Short Report: Group 1 *Vaccinia virus* Zoonotic Outbreak in Maranhão State, Brazil

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Abstract. In Brazil, several exanthematic autochthonous *Vaccinia virus* (VACV) outbreaks affecting dairy cattle and rural workers have been reported since 1999. Although outbreaks had been first described in the Brazilian Southeast, VACV outbreaks were notified in all Brazilian regions in < 10 years. However, in this context, VACV outbreaks had not been described in some Brazilian States, likely because of a lack of notification, or yet unknown epidemiological reasons. Here, we describe the first VACV outbreak in Maranhão State, northeastern Brazil. The virus isolated from this outbreak showed several biological and molecular features that resemble other Group 1 Brazilian VACV, including a deletion signature in the A56R gene. This study raises new questions about diversity and epidemiology of Brazilian VACV.

After centuries of epidemics and deaths, smallpox was declared eradicated in 1980, after an intensive vaccination campaign promoted by the World Health Organization (WHO).^{1,2} The smallpox vaccines used in the WHO campaign were, in fact, strains of the *Vaccinia virus* (VACV), a species belonging to the genus *Orthopoxvirus* (OPV), which induced serological cross-reactivity against other OPV members, including *Variola virus* (the agent of smallpox).^{1,2} Despite the immune protection provided by VACV, several cases of adverse manifestations caused by vaccination were reported, which led to the suspension of the vaccination after the disease eradication.³ However, the suspension of the smallpox vaccination led to the emergence of a generation that is susceptible to infection by other OPV species, such as 1) *Cowpox virus* (CPXV) in Europe; 2) *Monkeypox virus* (MPXV), which occurs naturally in Africa with one introduction event reported from the United States; and 3) VACV in Asia and South America.^{4–6}

In Brazil, several exanthematic autochthonous VACV outbreaks affecting dairy cattle and rural workers have been reported since 1999.^{4,7} The disease has a great impact on the Brazilian milk industry and public health services.^{4,7} During these outbreaks, infected dairy cattle usually presented ulcerative lesions on their teats and udders and had decreased milk production.^{8,9} Rural workers who were infected with VACV, most likely from occupational contact with infected cattle, usually presented lesions on their hands and arms, lymphadenopathy, high fever, and prostration, among other symptoms.^{8,9} Since early reports of VACV outbreaks in Brazil, several VACV isolates have been characterized.^{4,7–9} Molecular and biological studies have shown that Brazilian VACV isolates can be divided into two distinct groups: Group 1 and Group 2.^{10,11} The Group 1 VACV-BR comprises Cantagalo, Araçatuba, Passatempo, GuaraniP2, Mariana, Pelotas2, DMTV, and other isolates; Group 2 VACV-BR includes GuaraniP1, Pelotas1, SH2V, Bean58058, and other isolates.^{10,11} Although outbreaks had been first described in Brazilian Southeast, VACV had spread to all Brazilian regions in < 10 years. In this context, VACV outbreaks had not been described in some Brazilian States, likely because of lack of notification, or yet unknown epidemiological reasons. Here,

we describe the first VACV outbreak in Maranhão State, northeastern Brazil.

In February 2009, an exanthematic VACV outbreak was reported in the rural region of Açailândia County (04°56'49"S 47°30'18"W), Maranhão State, Brazil. This region is characterized by the presence of small rural properties, where cattle are kept for milk production. During this outbreak, several animals (42 bovine cattle) and farm workers (6 patients) from neighboring properties presented exanthematous lesions similar to those reported during other Brazilian bovine VACV outbreaks.^{4,7–9} A total of 9 farms were affected by this bovine vaccinia outbreak. The origin of this outbreak is unknown, but workers were most likely infected while milking infected animals and transmitted the virus by direct contact with healthy animals at the other properties. Although previous studies had shown the importance of peridomestic rodents in bovine vaccinia outbreaks,⁷ the capture of rodents was not performed during the outbreak. A total of three farms that were affected during the outbreak were visited, and epithelial samples (three dried scabs) from three infected dairy cows were collected (a sample was collected per farm) with tweezers, kept under refrigeration, and sent to our laboratory for etiological agent identification. The scabs were macerated using a homogenizer (Politron, Littau, Switzerland) in phosphate buffered saline (PBS), which contained 200 U/mL penicillin, 4 µg/mL amphotericin B, and 100 µg/mL gentamicin (0.1 g scab/0.9 mL PBS), and centrifuged at 2000 × g for 3 min. The resulting supernatants were used for diagnostic purposes, viral isolation, and molecular assays.¹²

To confirm if the etiological agent of the outbreak was an OPV, the sample supernatants were diluted 1:100 in PBS and used as templates for a polymerase chain reaction (PCR) that targeted a partial region of viral growth factor gene (vgf). The reactions were carried out by adding 2 µL of the template to 18 µL of the PCR reaction mixture that contained 0.4 mM of vgf primers, as previously described.¹³ The PCR products were electrophoresed in 8%-PAGE gels and silver stained.¹⁴

For viral isolation, 300 µL of the sample was added onto BSC-40 cell monolayers that were grown in a six-well plate and incubated at 37°C for 72 hours or until detection of the cytopathic effect (CPE). After CPE observation, the cells were harvested and new BSC-40 cell monolayers were reinoculated for viral amplification. The resulting viruses were purified in a sucrose gradient and titrated as described.^{15,16} For plaque phenotype assays, BSC-40 cell monolayers at 90–95% confluency were infected with a multiplicity of infection

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(MOI) of 0.01 of the new isolate, GP1V (as a large-plaque control) and GP2V isolates (as a small-plaque control). The VACV-WR strain and PBS were used as additional controls. Forty-eight hours after infection, the cells were fixed with paraformaldehyde and stained with crystal violet for plaque size analysis.

Attempts at viral isolation revealed typical pox-like CPEs in BSC-40 cells that were inoculated with supernatants from the three tested scabs. No cellular changes were observed in the BSC-40 monolayer that was inoculated with PBS control. In parallel, the nested-PCR assay that targets C11R¹³ resulted in the amplification of OPV-specific fragments in all tested

samples (three scabs) that were also present in the VACV-WR positive control. Because all tested samples were identical in biological and molecular tests, the isolate was named Maranhão virus (MRV).

After amplifying and purifying MRV, plaque phenotype assays were performed to evaluate the biological cluster of this new isolate. Previously, Brazilian OPV isolates have been clustered into two distinct groups, Group 1 and Group 2. Compared with GP1V (Group 2) and GP2V (Group 1) in plaque assays, MRV plaques were small and similar to those formed by GP2V, which provides supporting evidence to classify MRV as a member of Group 1 VACV. As expected,

FIGURE 1. (A) Phylogenetic tree generated from OPV TK nucleotide sequences, including the Brazilian *Vaccinia virus* (VACV) isolates. The colored boxes are species-related (VACV, orange; VARV, green; CPXV, blue; and MPXV, pink). The OPV clusters (colored boxes) are strongly supported by high bootstrap *P* values. The Maranhão isolate is depicted as a black dot. (B) Phylogenetic tree generated from OPV A56R nucleotide sequences, including the Brazilian VACV isolates. A56R phylogenetic analyses showed that the Brazilian VACV isolates are split into two different branches (Groups 1 and 2). Maranhão isolate clustered with Group 1 VACV isolates. Maximum likelihood trees were reconstructed using different data sets containing sequences from the genes listed previously, using MEGA 4.0. Gamma categories were used to model evolutionary rate differences among sites and the reliability of branching patterns was tested through 1,000 bootstrap sampling. (C) Nucleotide sequence alignment of A56R coding DNA sequences. Sequences were retrieved from GenBank and aligned using the ClustalW method as implemented in the MEGA 4.0 program. The Maranhão isolate showed an 18-nt deletion. The same deletion was observed in the Brazilian VACV Group 1 isolates. Nevertheless, the Brazilian Group 2 isolates had no deletion in A56R. The deletion site is highlighted with an asterisk.

VACV-WR produced large plaques similar to those associated with GP1V (data not shown).

For molecular characterization, viral genes such as the highly conserved TK gene (thymidine kinase)¹⁷ and the variable A56R gene (hemagglutinin, HA)^{18,19} were amplified and sequenced for phylogenetic analysis. The chemistry and thermal conditions of these PCR reactions were similar to those used for the vgf nested-PCR; however, the annealing temperatures of the specific primers were changed. The PCR fragments obtained from this study were sequenced in both orientations and in triplicates (Mega-BACE 1000 sequencer) (GE Healthcare, Buckinghamshire, UK). The sequences were aligned with previously published OPV sequences from GenBank using the ClustalW method, and the alignments were checked manually with the MEGA version 4.0 software (Arizona State University, Phoenix, AZ). Accession numbers for the analyzed sequences can be found in their respective figures. Maximum likelihood trees were reconstructed using different data sets containing sequences from the genes listed previously, using MEGA 4.0. Using the ModelTest Server,¹⁹ the nucleotide substitution model of Tamura 1992 was selected as the best one fitting the data. Rates of variation among sites were estimated for each data set and two discrete Gamma categories were used to model evolutionary rate differences among sites and the reliability of branching patterns was tested through 1,000 bootstrap sampling. The MRV sequences obtained in this study were deposited in GenBank: 1640002 and 1639939 (provisional).

The TK sequences of MRV indicate that the isolate clusters with other VACV isolates (Figure 1A). Because of the conservation of the nucleotide sequences, TK can serve as a genetic marker for OPV species identification; however, it provides limited information for VACV sub-cluster analysis. Phylogenetic analyses of A56R sequences clustered MRV with Group 1 Brazilian VACV isolates (Figure 1B), confirming our

biological data. The hemagglutinin gene nucleotides were analyzed by alignment with similar sequences from other OPV isolates deposited in GenBank. The MRV A56R sequence contained a signature deletion (Figure 1C) also present in the sequences of other Brazilian Group 1 VACV isolates, such as Araçatuba virus (ARAV), Cantagalo virus (CTGV), Serro virus (SV2), GuaraniP2 virus (GP2V), Muriae virus (MURV), and others, but absent in Group 2 VACV, such as GuaraniP1 virus (GP1V), Belo Horizonte virus (VBH), BeAn58058 virus (BAV), and SPAn232 virus (SAV). Although some Group 1 VACV present exclusive nt substitutions, phylogenetic analysis do not support the prediction of a progenitor (sub-branches with low bootstrap *P* values—data not shown).

Taken together, our results showed that MRV is a new Group 1 Brazilian VACV strain that was isolated from a zoonotic vaccinia outbreak in the northeastern region of Brazil. Farming and dairy production are important activities in this region and were negatively impacted by the bovine vaccinia outbreaks. The infection of rural workers has public health implications. This is also the first characterization of a VACV isolated during an outbreak in Maranhão State. Transmission of VACV in northeastern Brazil has been reported, and outbreaks have been notified in Pernambuco, Tocantins, Bahia, and Pará States²¹, which are close to Maranhão (Figure 2). Although our team was not able to screen the source of this outbreak, we believe that MRV could derive from the virus(es) that circulate(s) in those neighbors States. The VACV has also been detected in wild monkeys in the Brazilian Amazon,²¹ therefore the role of wild life in VACV spread and maintenance could not be neglected, especially in states where the forest is present, such as Maranhão State. Some of these viruses may be related to that isolated in this study because some VACV isolates have the same signature deletion in the HA gene as MRV. We believe that veterinary surveillance, farm worker education, inspection of farm

FIGURE 2. Map highlighting Group 1 and Group 2 VACV circulation in Brazil. MRV was isolated from Maranhão State.

workers and cattle before milking, and quarantine of sick animals are *sine qua non* conditions for controlling viruses. New ecological and epidemiological studies could also help to explain why VACV has spread in the last decade State-by-State, through Brazilian rural areas.

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