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1 **Title: Absence of Vaccinia virus detection in a remote region from Northern Amazon forests,**
2 **2005-2015**

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16

17 **Abstract**

18 *Vaccinia virus* (VACV) circulates in Brazil and other South America countries, being responsible
19 for a zoonotic disease that usually affects dairy cattle and humans, causing economic losses and
20 impacting animal and human health. Furthermore, VACV was already detected in wild areas from
21 Brazilian Amazon. To better understand the natural history of VACV, we investigated its
22 circulation in wildlife from French Guiana, a remote region in Northern Amazon forests. ELISA
23 and plaque reduction neutralization test were performed to detect anti-*Orthopoxvirus* antibodies.
24 Real-time and standard PCR targeting C11R, A56R and A26L were applied to detect VACV DNA
25 in serum, saliva and tissue samples. VACV was not evidenced in any samples tested. These

26 findings provide additional information on the VACV epidemiological puzzle. The virus could
27 nevertheless be circulating at low levels that were not detected in areas with no human or cattle
28 presence.

29

30

31 *Vaccinia virus* (VACV) is the prototype of the *Orthopoxvirus* (OPV) genus, *Poxviridae*
32 family, and was used as a live vaccine around the world to eradicate smallpox. VACV circulates in
33 zoonotic cycles involving bovine herd and dairy workers in rural areas from Brazil, causing a
34 disease named Bovine Vaccinia (BV) which accounts for several economical losses and increasing
35 the public health concerns [1].

36 In the last seventeen years, VACV spread throughout all Brazilian territory [1], though its
37 occurrence varies among different regions. Furthermore, VACV has been detected in countries
38 bordering Brazil, such as Argentina [2], Uruguay [3], and Colombia [4]. Although since 1999,
39 several BV outbreaks affecting mainly dairy cattle and humans have been reported [1], natural
40 VACV circulation had already been described in a rodent of *Oryzomys* genus captured in the 60's in
41 the Amazon region [5].

42 During these last years in which reports of BV outbreaks have been observed, VACV
43 circulation has been described in the Amazon region, in mammal species such as bovines, primates,
44 coatis, and also humans [6–12]. Nevertheless, there is little information on a possible VACV
45 sylvatic cycle and on its natural reservoirs since the origin of VACV is still unknown and its natural
46 reservoir is still unidentified [13–16]. Given that VACV has a broad host range and that wild
47 animals might be implicated in its transmission chain, we decided to search for VACV circulation
48 in wildlife animals (rodents, marsupials, and bats) captured in pristine forest areas with the absence
49 or limited anthropogenic impacts of Northern Amazon forests.

50 We analyzed a total of 2,365 wild mammals from French Guiana, captured between 2005
51 and 2015 in tropical upland rain forest, Amazon region [17, 18]. Captured sites were located in
52 remote forests, slightly disturbed forests (facing selective logging, hunting pressure, and low
53 fragmentation level), secondary forests and edge between forest and open habitats (Figure 1). Wild
54 mammals included rodents (n = 420), marsupials (n = 606) and Chiroptera (n = 1,339) (Table 1).
55 All samples underwent molecular testing for VACV. To detect VACV DNA we used real-time PCR
56 targeting the C11R and A56R genes, and semi-nested PCR targeting C11R and A26L genes [19]. A
57 molecular approach was performed in serum and tissue samples from rodents and marsupials, and
58 in DNA extracted from saliva and blood clots of bats. The positive controls used on molecular assays
59 were DNA extracted from serum and liver of experimental infected Balb/c mice with *Vaccinia virus* Western
60 Reserve (VACV-WR) strain and Brazilian Vaccinia (Br-VACV) group 1 and 2 viruses [20]. For those for
61 which we were not able to have an experimental infected animal (marsupials and bats), we provided
62 an experimentally contamination of serum and liver samples as in the protocol previously described
63 by Dutra and co-authors [21]. Briefly, it was added different concentrations of VACV-WR and Br-
64 VACV strains (with a range from 10^4 pfu/ μ L to 1 pfu/ μ L, respectively). The experimentally
65 contaminated samples were tested by PCR, compared to the Balb/c controls, and then used as
66 positive controls for marsupials and bats.

67 In addition, serum samples from the rodents and marsupials were screened for anti-OPV IgG
68 by ELISA and neutralizing antibodies by $\geq 50\%$ plaque reduction neutralization test (PRNT₅₀) [19].
69 Sera were grouped into pools of 2–5 serum samples from animals belonging to the same species
70 that were from the same collection area. No serum samples from bats were available for serological
71 tests. For serological assays, the positive controls were also those provided by experimental infected
72 mouse with VACV-WR and Br-VACV strains [20]. For the ELISA assay particularly, we used
73 purified recombinant A/G protein peroxidase conjugated as secondary antibody. We run rodent and

74 marsupial samples together in the same plate (for ELISA and PRNT), comparing the results with
75 known positive and negative rodents' serum samples.

76 Most of the rodents captured belong to the species *Proechimys guyannensis* (46,4%),
77 followed by *Proechimys cuvieri* (11,2%) and *Zygodontomys brevicauda* (9,5%). The main
78 marsupials studied were *Didelphis marsupialis* (34,1%) and *Marmosa murina* (26,2%). For bat
79 species, most of them were *Desmodus rotundus* (32,3%), *Carollia perspicillata* (17,2%) and
80 *Pteronotus* spp. (14,0%). All animals tested by both serological and molecular techniques were
81 negative for VACV.

82 In this study, we assessed numerous forest mammal species and found no serological or
83 molecular evidence for VACV circulation among them. Increasing efforts have been done regarding
84 VACV ecology in South America, and some studies have highlighted the importance of wild
85 animals and peridomestic rodents as VACV carriers [7, 22, 23, Miranda JB, personal
86 communication/in peer review]. Our negative results contrast with the above-mentioned ones and
87 some hypotheses can be considered in explaining the lack of evidence for VACV circulation in wild
88 animals in French Guiana during the 2005-2015 period. One hypothesis is that wildlife was
89 investigated in a highly preserved Northern Amazon region, where natural geographic barriers of
90 unfavorable forest habitats exist, preventing VACV dissemination from Brazil to French Guiana.
91 This could also be emphasized by the "dilution effect" hypothesis, taking into account that in a
92 highly preserved environment, the maintenance of a highly diverse mammal community may
93 prevent infection of putative more sensitive species. A great diversity of mammal species was
94 studied, but with only a small sampling of species from which VACV has been detected in Brazil,
95 such as *Mus musculus* [20], *Oryzomys sp* [5], and *Caluromys philander* [Miranda JB, personal
96 communication/in peer review]. Furthermore, the species *Didelphis albiventris* from which VACV
97 was recently detected in São Paulo State, Southeast region of Brazil [24], is not present in French

98 Guiana. Nevertheless, two closely related species *Didelphis imperfecta* (n = 2) and *D. marsupialis*
99 (n = 207) were tested.

100 Another explanation for the absence of VACV detection in this study could be the extremely
101 low number of bovines in French Guiana. One hypothesis for the broad circulation of VACV in
102 Brazil is that bovines are acting as VACV amplifiers, excreting high loads of viable particles, being
103 a source of infection for themselves and other animals [25]. VACV can be detected in animals
104 without clinical disease since it has been detected in blood samples and feces in animals without
105 lesions but presenting neutralizing antibodies [25]. Due to the potential role of cattle as a viral
106 amplifier, the presence of bovines could facilitate VACV persistence and dissemination into the
107 environment.

108 VACV has recently been identified at the borders of Brazil, in countries such as Argentina
109 [2], Uruguay [3], and Colombia [4]. Even though the circumstances of VACV detection in South
110 American countries are still uncertain, the cattle trade and translocation has to be considered, as
111 highlighted in Amazon region (Mato Grosso and Rondônia states) [9]. Furthermore, some authors
112 have hypothesized that the seroprevalence of OPV-antibodies in human populations from the
113 Amazon basin (Acre state) could be linked to agriculture and livestock practices, reinforcing the
114 role of cattle on VACV maintenance [8].

115 Compared to other South American countries where VACV has been detected, a different
116 situation is observed in French Guiana where a lower deforestation rate is observed and where only
117 a few cattle or horses are present [26] (<http://www.globalforestwatch.org/>) (Figure 1). Nevertheless,
118 our findings do not definitively establish that VACV is not circulating, and also do not exclude the
119 possibility that small mammals can act as its reservoir in nature. However, data presented here
120 strongly suggest the absence of circulation of VACV, or at low levels that were not detected, in
121 remote Amazonian areas with no human impact over a long period of time. The monitoring and

122 surveillance of VACV circulation is important to ensure early warning of zoonotic outbreaks for
123 vulnerable human populations.

124

125

126 **Legend for figure:**

127 An overview of French Guiana with the location of the studied regions, where were implemented
128 several trapping sites. The background of the map highlights the human footprint index expected to
129 summarize the anthropic threats on biodiversity. These threats (accessibility, land use, human
130 density) are summed and allowed producing an index with lower pressures strenght in light and
131 higher pressures darker. For each studied region, the dominant habitat is given.

132

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144

145 **Conflict of interest**

146 Authors declare no conflict of interest.

147

148 **Ethical approval**

149 Captures of rodents, bats and marsupials do not require ethic committee in French Guiana.

150

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