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Co-circulation of vaccinia and monkeypox viruses in rural areas of Brazil: Importance of differential molecular diagnosis

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Monkeypox virus (MPXV), an orthopoxvirus (OPXV) of the *Poxviridae* family, is the etiologic agent of Mpox. The disease has spread to more than 100 countries through sustained human-to-human transmission since May 2022. Atypical clinical presentations have been observed with single or few body lesions, as well as more severe cases with extensive genital and perianal lesions associated with rectal pain and proctitis [1].

Until March 24, 2023, more than 10,800 cases and 15 deaths were reported in Brazil. Cases were initially concentrated in the main urban centers with intense international traffic and, later, other states also reported cases, mainly in urban environments, in association with domestic travelers who participated in mass gathering events. The establishment of community transmission led to the detection of Mpox cases in more peripheral communities and underprivileged regions. However, the detection of Mpox cases in rural and agricultural areas of Brazil has not been common [2].

Rural and farming regions of Brazil harbor an endemic circulation of the vaccinia virus (VACV; *Orthopoxvirus* genus), which has also been reported in Colombia and India [3]. Infection causes fever, lymphadenopathy, and pustular lesions similar to those generated by Mpox on the hands and arms of milkers and on the teats and udders of dairy cows and buffaloes. Since 1999, our group and others have detected the circulation of the VACV strain Cantagalo (CTGV) in several states of Brazil, including regions with intense agricultural activity in the Midwest region of Brazil [3–5].

Goiás is a state in the Midwest with extensive rural areas located a short distance from urban centers. It is the first milk producer in the Midwest and an important region for rural tourism and national and international ecotourism. Since July 2022, Goiás has notified 563 cases of Mpox in different cities, some in rural municipalities such as Uruana (Fig. 1A). In August 2022, the Coordination of Zoonosis and the Central Laboratory of Public Health of the state of Goiás (LACEN/GO) reported four cases of uncertain clinical diagnosis suspected of Mpox or vaccinia. The patients were rural workers from three municipalities: Faina, Cocalzinho de Goiás, and Uruana (Table 1). Patients had isolated pustular lesions in the hands and arms that were clinically compatible with MPXV or VACV infection. Fever, asthenia, malaise, local edema, and muscle ache were reported.

Skin fragments and/or lesion swabs were collected, and DNA was extracted to be used in a 4-plex real-time PCR assay (Kit Molecular 4Plex OPXV/MPXV/VZV/RP, Bio-Manguinhos/Fiocruz, Brazil). All samples were negative for MPXV and positive for OPXV (Table 1). The DNA samples were then used for conventional PCR to detect OPXV by amplification of the hemagglutinin gene [4], followed by nested PCR to specifically detect VACV strain Cantagalo by the amplification of a target fragment of the CTGV hemagglutinin gene, as described elsewhere [6]. The four clinical samples were again positive for OPXV detection (Fig. 1B, top panel), confirming the 4-plex diagnosis. As expected, control DNA samples from VACV strains WR and CTGV CM-01 were also positive. However, in the nested PCR specific for CTGV detection, only the control CTGV CM-01 and the four clinical samples from Goiás were amplified (Fig. 1B, bottom panel). These results confirmed that the four rural workers were infected with VACV strain Cantagalo and not with MPXV.

To analyze the viral protein profiles, we infected BSC-40 (African Green Monkey) cells with each swab eluate from the four clinical samples, as described elsewhere, or with control viruses CTGV CM-01 or a MPXV isolate from Goiás. Manipulation of MPXV and VACV samples was performed in biosafety level (BSL)-3 and BSL-2 containment laboratories, respectively. After 24 hours of infection, cells were resuspended in SDS-containing buffer and analyzed by 11% polyacrylamide-SDS gel electrophoresis, followed by Western blot using antibody against structural VACV proteins [4]. A typical pattern of viral structural proteins was detected in the CTGV samples from Goiás, similar to the CTGV CM-01 used as the control virus (Fig. 1C). The anti-VACV antibody also detected MPXV structural proteins, as expected, due to the well-known cross-reactivity among orthopoxviruses (Fig. 1C). Interestingly, the protein profile of MPXV was almost indistinguishable from that of VACV, reinforcing that immunological methods, such as Western blots or even ELISA, are not suitable for the diagnosis of Mpox as they do not distinguish MPXV from other orthopoxviruses, particularly VACV, as shown in Fig. 1C.

DNA was recovered from cytoplasmic extracts of cells infected with each of the four clinical isolates of Goiás and used for whole genome

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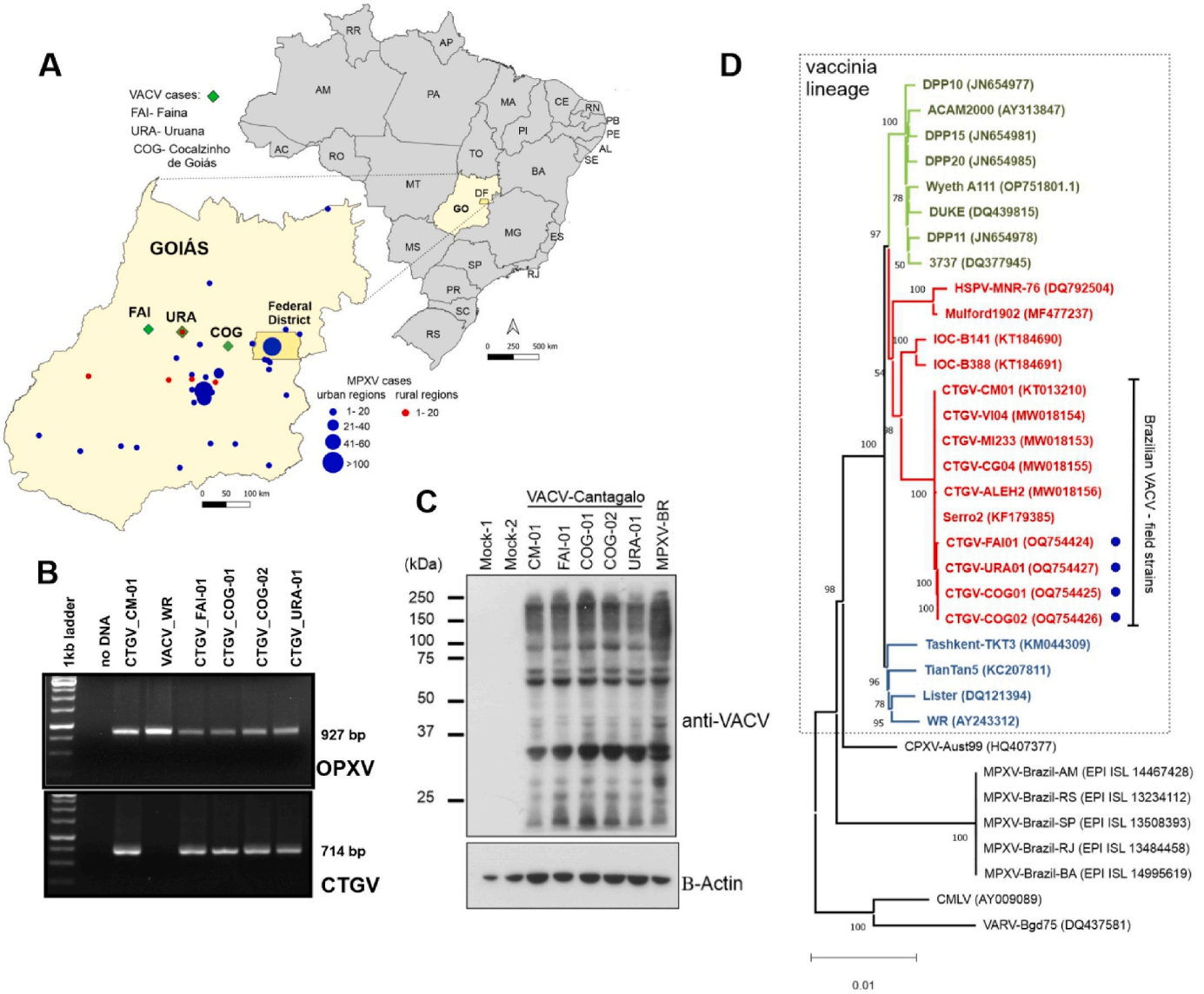

Fig. 1. Detection of vaccinia virus strain Cantagalo in rural areas of Goiás, Brazil. A) Map of Brazil highlighting the state of Goiás (QGIS 3.30.0 ″s-Hertogenbosch', https://qgis.org/). Green diamonds indicate cases of VACV. Blue and red circles indicate cases of MPXV in urban centers and rural areas, respectively. B) PCR detection of the hemagglutinin gene (927 bp) in the upper panel. Nested PCR for specific detection of a fragment of VACV strain Cantagalo (CTGV) hemagglutinin gene (714 bp). C) Western blot analysis of CTGV and MPXV (a clinical isolate from Goiás) structural proteins using antiserum raised against VACV structural proteins. B-Actin was used as loading control. Mock-1 and Mock-2 refer to two plates of non-infected cells used as control. D) Neighbor-joining phylogenetic tree (Mega 11 vs 11.0.10) of the central conserved region (approximately 90,000 bp) from 34 orthopoxvirus genomes, opting for kimura-2p and 1000 replicates of bootstrap. The dotted box indicates the vaccinia lineage, showing the three clades in green, red, and blue. Blue circles indicate the genomes from the clinical samples analyzed in this work. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Clinical isolates from Goiás, Brazil, used in this study.

Municipality	Faina	Cocalzinho de Goiás		Uruana
Laboratory designation	FAI-01	COG-01	COG-02	URA-01
MPXV Ct values	Negative	Negative	Negative	Negative
OPXV Ct values	17.84	25.85	14.87	20.49
Genome size (bp)	186,425	187,256	186,884	187,259
genome coverage	3057 x	404 x	497 x	445 x

sequencing using the Illumina platform, as described elsewhere [7]. Sequencing statistics are described in Table 1. The phylogenetic tree based on the central conserved region of the genomes from 34 orthopoxviruses shows that the four clinical isolates from Goiás group with other CTGV isolates from different regions of Brazil. As expected, all CTGV genomes lie within the vaccinia lineage and, therefore, distant from the MPXV isolates (Fig. 1D).

In conclusion, our work shows that the VACV strain Cantagalo co-circulates with MPXV in the state of Goiás, which may occur in other rural regions of Brazil, as CTGV has been detected in several states where positive cases of Mpox have been reported. This fact raises a warning that specific diagnostic tests that differentiate MPXV from other OPXV should always be used in countries where the circulation of non-monkeypox OPXV occurs, such as Brazil, Colombia and India. Our data also reinforce that Western Blot or other immunological tests are not adequate for the diagnosis of MPXV. Travelers should be alert if Mpox-like lesions appear in the hands and arms after visiting dairy farms with sick animals and seek for differential diagnosis.

Author contributions

M.N.L., R.L.O., D.S.N., L.M.H. and C.R.D designed the experiments; A.F.M., L.A.P., and F.S. identified the cases and collected samples; T.M. P.P.C., A.T. and C.R.D. provided resources and funding; S.H., R.L.O., D. S.N. and L.M.H. conducted virus isolation and propagation; D.S.N. performed Western blot assays; M.N.L., S.H., and R.L.O. performed PCR tests for OPXV and VACV; M.N.L. performed sequencing, sequence assembly, genome annotation, and phylogenetic analysis; C.R.D. wrote the original draft and edited the final version; C.R.D., L.M.H, D.S.N., R.L.O, M.N.L., T.M.P.P.C. and A.T. reviewed and edited the manuscript with inputs from all authors.

Ethical statement

This study was approved by the Research Ethics Committee of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro, under the protocol number CAAE 62281722.5.0000.5257.

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Declaration of competing interest

The authors declare no competing interests.

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