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Short communication: Survival of *Vaccinia virus* in inoculated cheeses during 60-day ripening

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ABSTRACT

Bovine vaccinia is a neglected zoonosis caused by *Vaccinia virus* (VACV) and has a major economic and public health effect in Brazil. Previous studies showed infectious VACV particles in milk from either experimentally or naturally infected cows and in fresh cheeses prepared with experimentally contaminated milk. Ripening is a process that leads to major changes in the physical and chemical characteristics of cheese, reducing contamination by spoilage, pathogenic microorganisms, or both. However, it is not known if VACV infectious particles persist after the ripening process. To investigate this issue, viral infectivity at different ripening times was studied in cheeses manufactured with milk experimentally contaminated with VACV strain Guarani P2 (GP2). Cheeses were analyzed at 1, 7, 14, 21, 45, and 60 d of ripening at 25°C. Viral DNA was quantified by real-time PCR, and VACV isolation and titration were performed in Vero cells. The whole experiment was repeated 4 times. Analysis of the mean viral DNA quantification and infectivity indicated a reduction of approximately 2 logs along the ripening process; however, infectious viral particles (1.7×10^2 pfu/mL) could still be recovered at d 60 of ripening. These findings indicate that the ripening process reduces VACV infectivity, but it was not able to inactivate completely the viral particles after 60 d.
Key words: cheese ripening, raw milk inoculated, *Vaccinia virus*, viral infectivity

Short Communication

Bovine vaccinia (**BV**) is a neglected zoonotic disease caused by *Vaccinia virus* (**VACV**), *Orthopoxvirus* (**OPXV**) genus. Bovine vaccinia is an important disease in Brazil, due mainly to its implications in public health and economic losses in the dairy chain (Lobato et al., 2005). *Vaccinia virus* infectious viral particles were detected in raw milk from naturally and experimentally infected cows (Abrahão et al., 2009; de Oliveira et al., 2015) and in fresh cheeses produced with experimentally contaminated raw milk (de Oliveira et al., 2010). The production of artisanal cheeses with raw milk is a traditional activity in Brazil and artisanal cheese is a very important product for the local economy. However, the use of raw milk poses a potential risk because it may be contaminated by infectious agents (Machado et al., 2004). These previous data are alarming because they suggest the possibility that contaminated milk and artisanal cheeses may be a source of VACV transmission to humans by handling or consuming these products. Therefore, the aim of this study was to produce cheese with experimentally VACV-inoculated raw milk and analyzed the viral infectivity during the 60-d ripening process.

Twenty-four liters of raw milk was used for cheese production. Twelve liters was inoculated for a final concentration of 10^5 pfu of VACV per milliliter (de Oliveira et al., 2010). The other 12 L of raw milk was used to produce the noncontaminated control cheeses. Each cheese was manufactured using 2 L of raw milk with the addition of 2% commercial natural yogurt from Nestlé (Ibia, Minas Gerais State, Brazil) as a source for fermenting microorganisms. Additionally, for enzymatic coagulation, 0.08% (vol/vol) of commercial liquid rennet HA-LA (Chr. Hansen SA, São Paulo, Brazil) was added. The cheeses, weighing on average 250 g, were removed from the molds after 24 h, and they were stored in a biochemical oxygen demand incubator at 25°C for up to 60 d. The cheeses were produced in a laboratory scale, in a way similar to the process of Minas cheese manufacturing. They were ripened for 60 d due to the requirement of the Brazilian legislation that establishes a minimum of 60 d of ripening for cheeses made of

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raw milk. The ripening temperature was 25°C to mimic the conditions in which artisanal cheeses are ripened. Six contaminated and 6 noncontaminated cheeses were produced. One cheese was randomly selected at 1, 7, 14, 21, 45, and 60 d of ripening. Additionally, during the cheese production process, contaminated and noncontaminated milk, whey, and curd samples were collected. To improve the repeatability of the viral infectivity in ripened cheese at different ripening times, the experiment was repeated 4 times, totaling 4 cheeses per ripening time. Noncontaminated and contaminated cheeses were collected randomly at each established ripening time (1, 7, 14, 21, 45, and 60 d of ripening). The samples were macerated, diluted 1:10 in PBS, and centrifuged. The supernatant fluids were collected and stored at −80°C until being submitted for real-time PCR (de Oliveira et al., 2015) and viral titration in Vero (*Cercopithecus aethiops*, ATCC: CCL-81) cells (de Oliveira et al., 2010). The effect of ripening times on the viral titration and DNA quantification was tested by a simple or quadratic linear regression model using orthogonal polynomials.

Cheese samples from the d 1 to 60 of ripening were infective when inoculated in Vero cell monolayers. Milk, curd, and whey, products from the cheese manufacturing process, were also infective ($P \leq 0.05$; Table 1). The DNA quantification during all the ripening period did not show a significant difference ($P > 0.05$). However, a decreasing tendency in viral DNA during cheese ripening was verified (Table 2).

This work showed that VACV remains viable in the cheese for at least 60 d of ripening at 25°C. Moreover, the real-time PCR technique showed a decrease in viral DNA, maybe due to degradation/denaturation, during the ripening process. Viral DNA to degradation/denaturation could have been caused by proteolytic enzymes produced during the cheese ripening process. Two factors may explain this viral vulnerability to degradation/denaturation during ripening. The first factor may be related to the presence of many intracellular mature virion particles in the inoculum. These viral particles have an outer lipoprotein membrane (Moss, 2013), which may have been denatured by lipolysis and proteolysis processes that occur during ripening.. The second factor may be that VACV was not associated with milk cells as it was inoculated directly in milk, contributing to the viral particle degradation during ripening. The association of VACV with milk somatic cells of experimentally infected dairy cows was suggested by de Oliveira et al. (2015). The VACV associated with cells could be protected in contaminated commercial artisanal cheeses and could remain viable in cheeses for a longer time, increasing the risk of viral transmission after consumption of these products.

Table 1. Mean value of *Vaccinia virus* (VACV) strain Guarani P2 (GP2) titer (pfu/mL or pfu/g) in experimentally contaminated milk, whey, curd, and cheese samples, ripened for 60 d at 25°C

Item	Milk	Whey	Curd	Ripening time[1]							*P*-value[2]		
				C1	C7	C14	C21	C45	C60		Days	L	Q
Mean value of VACV-GP2 titer (pfu/mL or pfu/g)	1.0×10^5	2.2×10^3	8.7×10^4	1.6×10^4 (±0.076)	1.8×10^3 (±0.037)	2.9×10^3 (±0.044)	9.3×10^2 (±0.37)	2.3×10^2 (±0.112)	1.7×10^2 (±0.076)		<0.0001	<0.0001	0.103

[1]C1, C7, C14, C21, C45, and C60 represent cheese samples analyzed during ripening up to 60 d at 25°C. Intervals represent SEM for each day of ripening.
[2]L = linear effect; Q = quadratic effect.