Ciência Rural, Santa Maria, v.48:05, e20170723, 2018 http://dx.doi.org/10.1590/0103-8478cr20170723
ISSNe 1678-4596
MICROBIOLOGY

Bovine Vaccinia in dairy cattle and suspicion of vesicular disease on milkers in Brazil

Thaís Garcia da Silva[1*] Michele dos Santos Lima[1] Alessandra Marnie Martins Gomes de Castro[2] Maira de Souza Nunes Martins[1] Vivian Cardoso Castiglioni[1] Claudia Del Fava[1] Liria Hiromi Okuda[1] Edviges Maristela Pituco[1]

[1]Instituto Biológico, Centro de Pesquisa e Desenvolvimento de Sanidade Animal, Laboratório de Viroses de Bovídeos, Av. Conselheiro Rodrigues Alves, 1252, 04014-002, São Paulo, SP, Brasil.
[2]Complexo Educacional Faculdades Metropolitanas Unidas, São Paulo, SP, Brasil. E-mail: zoothais@hotmail.com. *Corresponding author.

ABSTRACT: *Bovine vaccinia (BV) is a vesicular disease induced by the Vaccinia virus (VACV) that affects milk production and is an occupational zoonosis. This research had the following objectives: (i) detection of VACV by qPCR in cattle with clinical suspicion of vesicular disease; (ii) symptoms characterization in animals and milkers with clinical suspicion of the disease and virus detection in humans; and (iii) identification of risk factors for infections of VACV in herds from several Brazilian states. A total of 471 bovine epithelial samples from dairy farms, in 15 Brazilian states, were evaluated between 2007 and 2012. The samples were tested by quantitative PCR (qPCR) using SYBR Green® reagents, validated with a lower limit of detection of 10^0 $TCID_{50}$/50µL ($1.7x10^0$ viral particles), and 45.1% of VACV positive samples were detected. Using official forms for epidemiological investigation (FORM-IN), the risk factors for VACV infections in cattle were determined to be farms with a lack of technological facilities (P=0.029) and the presence of rodents (P=0.001). There was an effect of seasonality in cattle with a higher occurrence of BV during the dry season. A total of 420 epidemiological questionnaires were applied at public health care centers, where 100% of the milkers had vesicular lesions on their hands (98.1%) and on their arms (6.9%). The most frequent clinical symptoms in humans were: local swelling (74.2%), headache (20.7%), fever (10.4%) and inguinal lymphadenopathy (74.2%). Only 19.98% of milkers aged between 39 and 58 years were seroreactive to VACV and were immunized with the human anti-smallpox vaccine. There was an increase in the frequency of BV in older individuals due to their natural decrease in specific immunity. It has been shown that the implementation of zootechnical management techniques and health planning are important for the prevention of BV in animals and humans.*
Key words: *Orthopoxvirus; Poxviridae; quantitative PCR; risk factor; VACV; zoonosis.*

Vaccinia bovina em gado leiteiro e suspeita de doença vesicular em ordenhadores no Brasil

RESUMO: *Vaccinia bovina (VB) é uma doença vesicular induzida pelo Vaccinia virus (VACV) que afeta a produção de leite e é uma zoonose ocupacional. Este trabalho teve os seguintes objetivos: (i) detecção de VACV por qPCR em bovinos com suspeita clínica de doença vesicular; (ii) caracterização dos sintomas apresentados por animais e ordenhadores com suspeita clínica da doença e detecção do vírus em humanos; e (iii) identificação de fatores de risco para infecção por VACV em rebanhos de* vários estados brasileiros. *Um total de 471 amostras de epitélio bovino de fazendas leiteiras, em 15 estados brasileiros, foram avaliados entre 2007 e 2012. As amostras foram testadas por PCR quantitativa (qPCR) usando reagentes SYBR Green®, validados com um limite inferior de detecção de $10^0$$TCID_{50}$/50µL ($1,7x10^0$ partículas virais) e 45,1% das amostras positivas de VACV foram detectadas. Usando formulários oficiais de investigação epidemiológica (FORM-IN), os fatores de risco para infecções por VACV em bovinos foram determinados como fazendas com falta de instalações tecnológicas (P=0,029) e presença de roedores (P=0,001). Houve um efeito da sazonalidade no gado com maior ocorrência de VB durante a estação seca. Um total de 420 questionários epidemiológicos foram aplicados nos centros públicos de saúde, onde 100% dos ordenhadores apresentaram lesões vesiculares nas mãos (98,1%) e nos braços (6,9%). Os sintomas clínicos mais frequentes em humanos foram: inchaço local (74,2%), cefaleia (20,7%), febre (10,4%) e linfadenopatia inguinal (74,2%). Apenas 19,98% dos produtores de leite com idade entre 39 e 58 anos foram sororreagentes ao VACV e foram imunizados com a vacina contra a varíola humana. Houve um aumento na frequência de BV em indivíduos mais velhos devido à sua diminuição natural na imunidade específica. Demonstrou-se que a implementação de técnicas de gestão zootécnica e planejamento sanitário são importantes para a prevenção da VB em animais e seres humanos.*
Palavras-chave: *Poxviridae; quantitative PCR; risk factor; VACV; zoonosis.*

INTRODUCTION

From the sanitary and economic standpoints, differential diagnosis of vesicular diseases in cattle is essential in countries where the milk and meat production chains represent a significant source of income and employment. Brazil is heading toward the eradication of foot-and-mouth disease (FMD). For this reason, the epidemiological surveys and differential diagnosis approaches of other diseases with vesicular

Received 10.09.17 Approved 03.02.18 Returned by the author 08.04.18
CR-2017-0723.R2

symptomatology that affect bovine herds are required (BRASIL, 2007; BRASIL, 2009).

Bovine vaccinia (BV), caused by the *Vaccinia virus* (VACV), a DNA virus of the family *Poxviridae,* Genus *Orthopoxvirus* (OPV), stands out as an important re-emergent zoonosis in Brazil (DAMASO et al., 2000; TRINDADE et al., 2007; ICTV, 2013). This agent spreads easily across herds, causing vesicular lesions in teats and udders, in addition to affecting the gums and tongues of lactating calves (LOBATO et al., 2005). BV is an important occupational zoonosis that occurs mainly in small milk farms that do not implement appropriate sanitary measures or technical assistance by professionals, which helps maintain the virus in the environment and spreads the disease (PITUCO et al., 2008; MEGID et al., 2012; PERES et al., 2013).

The human smallpox virus was eradicated in 1980, after a global vaccination campaign promoted by the World Health Organization (WHO). Vaccine had strains of VACV, that cross reacts with other members of the *Poxviridae* family, including the smallpox virus (DAMON, 2007; FENNER et al., 1988). At the end of this vaccination campaign, a generation of people susceptible to infection caused by several strains of OPV emerged (DAMASO et al., 2000; REYNOLDS et al., 2006).

This has been related to the occurrence of BV in several Brazilian states, including large dairy herds in Minas Gerais and São Paulo, affecting humans and it is of importance for public health (LEITE et al., 2005; ASSIS et al., 2013; REHFELD et al., 2017).

This study had the following objectives: (i) detection of VACV infections by qPCR in cattle with clinical suspicion of vesicular disease; (ii) characterization of the symptoms presented by animals and milkers with clinical suspicion of the disease and the detection of the disease in humans; and (iii) the identification of risk factors for VACV infection in herds in several Brazilian states.

MATERIALS AND METHODS

A total of 471 bovine epithelium samples with clinical suspicious of vesicular disease, collected in 15 Brazilian states between 2007 and 2012, were frozen at -20°C before being sent and analyzed in the Bovine Virus Laboratory (LVB) of the Centro de Pesquisas em Saúde Animal, Instituto Biológico de São Paulo, accredited by the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) to carry out the diagnosis of vesicular diseases (MAPA, 2014). After ruling out the presence of the FMD virus at Lanagro Minas Gerais, the LVB continued investigations to detect other viral agents causing vesicular diseases. These clinical specimens were collected and sent by veterinarians of the Animal Health Defense Service from several Brazilian states, with clinical and epidemiological data on foci of bovine vesicular diseases, detailed on the Form for Initial Epidemiological Investigation (FORM-IN).

The molecular analysis, quantitative PCR (qPCR) for VACV, was performed on samples macerated in 20% (w:v) suspension in Minimal Eagle Medium (MEM), and 2% antibiotics (Potassium Penicillin G 11,200IU/mL; Streptomycin 0.01g/mL, Gentamicin 0.01g/mL, L-Glutamine 0.029g/mL and Amphotericin B 0.5mg/mL). DNA samples were extracted using Guanidine Isothiocyanate (GT) according to the manufacturer's instructions, and stored at -20°C.

The standard virus used as a positive control was the Araçatuba vaccinia strain (ARAV, GenBank accession number AF503169.1), in order to detect the hemagglutinin (HA) gene (TRINDADE et al., 2003). The standard curve quantification for the qPCR was constructed using ARAV extracted and amplified by conventional PCR using generic HA forward and reverse primers, anneal on the nucleotides 156.705 to 156.730 (primer F-HA) and 156.848 to 156.870 (primer R-HA) (TRINDADE et al., 2008). DNA fragments with approximately 183 base pairs (bp) were successfully amplified, purified from agarose gel, using the Wizard PCR Clean-Up kit (Promega™) following manufacturer's instructions.

In order to determine qPCR standardized sensitivity, purified viral DNA concentration was obtained using the apparatus *QuantiFluor™ Promega dsDNA*, according to manufacturer's recommendations. Concentration value (35ng/µL) was transformed in number of DNA copies per microliter ($1{,}7x10^{11}$ copies of DNA/µL) <http://www.scienceprimer.com/copy-numbercalculator-for-realtime-pcr>. From this value, ten-fold serial dilutions ($10^5 – 10^0$ DNA copies/µL) of ARAV (5µL of the purified virus diluted in 45µL of the macerated bovine epithelium negative to VACV) were done.

For qPCR, generic HA forward (5' CAT CAT CTG GAA TTG TCA CTA CTA AA 3') and reverse (5' ACG GCC GAC AAT ATA ATT AAT GC 3') primers (TRINDADE et al., 2008) were used. A commercial LightCycler 480 SYBER Green I Master Kit (Roche Molecular Systems) was used with 10µL Master Mix (2X concentrated). Reactions were performed in a total volume of 20.0µL, in the presence of 1.0µL (10nM/µL) of each primer, 6.0µL nuclease-free water and 2.0µL

DNA template. Reaction conditions were adjusted for pre-incubation at 95°C for 10min, following 45 amplification cycles at 95°C for 10s, 58°C for 40s, and 72°C for 10s, with a melting curve at 95°C for 5s, 60°C for 1min, 97°C for 5s, and cooling at 40°C for 10 s. The Xeno® DNA VetMAX®-PlusqPCR Master Mix[e] was used as the external control for qPCR with 2µL of this synthetic DNA added to each ARAV-negative sample, following the manufacturer›s instructions.

The standard virus and the extracted DNA of epithelium negative for VACV were submitted to three replications in three days, for validation and confirmation of assay results. The viral sample load was determined by comparing to the standard curve, expressed as DNA copies per tissue gram.

Epidemiological forms (FORM-IN), 471, structured with risk factors for VACV infections were administered to each farm at the time of epithelium collection. The variables of the questionnaires analyzed for the calculation of risk factors were: animal category, sex, milking management, breeding system, type of farm, type of farm, main activity, origin of animals, probable origin of the disease, cases of mastitis, destination of milk, origin of reports, and presence of rodents. These data underwent a first exploratory (univariate) analysis by the chi-square test (X^2) or Fisher's Exact Test (when necessary) and only significant variables ($p<0.05$) were selected for logistic regression analysis by the forward Stepwise method, in order to calculate the *Odds Ratio* ($P<0.05$).

Questionnaires (420) were applied at local public health care centers in different regions of Brazil in order to obtain clinical and epidemiological information on VACV infections in milkers from the same properties where the bovine samples were collected, from which a descriptive epidemiological analysis was carried out.

RESULTS

The analytic sensitivity of the qPCR, performed with experimental contamination of bovine epithelium samples, was validated with a threshold detection of 10^0 ($1.7x10^0$ viral particles), corresponding to 1 copy of DNA/µL, with a cycle threshold (Ct) of approximately 35 of the last dilution detected. The assay showed the following reaction values: Error = 0.0233; Efficiency = 2.001; Slope =-3.320. The Melting curve exhibited a single peak with a temperature (*Tm*) of 78.33°C. VACV positive samples showed viral load of $5.0x10^3$ to $5.0x10^{-1}$ DNA copies/0,5mg of tissue, detected between Ct values of 19.88 to 35.94 (data not shown).

Of the collected bovine epithelium samples, 45.1% (212/471) were positive for VACV detected by qPCR. Highest frequencies of VACV infection in cattle and highest VACV reports in humans were observed in the states of Minas Gerais (MG), São Paulo (SP), Maranhão (MA), Mato Grosso (MT) and Rondônia (RO) (Figure 1).

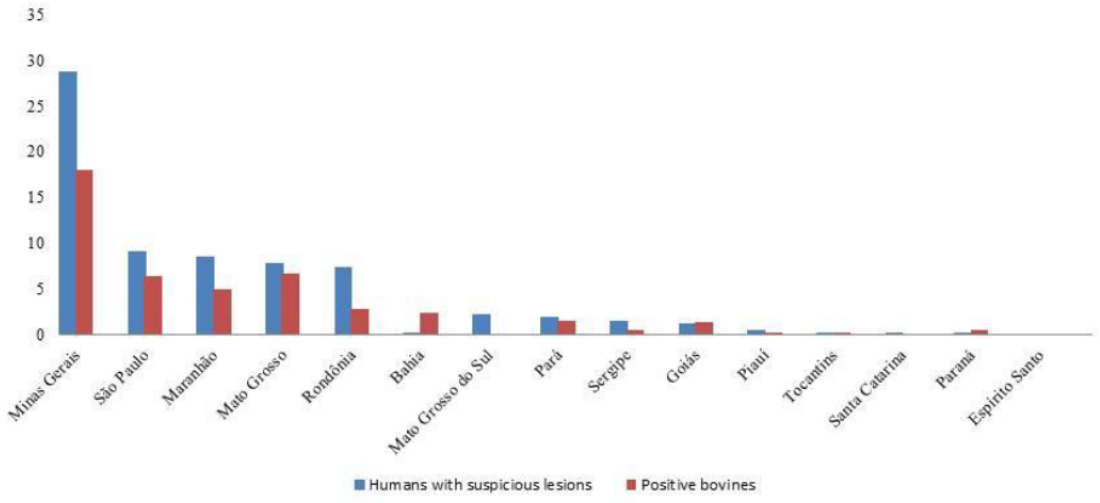

Figure 1 - Percentage of cattle infected by VACV (qPCR positive), and percentage of humans with suspected VACV lesions in the farms evaluated from several Brazilian states.

The mean monthly distribution of BV cases in cattle was concentrated between June to October, considered to be the dry months in the states of Minas Gerais and São Paulo. This indicates an occurrence of outbreaks during the dry winter season in the southeastern region of Brazil (Figure 2).

Reports of disease outbreaks in cattle in 71.5% (323/452) of the cases were made by veterinarians of the Animal Health Defense Service, detected during epidemiological surveillance actions. Other reports were made by owners 26.5% (120/452), and by third parties 2% (9/452). Timelines involved in bovine outbreaks showed that the mean time between the beginning of an outbreak and communication of the suspected vesicular disease to the health authorities was 15 days (15.33), the mean time between the onset of the outbreak and the initial visit was 21 days (20.63), and the mean time between the onset of the disease and the reception of a sample at LVB/IB was 21 days (20.63).

According to the FORM-IN information, the properties were small and medium-sized, lacking technical facilities in which dairy farming represented the main activity associated with production of other livestock such as poultry, swine and sheep. Animals were mainly dairy cows, 94.9% (447/471), followed by beef cattle, 2.5% (12/471), and mixed cattle 2.5% (12/471). Considering the animal category, cows were the most affected by VACV, 96.6% (455/471), followed by calves 3.4% (16/471). In cows, vesicular lesions were located in teats, 54.5% (257/471), udder and non-teats, 47.7% (225/471), and in calves, 100% of lesions were in the mouth and gingiva. In all the cases studied, only cows in lactation and lactating calves that suckled directly from cows with teat and udder lesions became clinically ill. Regarding milking processes, 86% (405/471) of the farms used a traditional manual milking system. In 71.0% (308/434) of the cases it was determined that the cattle had mastitis.

The presence of rodents was reported on 74.9% (304/406) of the farms. The probable origin of the disease was considered to be: proximity between neighboring properties, 50.0% (207/414), of an unidentified origin 45.2% (187/414), and newly acquired animals 4.8% (20/414).

Table 1 shows the results of the univariate analysis and the multivariate model. The final multivariate model indicated two risk factors (odds ratio, 95% confidence interval): farms with lack of technology (2.64, 1.10- 6.34) and presence of rodents (2.38, 1.45-3.89).

According to information from the questionnaires applied by the health care centers, 44.04% (185/420) of the suspicious VACV cases in humans showed anti-Vaccinia antibodies indicating infections by virus neutralization tests (NEWMAN et al., 2003). Of the individuals with suspected disease, 27.61% (116/420) did not undergo laboratory diagnosis, so that the causative agent of the lesion was not identified. Subjects with suspected disease were aged between 19 and 57 years old and only 9% (37/420) were vaccinated against smallpox (Table 2).

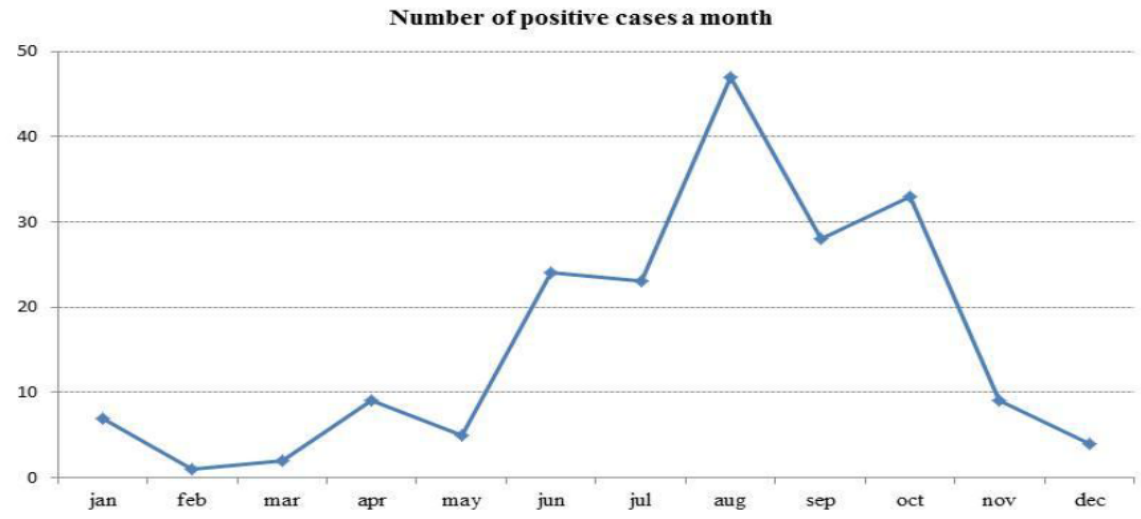

Figure 2 - Monthly distribution of VACV in cattle between 2007 and 2012 totaling 192 cases.

Table 1 - Univariate analysis of the variables related to the presence or not of VACV infection in cattle (* P < 0.05).

VARIABLES		NEGATIVE		POSITIVE		VALUE P
		TOTAL	%	TOTAL	%	
Cattle	Meat	5	2.4	7	3.3	0.846
	Milk	205	97.2	204	96.2	
	Mixed	1	0.5	1	0.5	
Animal category	Cows	203	96.2	207	97.6	0.393
	Calves	8	3.8	5	2.4	
Sex	Male	0	0.0	1	0.5	0.494 (F)
	Female	211	100	205	99.5	
Milking management	Manual	182	87.5	181	87.9	0.910
	Mechanical	26	12.5	25	12.1	
Production system	Breeding	135	64.9	146	70.9	0.110
	Rearing	59	28.4	41	19.9	
	Subsistence	14	6.7	19	9.2	
Operations	Milking	194	93.3	193	93.7	0.863
	Mixed	14	6.7	13	6.3	
Farm with technological aids	No	181	87.0	187	93,0	0.034*
	Yes	22	10.6	14	7.0	
	No information	5	2.4	0	0.0	
Primary activity	No	50	24.0	39	19.3	0.245
	Yes	158	76.0	163	80.7	
Origin of sick animals	Own	181	99.5	183	98.9	>0.999 (F)
	Introduced	1	0.5	2	1.1	
Probable origin of disease	Not identified	91	49.7	81	42.9	0.289
	Neighboring farm	81	44.3	99	52.4	
	Newly acquired animals	11	6.0	9	4.8	
Cases of mastitis	No	74	38.1	49	24.7	0.004
	Yes	120	61.9	149	75.3	
Destination of contaminated milk	Discarded	82	45.1	72	38.9	0.234
	Used as animal feed	100	54.9	113	61.1	
Notification by	Owner	52	25.0	62	30.7	0.124
	Surveillance	154	74.0	134	66.3	
	Third parties	2	1.0	6	3.0	
Presence of rodents	No	64	35.8	35	18.9	<0.001*
	Yes	115	64.2	150	81.1	

It was seen that 100% of the infected persons worked with cattle, and presented vesicular lesions on their fingers/hands, 98.1% (412/420), and on their arms, 6.9% (29/420).

The most frequent clinical symptoms in humans were: local swelling in 74.2% (312/420), headache in 20.7% (87/420), fever in 10.4% (44/420), and inguinal lymphadenopathy in 74.2% (312/420).

The most frequent treatments in the 420 humans were: analgesics 80.71% (339), anti-inflammatory drugs, 11.4% (48), and antibiotics 7.8% (33). The type of medical care consulted by the 420 patients was public, 72.6% (305), private, 7.1% (30), or both, 20.2% (85). The clinical outcome or duration of clinical symptoms in the affected individuals was on average two weeks long.

Although, VB is a self-limiting disease, that weakens the patient and prevents him/her from working, only 0.9% (4/420) of the patients were admitted to the hospital and temporary absence from work was not reported.

DISCUSSION

Differential diagnosis of vesicular diseases is fundamental for the epidemiological surveillance

Table 2 - Distribution of suspected human cases with VACV by VN method according to age and whether vaccinated or not vaccinated against smallpox.

Age group	Vaccinated		Not vaccinated	
	N	%	N	%
19-23	0	0	8	4.4
24-28	0	0	20	11
29-33	0	0	38	20.5
34-38	0	0	82	44.3
39-43	2	1.1	0	0
44-48	9	4.8	0	0
49-53	12	6.4	0	0
54-58	14	7.5	0	0
Subtotal	37	19.8	148	80.2
Total	185 (100%)			

system (LANGUARDIA-NASCIMENTO et al., 2016). Due to the impact of these diseases, Brazil has a laboratory network in support of the PNEFA (National Program for Eradication of Foot-and-Mouth Disease), accredited by MAPA.

Quantitative PCR, in this research, showed high sensitivity as reported in previous studies (TRINDADE et al., 2008; YANG et al., 2007). The use of SYBR Green in the diagnosis of VACV demonstrated specificity in the differential diagnosis of vesicular diseases, and speed and viability in diagnostic laboratory routines.

Analyzing outbreak timelines, it was seen that the average time between the beginning of the outbreak and the arrival of a sample at the laboratory was 20.63 days, which PNEFA considered late (BRASIL, 2007). This delay was probably caused by late reporting, evidencing the need for health education among farmers in order to raise awareness of the importance of immediate reporting of suspected cases of vesicular disease, making it possible to find typical VACV lesions in the initial phase of clinical disease. The critical point for epidemiological surveillance systems of animal diseases is the early detection of the pathogen to confirm the case, making it possible to carry out emergency sanitary actions to contain vesicular diseases (BRASIL, 2007). Conversely, 71.5% of reports were made by official veterinarians of the Animal Health Defense Service, indicating that active epidemiological surveillance in these regions has been carried out.

In the present research, cases of VACV in cattle and humans were predominantly confirmed in the states of Minas Gerais and São Paulo, regions that are heavily focused on dairy farming, in which several outbreaks of VACV have been reported in dairy herds, domestic animals and humans (MEGID et al., 2008; 2012; PERES et al., 2013; ABRAHÃO et al., 2015). These findings confirmed the presence of several strains of VACV, raising important questions about the emergence and distribution of the virus in Brazil, in the last decades, variants of VACV, from several states of Brazil, have been identified and characterized as Cantagalo Virus (CTGV) (DAMASO et al., 2000), Passatempo Virus (PSTV) (LEITE et al. 2005), Guarani Virus (TRINDADE et al., 2006) and Muriaé Virus (TRINDADE et al., 2007).

The farms affected by BV suffered economic losses, including a reduction in milk production, farm interdiction, animal drug costs, and the hiring of temporary staff to replace sick workers (DONATELE et al., 2007). One of the main consequences observed in the present research was mastitis, which can be characterized as a secondary infection associated with poxvirus. Mastitis, in addition to causing direct losses to producers, also causes a decrease in the quantity and quality of milk (REHFELD et al., 2017). As the disease is transmitted horizontally by direct contact, mainly cows and calves were the most affected categories. Lesions occurred on the teats and oral mucosa of the cows and muzzle region of lactating calves, as also occurred in other outbreaks of the disease (DONATELE et al., 2007; SIMONETTI et al., 2007).

Loaning employees to neighbors, permitting the access of itinerant milkers to herds, and an unrestricted movement of people and animals between neighboring farms are common in the rural areas studied and pose a risk of transmission from one herd to another, corroborating findings (KROON et al., 2011). Farms lacking technological aids (odds ratio 2.64 and $P = 0.029$) and with the presence of rodents (odds ratio 2.38 and $P = 0.001$) were more likely to present VACV infection. Lower VACV attack rates on farms using mechanical milking in comparison to manual milking was also verified by TRINDADE et al. (2007). It should be emphasized that the presence of rodents was highly significant, leading to the assumption that these animals can act as vectors of VACV. A serological study detected neutralizing antibodies against VACV in *Rattus rattus* captured in the metropolitan region of São Paulo/SP/Brazil, evidencing that VACV circulates in this species in synanthropic conditions (BABOLIN et al., 2016). However, it was detected, in rural properties of São Paulo, a high sero-prevalence among domestic animals (cows, horses, sheep, pigs,

dogs and cats) and humans, and there was no positive result for wild rodents, emphasizing the involvement of other species that act as reservoirs in the VACV transmission cycle (PERES et al., 2013). A study of rodents on rural properties is justified, in order to verify their importance as VACV vectors.

The occurrence of outbreaks in cattle was concentrated between the months of June to October, during the dry season, indicating the existence of seasonality. Therefore, there is a probable favorable climatic condition for the disease. Similar results were reported (LOBATO et al., 2005; ASSIS et al., 2015). Conversely, research carried out by other authors disagree that there is seasonality, and consider VB as an epidemic disease that occurs throughout southeastern Brazil from January to December (SILVA-FERNANDES et al., 2009).

Due to the fact that BV is a zoonosis, when there was an outbreak of BV in cattle, there were also reports of human cases. For this reason, milkers, who came into constant and direct contact with animals, were the most infected profession, a fact also described by other authors (NAGASSE-SUGAHARA et al., 2004; ABRAHÃO et al., 2010).

According to the information present in the 420 questionnaires applied at human health care centers, the clinical symptoms presented by the milkers were characteristic of poxviruses (SANT'ANA et al., 2013). However, in 27.61% of human cases with vesicular lesions suspected of being VACV, there was no laboratory confirmation, due to the absence of local health care centers able to perform the analysis, showing a deficiency in the human health care system regarding the assistance of this occupational zoonosis (SILVA-FERNANDES et al., 2009).

Human medical care consulted was predominantly public, probably due to the fact that it is free. VB does not have a specific treatment, so the treatment used by the patients consisted of cleaning and local hygiene of the lesions associated with medication such as antibiotics and analgesics, a palliative treatment to alleviate pain and secondary infections, as described by other authors (TRINDADE et al., 2007). Both vaccinated and unvaccinated patients against the smallpox virus had the same symptoms and vesicular lesions caused by VACV (SILVA-FERNANDES et al., 2009).

The last case of human smallpox occurred in Somalia in 1977, and the disease was considered eradicated by the WHO in 1980. As a result, mass immunization with attenuated VACV was discontinued, allowing for a decline in immunity and resulting in increased *Orthopoxvirus* in humans (WOLFS et al., 2002). The immunological state of the population against OPV is an important risk factor for re-emergence and cause of frequent VACV infections.

In this study, only 19.98% of the infected milkers reported having been immunized with the human anti-smallpox vaccine. These were aged between 39 and 58 years, and there was increased frequency of smallpox in older individuals. This can be explained by the natural decrease of the specific immunity against the pathogen with advancing age. It was also reported an increase in the clinical frequency of VACV in older vaccinated humans, because the titers of vaccine antibodies decreased with advancing age (SILVA-FERNANDES et al., 2009; ABRAHÃO et al., 2015). This observation is of concern since it demonstrates that individuals vaccinated against the human smallpox virus may not be protected against circulating strains of VACV in Brazilian territory and in other regions of the world.

Our data confirmed that the self-limiting nature of the infection means that the milker does not seek adequate treatment in specialized health units, leading to frequent under-reporting of cases. BV is an occupational disease of public health importance and should be notified.

CONCLUSION

Our epidemiological studies confirmed the presence of VACV in Brazilian bovine herds, including infections of the milkers. It was identified as risk factors the absence of technology on farms and the presence of rodents, demonstrating that it is necessary to implement technology on zootechnical herd management along with sanitary planning, with the aim of preventing VACV in animals and humans in Brazil.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This research was carried out following the ethical guidelines adopted by the Sociedade Brasileira de Ciências de Animais de Laboratório (SBCAL) and the Colégio Brasileiro de Experimentação Animal (COBEA), and was approved by the Comitê de Ética do Instituto Biológico de São Paulo, Protocol Number 66/08.

ACKNOWLEDGEMENTS

The authors are grateful to the Agricultural Defense for their assistance in the conduction of this study, to the workers of health care centers, who made it possible to assess the implications of the disease for public health. The authors are grateful to Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarships for T.G. Silva, M.S. Lima, M.S.N. Martins and V.C. Castiglioni.

DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

ABRAHÃO, J.S. et al. Human *Vaccinia virus* and *Pseudocowpox virus* co-infection: Clinical description and phylogenetic characterization. **Journal of Clinical Virology**, v. 48, n.1, p. 69-72, 2010. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/20207192?dopt=Abstract>. Accessed: Nov. 12, 2016. doi: 10.1016/j.jcv.2010.02.001.

ABRAHÃO, J.S. et al. Outbreak of severe zoonotic vaccinia virus infection, Southeastern Brazil. **Emerging Infectious Disease**, v. 21, n. 4, p. 695–698, 2015. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378504/>. Accessed: Dec. 15, 2016. doi: 10.3201/eid2104.140351.

ASSIS, F.L. et al. J.S. Horizontal study of *Vaccinia virus* infections in an endemic area: epidemiologic, phylogenetic and economic aspects. **Archives Virology**. v. 160, p. 2703-2708, 2015. Available from: <https://www.researchgate.net/publication/280693749>. Accessed: Jan. 10, 2016. doi: 10.1007/s00705-015-2549-1.

ASSIS, F.L. et al. Reemergence of Vaccinia virus during zoonotic outbreak, Pará State, Brazil. **Emerging Infectious Disease**, v.19, n. 12, p. 2017-2020, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3840876/>. Accessed: Dec. 10, 2015. doi: 10.3201/eid1912.130589.

BABOLIN, L.S. et al. **Zoonosis associated to *Rattus rattus* and the impacts of the public actions to control the species. Arquivos do Instituto Biol**ógico, São Paulo, v.83, p. 1-7, 2016. Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S180816572016000100208>. Accessed: Nov. 10, 2015. doi: 10.1590/1808-1657000832014.

BRASIL. **Plano de ação para febre aftosa**: Ministério da Agricultura, Pecuária e Abastecimento, Secretaria de Defesa Agropecuária, Departamento de Saúde Animal Vigilância. Brasília. 2009. 96p.

BRASIL. **Programa Nacional de Erradicação de Febre Aftosa**: Secretaria de Defesa Agropecuária, Departamento de Saúde Animal Vigilância, Vigilância Veterinária de Doenças Vesiculares - Orientações Gerais. Ministério da Agricultura, Pecuária e Abastecimento. Brasília, 2007. 49p.

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: <https://www.ncbi.nlm.nih.gov/pubmed/11080491>. Accessed: Nov. 10, 2000. doi: 10.1006/viro.2000.0603.

DAMON, I. (eds). Fields Virology. Poxviridae and their replication. Knipe DM, Howley PM. New York: Raven Press, 2007, p. 2079-2081.

DONATELE, D.M. et al. Epidemiology of bovine poxvirosis in the state of Espírito Santo, Brazill. **Brazilian Journal of Veterinary Research and Animal Science**, v.44, n.4, p. 275-282, 2007. Available from: <http://www.revistas.usp.br/bjvras/article/view/26628>. Accessed: Dec. 16, 2015.

FENNER, F. et al. **Smallpox and Its Eradication**. Geneva: World Health Organization Press, 1988.

ICTV, 2013. **International Committee on taxonomy on viruses**. Version=2013. May 2017. Available from: <http://www.ictvonline.org/virustaxonomy.asp?>. Accessed: Dec. 16, 2015.

KROON, E.G. et al. Zoonotic Brazilian *Vaccinia virus*: from field to therapy. **Antiviral Research**, v. 92, p. 150–163, 2011. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/21896287>. Accessed: Dec. 15, 2015. doi: 10.1016/j.antiviral.2011.08.018.

LANGUARDIA-NASCIMENTO, M. et al. Detection of multiple viral infections in cattle and buffalo with suspected vesicular disease in Brazil. **Journal of Veterinary Diagnostic Insvestigation**, v. 28, n. 4, p. 377–381, 2016. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/27154321>. Accessed: Dec. 20, 2016. doi: 10.1177/1040638716645836.

LEITE, J.A. et al. Passatempo Virus, a Vaccinia Virus Strain, Brazil. **Emerging Infectious Disease**, v.11, n. 12, p. 1935–1938, 2005. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3367646/>. Accessed: Nov. 20, 2016. doi: 10.3201/eid1112.050773.

LOBATO, Z.I.P. et al. Outbreak of bovine smallpox caused by the vaccinia virus in the region of Zona da Mata Mineira. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 57, 423-29, 2005.

MAPA (2014) – **Ministério da Agricultura, Pecuária e Abastecimento**. Portaria DSA n. 189, de 28 de julho de 2014, DOU 143 de 29/07/14, seção 1, p.4. Escopo de Credenciamento do Instituto Biológico.

MEGID, J.et al. Short report: Vaccinia virus in humans and cattle in southwest region of São Paulo state, Brazil. **American Journal of Tropical Medicine and Hygiene**, v. 79, n. 5, p. 647-51, 2008. Available from: <http://www.ajtmh.org/content/journals/10.4269/ajtmh.2008.79.647>. Accessed: Nov. 10, 2015. doi: 10.4269/ajtmh.2008.79.647.

MEGID, J. et al. Vaccinia virus, zoonotic infection, São Paulo state, Brazil. **Emerging Infectious Disease**, v. 18, p. 189–191, 2012. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3310104/>. Accessed: Nov. 10, 2015. doi: 10.3201/eid1801.110692.

NAGASSE-SUGAHARA, et al. Human vaccinia-like virus outbreaks in São Paulo and Goiás states, Brazil: virus detection, isolation and identification. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 46, n. 6, p. 315-322, 2004. Available from: <http://www.scielo.br/scielo.php?pid=S0036466520040006000004&script=sci_abstract&tlng=pt>. Accessed: Nov. 20, 2015. doi: 10.1590/S0036-46652004000600004.

NEWMAN, F. K. et al. Improved assay to detect neutralizing antibody following vaccination with diluted or undiluted vaccinia (Dryvax) vaccine. **Journal of Clinical Microbiology**, v. 41, n. 7, p. 3154–3157, 2003. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC165337/>. Accessed: Dec. 15, 2015. doi: 10.1128/JCM.41.7.3154-3157.2003.

PERES, M.G. et al. Serological study of Vaccinia virus reservoirs in areas with and without official reports of outbreaks in cattle and humans in São Paulo, Brazil. **Archives Virology**, v. 158, p. 2433–2441, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3830743/>. Accessed: Dec. 20, 2016. doi: 10.1007/s00705-013-1740-5.

PITUCO, E.M. et al. **Bovine Poxviruses in Brazil**. In: Proceedings of the 17th International Poxvirus and Iridovirus Conference, Grainau, 2008.

REHFELD, I.S. et al. Subclinical bovine vaccinia: An important risk factor in the epidemiology of this zoonosis in cattle. **Research in Veterinary Science**, v. 114, p. 233–235, 2017.

Available from: <http://www.sciencedirect.com/science/article/pii/S0034528816303204?via%3Dihub>. Accessed: Oct. 01, 2017. doi: 10.1016/j.rvsc.2017.03.022.

REYNOLDS, M.G. et al. Clinical manifestations of human monkeypox influenced by route of infection. **Journal of Infectious Disease**, v. 194, n. 6, p.773–780, 2006. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/16941343>. Accessed: Dec. 20, 2016. doi: 10.1086/505880.

SANT'ANA, F.J. et al. Coinfection by Vaccinia virus and an Orf virus-like parapoxvirus in an outbreak of vesicular disease in dairy cows in midwestern Brazil. **Journal of Veterinary Diagnostic Insvestigation**, v. 25, p. 267–272, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/23404478>. Accessed: Dec. 20, 2016. doi: 10.1177/1040638713475799.

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. doi: 10.1016/j.jcv.2009.01.007.

SIMONETTI, B. et al. Animal infections by Vaccinia – like viroses in the state of Rio de Janeiro: 1- northwestern region. **Virus Reviews and Research**, v. 12, p. 32-36, 2007.

TRINDADE, G.S. et al. Araçatuba virus: A vaccinia like virus associated with infection in humans and cattle. **Emerging Infectious Disease**, v. 9, n. 2, p. 155-160, 2003. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2901946/>. Accessed: Nov. 15, 2015. doi: 10.3201/eid0902.020244.

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**, v. 75, n. 3, p. 486-490, 2006. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/16968926>. Accessed: Dec. 02, 2017. doi: 10.4269/ajtmh.2006.75.486.

TRINDADE, G.S. et al. Brazilian vaccinia viruses and their origins. **Emerging Infectious Disease**, v. 13, n. 7, p. 965-972, 2007. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2878226/>. Accessed: Dec. 20, 2015. doi: 10.3201/eid1307.061404.

TRINDADE, G.S. et al. Real-time PCR assay to identify variants of *Vaccinia virus*: Implications for the diagnosis of bovine vaccinia in Brazil. **Journal of Virological Methods**, v. 152, n. 1-2, p. 63-71, 2008. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/18602170>. Accessed: Oct. 20, 2015. doi: 10.1016/j.jviromet.2008.05.028.

YANG, Z. et al. Detection of PCV2 DNA by SYBR Green I-based quantitative PCR. **Journal of Zheijang University Science B**, v. 8, n. 3, p. 162-169, 2007. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1810386/>. Accessed: Dec. 20, 2016. doi: 10.1631/jzus.2007.B0162.

WOLFS, T.F. et al. Rat-to-human transmission of cowpox infection. **Emerging Infectious Disease**, v. 8, n. 12, p. 1495–1496, 2002. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2738512/>. Accessed: Dec. 15, 2016. doi: 10.3201%2Feid0812.020089.