Retraction

The editorial team of Ciência Rural announces the formal publication of the retraction:


Since it was proven duplicate publication of the article that was available in the previous edition of the journal Ciência Rural:


Rudi Weiblen
Editor in chief
Bovine Vaccinia in dairy cattle and suspicion of vesicular disease on milkers in Brazil

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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 cattle 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 10^5 TCID50/μL (1.7x10^3 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 seropositive 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.

RETRACTED ARTICLE

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RESUMO: Vaccinia bovina (VB) é uma doença vesicular induzida pelo Vaccinia virus (VACV) que afeta a produção de leite e é uma zoonose ocupacional. Neste 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 de vacinação do vírus em humanos; e (iii) identificação de fatores de risco para infecção por VACV em bovinos de fazendas leiteiras, em 15 estados brasileiros, foram avaliados entre 2007 e 2012. As amostras foram testadas por PCR quantitativa (qPCR) usando SYBR Green® reagentes, validados com um limite inferior de detecção de 10^5 TCID50/μL (1,7x10^3 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%) e febre (10,4%) e linfedema inguinal (74,2%). Apenas 19,98% dos produtores de leite em idade entre 39 e 58 anos foram soropositivos ao VACV e foram imunizados com a vacina contra a variola 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 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, 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 symptomatology that affect bovine herds are required (BRASIL, 2007; BRASIL, 2009).

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. Neste 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 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.
**Poxviridae**. Genus Orthopoxvirus (OPV), stands out as an important re-emergent zoonosis in Brazil (NAMSG 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 safety measures or technical assistance by professionals, which helps maintain the virus in the environment and spreads the disease (PITUÇO 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 the smallpox virus (DAMON, 2007; FENNER et al., 1988). At the end of this vaccination campaign, a generation of people susceptible to infection was caused by several strains of OPV emerged (DAMASO et al., 2000; TRINDADE et al., 2007; ICTV, 2013).

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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., 2006).

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 suspicion 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 Animal Health Research Center, Biological Institute of São Paulo, accredited by the Brazilian Ministry of Agriculture, Livestock and Food Supply (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 G11,200IU/mL; Streptomycin 0.0g/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/µL). 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 GAA TGG TCA CTG (CTT AAT GC 3’)) and reverse (5’ ACC GCC GAC AAT ATA TTG 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). Reaction 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 5s, 97°C for 5s, and cooling at 40°C for 30s. The Xeno® DNA VetMAX®-PlusqPCR Master Mix 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 ($T_m$) 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).

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 sucked 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 71.1% (308/434) of the cases it was determined that the cow 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 healthcare 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).

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

Table 1 - Univariate analysis of the variables related to the presence or not of VACV infection in cattle (* P < 0.05).
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 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., 2016).
et al., 2011). Farms lacking technological aids (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 factor 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., 2010). This observation is of concern since it demonstrates that individuals vaccinated against the human smallpox virus may not be protected against circulating viruses 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 milkers 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.

**ACKNOWLEDGMENTS**

The authors are grateful to the Agricultural Defense and 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.

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