cows and to study the kinetics of VACV circulation in the blood and shedding in the feces of these animals. To this end, eight crossbred lactating cows were used. Three teats of each cow were inoculated with the GP2V strain of VACV. All animals were monitored daily, and blood and fecal samples were collected for 67 days post-infection (dpi). After this period, four of these previously infected cows were immunosuppressed using dexamethasone. Viral DNA was continuously detected and quantified in the blood and feces of these animals in an intermittent way, even after the resolution of the lesions. At slaughter, tissues were collected, and viral DNA was detected and quantified in the mesenteric and retromammary lymph nodes, ileum, spleen and liver. The detection of VACV DNA in the feces for a longer period (67 dpi) and in the lymphatic organs provides new evidence about VACV elimination and suggests that BV could be a systemic infection with a chronic course and viral shedding through the feces.

1. Introduction

Vaccinia virus (VACV) is the etiological agent of an important zoonotic disease, bovine vaccinia (BV), which affects dairy cattle and humans in Brazil. VACV strains have been isolated since the 1960s in different Brazilian regions. VACV is epitheliotrophic and causes exanthematic lesions on the teats and sometimes the udders of lactating cows. The disease is transmitted to humans *via* direct contact with sick cows during milking, which is why BV is classified as an occupational zoonosis. In humans, the lesions are similar to those described in cattle, and they often occur on the hands (Damaso et al., 2000; De Souza Trindade et al., 2003; Lobato et al., 2005). Several animal models have been proposed to study the pathogenesis of orthopoxvirus (OPV) to understand the infections caused by VACV (Esteban and Buller, 2005). Currently, some aspects of the pathogenesis of Brazilian VACV strains isolated from outbreaks of BV have been studied in mice and rabbits (Ferreira et al., 2008a; Campos et al., 2011; Cargnelutti et al., 2012). In a study in which Balb/c mice were intranasally inoculated with a Brazilian VACV strain

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isolated from an outbreak of BV, the viral DNA could be detected in fecal, urine and saliva samples (Ferreira et al., 2008b). Using rabbits inoculated intranasally with a VACV strain isolated from horses with skin lesions, a study showed that VACV DNA could be detected in nasal secretions and feces (Cargnelutti et al., 2012).

Whether there are other ways that VACV is transmitted among bovines and humans in addition to direct contact is not known. One way to better understand VACV transmission pathways is to study the possible excretion routes. Studies have demonstrated that VACV DNA and infectious viral particles were detected in the milk of naturally infected cows (Abrahão et al., 2009a; Megid et al., 2012), although in both cases, determining if the virus in the milk was the result of a systemic infection or scab fragmentation during teat manipulation was not possible. The purpose of this study arose from the difficulty of conducting studies in naturally infected animals, especially when collecting samples at the early stages of the disease is necessary. Usually, an outbreak of BV is reported only when many animals and even humans are already affected. When a research team arrives at the farm, often the lesions are already in the healing phase, which makes monitoring the early stages of the disease, when viral shedding may be occurring, very difficult. This work aimed to experimentally infect dairy cows with a Brazilian VACV strain to obtain information about the pathogenesis of BV, such as the virus distribution in tissues and viral shedding, from the moment of infection and during the acute and convalescent phases of the disease.

2. Materials and methods

2.1. Animal housing and husbandry

Eight adult, cross-bred cows were housed in microbiologically secure (NB-2) animal pens at the National Agricultural Laboratory – Ministry of Agriculture, Livestock and Food Supply in Pedro Leopoldo, Minas Gerais, Brazil. The animals were fed a complete balanced diet and water *ad libitum* and all experimental procedures were approved by the Committee of the Ethics in Animal Experimentation, Federal University of Minas Gerais: process 167/2009.

2.2. Virus inoculation

The Brazilian VACV strain used in this study was the Guarani P2 virus (GP2V) isolated from a cow with characteristic BV lesions in southeastern Brazil (Trindade et al., 2006). The animals were inoculated intradermally in the teats, which were previously scarified with sand paper (number 4, JX-41, Lixas Doble A, Argentina), with 50 μl of inoculum per teat, containing 10^6 plaque-forming units (PFU) of GP2V. The left posterior teat was scarified but not inoculated and was used as a negative control.

2.3. Experimental design and sample collection

The eight cows were monitored daily, and blood and fecal samples were collected for 67 days post infection (dpi), daily during the first 15 dpi and on alternate days until the 67th dpi. After this period, four of these previously infected animals were submitted to an immunosuppressive treatment by the intramuscular administration of 0.1 mg of dexamethasone/kg bodyweight for five consecutive days (Iketani et al., 2002) to analyze possible changes in the shedding profile.

In the immunosuppressed animals, the samples were collected daily from the 69th dpi until the 81st dpi. Two aliquots of each clinical specimen were made for molecular tests and viral isolation.

At slaughter, the following tissues were collected: tonsil, mesenteric lymph nodes, ileum, spleen, retromammary lymph nodes, mammary glands and teats. Two fragments were removed from each tissue. One fragment was fixed in buffered 10% formalin for further analysis by immunohistochemistry, and the other fragment of the same tissue was stored at −70 °C for PCR and viral isolation.

2.4. Molecular assays: nested and real-time PCR

Blood, feces and tissues were submitted to DNA extraction using phenol:chloroform:isoamyl alcohol (25:24:1) (Sambrook et al., 1989). Then, the extracted samples were submitted to nested and real-time PCRs. The nested PCR amplified a target sequence of the VACV vaccinia growth factor gene (vgf). The reaction was designed to amplify a target region of 381 bp in the first reaction and then amplify a product of 181 bp in the second reaction (Abrahão et al., 2009b). Electrophoresis of the PCR products was performed on 8% polyacrylamide gel, which was then silver stained. Sterile ultrapure water and DNA extracted from feces of uninfected cattle were used as negative controls.

The samples were also tested in real-time PCR and quantified using a relative standard curve. Forward and reverse primers targeting the orthopoxvirus A56R gene (hemagglutinin) were used for VACV DNA quantification (Trindade et al., 2008). Exogenous control of the PCR reactions was used to quantify the B2L gene of parapoxvirus (Nitsche et al., 2006). Prior to the extraction of DNA, 100 ng of a pGEM-T plasmid containing the cloned B2L gene was added to all samples. VACV DNA (100 ng) was used as a positive control for both tests. Sterile ultrapure water and DNA extracted from feces of uninfected cattle were used as negative controls.

2.5. Viral isolation and identification

The isolation of infectious viruses was attempted using PCR-positive blood, fecal and tissue samples (diluted 1:10 [v/v] in phosphate-buffered saline). All diluted samples were inoculated in duplicate onto 80–90% confluent BSC-40 cell monolayers (ATCC, CRL-2761 Maryland, USA) grown on 6-well plates (Greiner Bio-one; Cellstar, Germany). The development of the VACV cytopathic effect (CPE) on the BSC-40 cells was monitored for a period of 72 h. The identification of viral CPE samples was performed using the immunoperoxidase method, in which the monolayer was incubated with primary antibody