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The detection of *Vaccinia virus* confirms the high circulation of *Orthopoxvirus* in buffaloes living in geographical isolation, Marajó Island, Brazilian Amazon

Ana Paula Moreira Franco-Luiz [a], Alexandre Fagundes Pereira [a], Cairo Henrique Sousa de Oliveira [b], José Diomedes Barbosa [c], Danilo Bretas Oliveira [a], Cláudio Antônio Bonjardim [a], Paulo César Peregrino Ferreira [a], Giliane de Souza Trindade [a], Jônatas Santos Abrahão [a], Erna Geessien Kroon [a,*]

[a] *Laboratório de Vírus do Departamento de Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil*

[b] *Setor de Medicina Veterinária Preventiva, Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, Goiânia, GO, Brazil*

[c] *Hospital Veterinário de Grandes Animais, Instituto de Medicina Veterinária da Universidade Federal do Pará, Castanhal, PA, Brazil*

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ABSTRACT

In Brazil, serologic evidence of Orthopoxvirus (OPV) circulation showed positivity around 20% in cattle, humans, monkeys and rodents. Although OPV seropositivity has been described in buffalo herds in southeastern Brazil, no *Vaccinia virus* (VACV) (member of genus OPV) outbreaks in buffalo herds have been described in this country. This study aimed to investigate the detection of anti-OPV antibodies and to study the OPV genome in Brazilian buffalo herds. Our results demonstrated a high OPV seropositivity in buffalo herds on Marajó Island and molecular data confirmed the circulation of VACV. The geographical isolation conditionmight be a *sine qua non* condition to explain our results.

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1. Introduction

Viruses belonging to the genus Orthopoxvirus (OPV), of which Monkeypox virus, Cowpox virus and *Vaccinia virus* (VACV) are infectious agents of zoonotic diseases worldwide. During the last few decades, reports about zoonotic OPV have increased [1]. In Brazil, VACV has been reported to affect mainly dairy cattle and rural workers [2]. A VACV variant, Buffalopox virus (BPXV), has been isolated from buffaloes (*Bubalus bubalis*). BPXV is circulating among buffaloes, cows and human mainly in India. Despite BPXV is closely related to VACV by phylogenetic analysis, each virus has distinct genetic signatures [3]. Serologic evidence of OPV circulation in Brazil showed a positivity around 20% in cattle, humans, monkeys and rodents [2,4,5]. Although OPV seropositivity has been described in buffalo herds in southeastern Brazil [6] no VACV outbreaks in buffaloeshave been described in this country. This study aimed to investigate the detection of anti-OPV antibodies and OPV genome in Brazilian buffalo herds. For the first time, we analyzed sera samples from buffalo herds living in geographical isolation, on the Marajó Island, northern Brazil.

2. Methods

In 2009, 2011 and 2012, sera were collected from 150 buffaloes (*B. bubalis*) from Cachoeira do Arari, Chaves, Salvaterra and Soure counties, Pará State, Brazil (Fig. 1). Marajó is an Amazonian island [7] the largest riverine island in the world, with an area of approximately 50,000 km^2. The buffalo's herds have not presented clinical sings of VACV at the time of serum collection and there were no previous VACV outbreaks notified in Marajó Island. To investigate the neutralizing antibodies in buffalo herds, sera

* Corresponding author at: Laboratório de Vírus, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Caixa Postal 486, CEP 31270-901, Belo Horizonte, Minas Gerais, Brazil.
E-mail addresses: anapaulamluiz@gmail.com (A.P.M. Franco-Luiz), alexandre210490@yahoo.com.br (A. Fagundes Pereira), cairo_henrique@yahoo.com.br (C.H.S. de Oliveira), diomedes@ufpa.br (J.D. Barbosa), danilobretas@yahoo.com.br (D.B. Oliveira), claudio.bonjardim@pq.cnpq.br (C.A. Bonjardim), peregrinopcp@hotmail.com (P.C.P. Ferreira), giliane@icb.ufmg.br (G. de Souza Trindade), jonatas.abrahao@gmail.com (J.S. Abrahão), ernagkroon@gmail.com, masc.egk@terra.com.br (E.G. Kroon).

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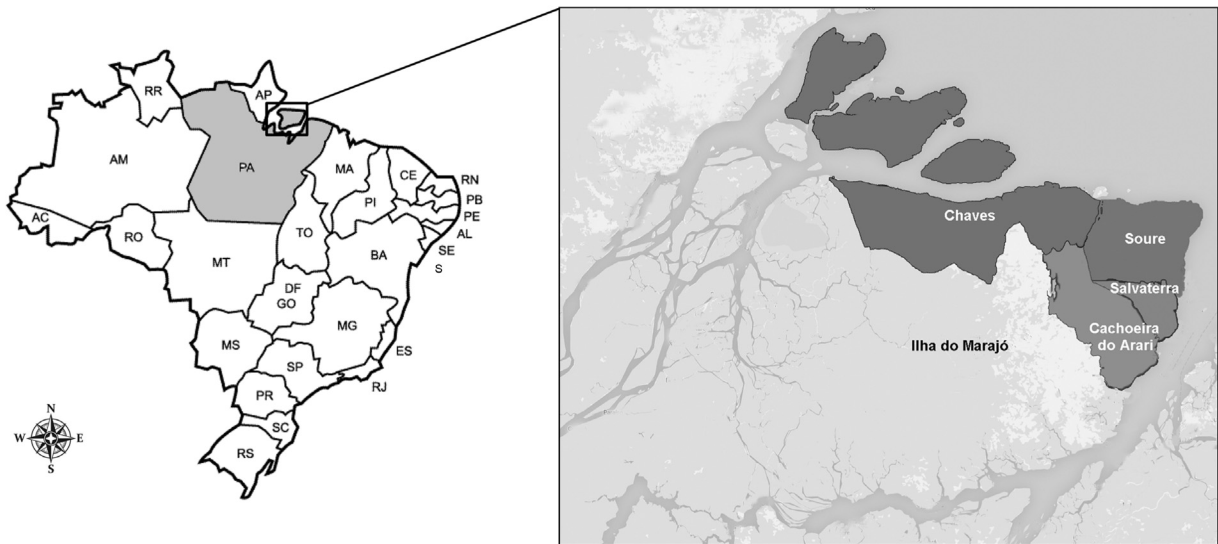

Fig. 1. Overview map of Brazil (left) and Marajó Island (right). The shaded regions represent counties where blood samples were collected. The region is located east of the island in the Amazonian Savannas.

from 53 dairy buffaloes and 97 beef buffaloes were submitted to an OPV plaque-reduction neutralization test (PRNT). PRNT assays were done in BSC40 cells seeded in 6-wells plates. The results were expressed as neutralizing units (NU)/ml wich correspond to the inverse of serum dilution that was able to neutralize at least 70% (PRNT70) of the viral plaques compared with seronegative controls, as previously described [6,8,9]. Bovine serum samples were used as positive and negative controls. On the basis of previous studies that have detected viral DNA in the sera of hosts both in the presence orabsence of clinical manifestations [8,10–12], a qPCR was used to amplify the highly conserved OPV vaccinia growth factor gene (C11R) [13]. This gene is routinely used as an OPV diagnostic tool by our group. The qPCRs were performed using the SYBr Green Mix (Applied Biosystems,USA), and the real-time PCR quality and sensitivity parameters were adjusted, including the efficiency (96.1%), R2 (1) and amplifies a target fragment of 85 bp (Alves et al., unpublished data). For molecular characterization, the non-conserved OPV hemagglutinin gene (A56R) DNA, which is a gene routinely used for this purpose, was amplified [14].

The PCR A56R DNAs were sequenced in both orientations and submitted to capillary electrophoresis (ABI3130, POP7-BigDye v3.1, Applied Biosystems). The sequences were aligned with previously published OPV sequences from GenBank using the ClustalW method, and the alignments were manually checked with MEGA version 4.0 software (Arizona State University, Phoenix, AZ, USA). The accession numbers for the analyzed sequences are given in the respective figures. Phylogenetic trees were constructed by the neighbor-joining method using the Tamura-Nei model of nucleotide substitutions and 1000 bootstrap replicates, as implemented in the MEGA 4.0 program. All field and laboratory clinical samples were manipulated separately to avoid cross-contamination, and both the serological and molecular tests were performed in two independent experiments.

3. Results

Of the 53 dairy buffalo serum samples, 37 (70%) contained OPV neutralizing antibodies; of these, 24 (65%) had titers of 100 neutralizing units (NU)/ml, five (13%) had titers of 200 NU/ml, 7 (19%) had titers of 400 NU/ml and 1 (3%) had titer of 800 NU/ml. Of the 97 beef buffaloes, 66 sera (68%) had antibodies to OPV; of these, 42 (64%) had titers of 100 NU/ml, 12 (18%) had titers of 200 NU/ml,7 (11%) had titers of 400 NU/ml, 4 (6%) had a titer of 800 NU/ml and one (1%) had a titer of 1600 NU/ml (Table 1).

To confirm that the high detected seropositivity was caused by VACV isolates in Brazil, and if the serum had detectable viral DNA, we performed a molecular screening. Of 150 serum samples, 32 (21%) were positive by the C11R-PCR assay, including 14 beef and 18 dairy buffaloes. Of 32 C11R-PCR-positive samples, 5 were also positive for the A56R gene DNA, including 3 from beef and 2 from dairy buffaloes (Table 1).

We were able to sequence two samples of A56 amplified DNA, which showed identity with group 1 VACV-BR isolates [2]. The A56R sequence from buffaloes of the Marajó Island was entitled VACV-BM2012. The sequence also showed a signature deletion of 18 nt in the A56R gene, and phylogenetic tree based on the nucleotide sequences of the A56R gene of OPV showed that the buffaloes VACV clustered together with VACV-BR group 1, but not with Group 2 VACV-BR [2] or with Indian BPXV (Fig. 2, panel A and B).

4. Discussion

Livestock farming has an economic importance in Marajó Island, and a BV outbreak could negatively impact this industry and also cause public health implications. Although no BV outbreaks have been described in animals in Marajó Island, VACV circulation in Pará State was previously described on two different and temporally isolated occasions: during the 1960s, the BeAn58058 virus was isolated from a wild rodent [15], and Pará virus (PARV) was isolated during a BV outbreak in 2010 [16]. This work has detected for the first time a high OPV seropositivity (70%) in serum from Brazilian buffaloes herds living in Marajó Island, Pará State. The molecular diagnosis for gene C11R revealed a presence of viral DNA in the sera samples (21%). Due to the conservation of duplicated gene C11R, it is useful as a genetic marker for OPV species identification however, it provides limited information for VACV sub-cluster analysis [13,14]. The molecular characterization using A56R gene revealed that VACV-BM is more closely related to PARV because both have an 18 nt deletion, similar to all isolates belonging to group 1 Brazilian VACV [2]. Furthermore, VACV-BM is genetically identical to Brazilian VACV, divergent from BPXV circulating in India and therefore not clustered together with Indian BPXV.

The immunological analyses demonstrated a high OPV seropositivity in Marajó Island, where 103 out of 150 samples were positive

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