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SHORT REPORT: ISOLATION OF TWO *VACCINIA VIRUS* STRAINS FROM A SINGLE BOVINE VACCINIA OUTBREAK IN RURAL AREA FROM BRAZIL: IMPLICATIONS ON THE EMERGENCE OF ZOONOTIC ORTHOPOXVIRUSES

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Abstract. Outbreaks of bovine vaccinia disease caused by circulation of *Vaccinia virus* (VACV) strains have been a common occurrence in Brazil in the recent years, being an important emergent zoonosis. During a single outbreak that took place in 2001, two genetically different VACV strains were isolated and named Guarani P1 virus (GP1V) and Guarani P2 virus (GP2V). Molecular diagnosis was done through restriction fragment length polymorphism (RFLP) of *ati* gene (A26L) and by sequence analysis of a group of five VACV genes including the *C11R*, *J2R*, *A56R*, *B18R*, and *E3L* genes. These findings confirmed the co-circulation of two different *Vaccinia virus* strains during the same outbreak, raising important questions about the origin, emergence, and circulation of VACV strains in Brazil.

Since 1999, an increasing number of episodes of eruptive skin disease affecting dairy cattle and humans have been reported in Brazil.[1–6] Our research team has been working on these zoonotic outbreaks, and in October of 2001, we studied an outbreak that occurred in Guarani town, Minas Gerais State, in the southeast region of the country.

An epidemiologic study was conducted on the affected area involving 72 visited properties, which comprised an area covered by ~5,700 km of roads. The study revealed that a total of 1,020 lactating cows presented lesions on the teats characterized by the presence of papules that evolved to ulcers. After some time, most of these lesions naturally progressed to healing. In 83% of the farms, human cases were registered, and ~110 persons were infected. Most sick humans were milkers that were contaminated after contact with lesions on cow's teats. These milkers presented pleiomorphic lesions on the hands (mainly papules and painful ulcers), fever, lymphadenitis with enlarged lymph nodes, and eventually secondary bacterial infection on the lesions. Also, in some farms, milkers reported transmission from person to person. The time-course of the infection was between 15 and 30 days. Initial diagnosis was done based on the observed clinical and epidemiologic features.[3]

To attempt laboratory diagnosis and virus isolation, two samples of dried scabs from two affected cows were collected. Each cow belonged to a different neighboring farm, and these farms were localized 10 km apart from each other. To avoid any possibility of laboratory cross-contamination, the different samples were never manipulated simultaneously. Two viral isolates were obtained and named Guarani P1 virus (GP1V) and Guarani P2 virus (GP2V). Virus isolation was performed using conventional methods including inoculation onto chorioallantoic membrane (CAM) of chicken embryonated eggs and plaque purification cloning on Vero cells to assure genetic homogeneity. These viruses were grown and purified in sucrose gradient as described elsewhere.[2,3]

Biologic diagnosis was based on pock morphology in CAM and a serum neutralization test using anti-VACV polyclonal rabbit serum as a positive control.[2,3] In addition, atomic force microscopy was also used to look for typical poxviruses particles on the samples (data not shown).[7]

Molecular diagnosis of the isolated viruses was performed by polymerase chain reaction (PCR) amplification of the A type inclusion body gene (*A26L*). The *A26L* gene analysis, based on partial amplification of this gene followed by digestion using *Xba*I, has been widely used as a tool for rapid screening and taxonomic differentiation of *Orthopoxvirus*.[8,9] The GP2V *A26L* gene was amplified, and the amplicon was similar in size to those described for Araçatuba virus (ARAV) and Passatempo virus (PSTV), other VACV strains isolated under similar circumstances in 1999 and 2003, respectively.[2,6] When the *A26L* amplicon of GP2V was digested with *Xba*I, it presented the same restriction pattern as in ARAV, which was also similar but not identical to those patterns obtained for known VACV strains, such as Western Reserve (WR) and Lister (LST) (Figure 1A). The main difference observed for the GP2V and ARAV *A26L* gene amplicon digests was that the larger fragment generated after *Xba*I digestion migrated faster on the agarose gel than the VACV-WR and VACV-LST analogous fragments (Figure 1A). Attempts to amplify the *A26L* gene of GP1V, using the ATI up and ATI low primers, were unsuccessful (data not shown).[8] The lack of this amplicon could be caused by a deletion at the *A26L* gene of the GP1V, a fact previously observed for other Brazilian VACV strains, such as BeAn58058 virus (BAV) and Belo Horizonte Virus (VBH).[10,11] To verify the extension of the probable GP1V *A26L* deletion, we performed a PCR amplification using oligonucleotides P4c1 (located within the *p4c1* gene) and RNApol, designed based on *Cowpox virus* Brighton Red (CPXV BR) as described elsewhere.[11] For CPXV, VACV-WR, and GP2V, a product of ~4.300 bp was obtained. However, for GP1V, a fragment of 300 bp (Figure 1B) was generated and sequenced (GenBank accession no. DQ363383), and it proved that, in GP1V, as well in VBH and BAV, a

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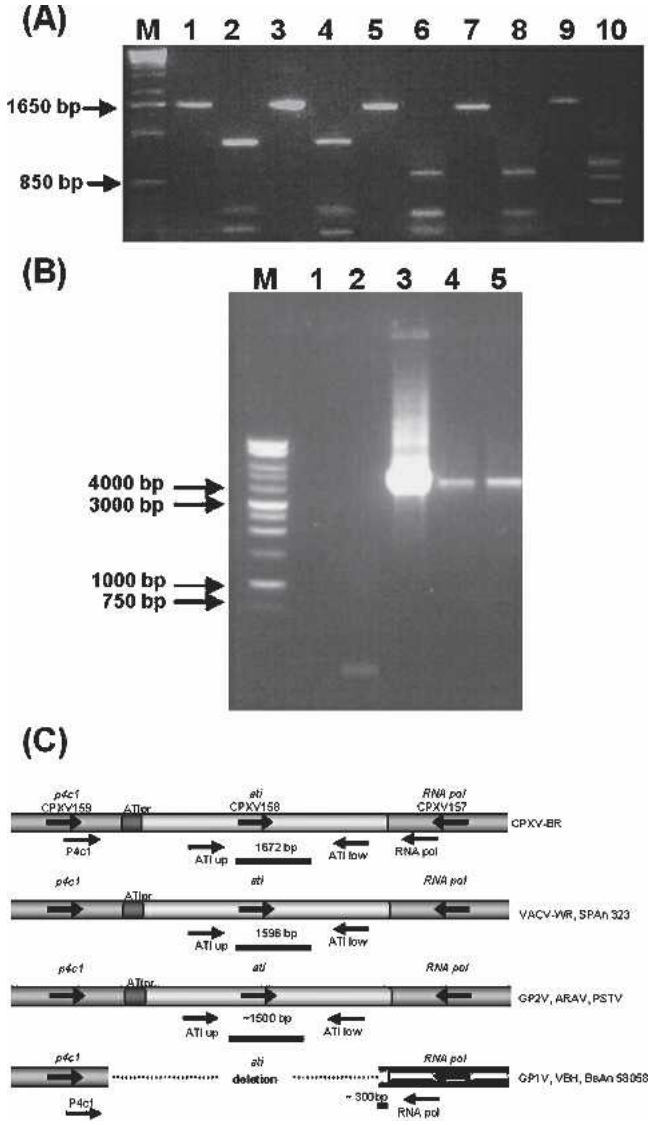

FIGURE 1. **(A),** PCR-RFLP analysis of A26L gene amplicons of VACVs isolated in Brazil. Lanes were grouped: odd numbers correspond to non-digested PCR and even numbers to RFLP (M, molecular size marker): 1–2, VACV-WR; 3–4, VACV-LST; 5–6, GP2V; 7–8, ARAV; 9–10, CPXV-BR. PCR amplicons digestions were performed using *Xba*I endonuclease. PCR and PCR-PFLP fragments were separated by electrophoresis in a 1% agarose gel stained with EtBr. **(B),** PCR amplification employing P4c1 and RNApol primers. Products were fractionated by electrophoresis in a 1% agarose gel stained with EtBr. Lanes (M, molecular size marker; 1-Kb DNA ladder; Promega): 1, negative control; 2, GP1V; 3, GP2V; 4, VACV-WR; 5, CPXV-BR. **(C),** Schematic comparison of sequenced regions of VAC-WR, GP2V, GP1V, VBH, and CPXV-BR based on work by Meyer and others.[8] Boxes, genes; thin arrows, primer positions; bold arrows, orientation of genes; dashed lines, deletion.

major portion of the *A26L* gene is missing (Figure 1C). Additionally, it is important to point out the usefulness of the *A26L* PCR-restriction fragment length polymorphism (PCR-RFLP) as a molecular diagnosis approach and for differentiation of Brazilian *Orthopoxvirus* species.[10,11]

Because GP1V and GP2V presented extensive differences in their *A26L* ORF, we decided to study other genes to evaluate genomic differences between GP1V and GP2V and other Brazilian VACV strains, such as ARAV, PSTV, Cantagalo virus (CTGV), BAV, and VBH.[1,2,6,11–13] The thymidine kinase (J2R), vaccinia growth factor (C11R), hemagglutinin (A56R), soluble alpha/beta interferon (IFN) receptor (B18R), and dsRNA-binding protein of VACV (E3L) were chosen for sequencing and analysis. *J2R*, *C11R*, and *A56R* are conserved genes in the *Orthopoxvirus* genus and have been used before as a taxonomic tool to study ungrouped poxvirus.[11,12,14] *B18R* and *E3L* genes, both involved in the viral mechanism of pathogenesis, were also amplified from GP1V, GP2V, and other Brazilian VACV strains.[15,16] Primers and the reactions conditions to amplify the *B18R* gene were those described by Marques and others.[10] The primers based on the *E3L* nucleotide sequence of VACV-WR were *E3L* sense (5′-AGGCGGATCCATGTCTAAAATCTATATC-3′) and E3L anti-sense (5′-TCGCAAGCTTTCAGAATCTAATGATGAC-3′), containing restriction sites for *Bam*HI and *Hind*III (underlined), respectively, and they were used to amplify a 589-bp DNA fragment. Annealing was carried out at 45°C, and PCR reactions were performed as described by Marques and others.[10]

Amplicons were cloned into pGEM-T vector (Promega Corp., Madison, WI) and sequenced in both orientations by the dideoxynucleoside method, using M13 universal primers and ET Dynamic Terminator for MegaBACE (GE HEALTHCARE, UK).[17] Nucleotide sequences were assembled using the CAP3 Sequence Assembling Program and deposited in GenBank.[18] Nucleotide and inferred amino acids sequences were aligned with other *Orthopoxvirus* sequences using CLUSTAL W and LALIGN.[19,20] *J2R* sequences of GP1V and GP2V (GenBank accession no. DQ206438 and DQ206439, respectively) were identical to each other, presenting high levels of similarity with VACV sequences. These sequences were also identical to VACV-WR and other Brazilian VACV strains, such as BAV, SPAn232, ARAV, and PSTV (data not shown).[2,6,12,13] C11R sequences of GP1V and GP2V (GenBank accession no. DQ206440 and DQ206441, respectively) presented 99.4% of identity compared with each other and high levels of similarity among other VACV strains.

B18R, E3L, and *A56R* sequences of GP1V and GP2V were also similar to other VACV strains sequences, although there was a higher level of genetic variance among them. GP1V and GP2V *B18R* sequences presented seven nucleotide substitutions that resulted in the two amino acid modifications in the inferred amino acid sequences. For GP1V, GP2V, ARAV, and BAV, in the position 346 of the B18R protein, a serine was found, whereas in VACV-WR, VACV-IOC, and CTGV, there was a threonine in the same position (data not shown). The GP1V and GP2V *E3L* genes presented 98.6% of identity to each other, with substitutions in eight nucleotide positions (Table 1). These mutations led to six amino acid differences observed between the GP1V and GP2V inferred amino acid sequences (data not shown).

However, when the GP1V and GP2V *A56R* sequences were compared, relatively lower levels of similarity were observed in both nucleotide (95.7%) and amino acid (92.9%) levels. Comparing the GP2V *A56R* sequence to other Brazilian VACVs, similarity values ranging from 99.1% to 99.3% were obtained (Table 1). As observed for ARAV, PSTV, CTGV, and VACV-IOC, GP2V also presented the same 18-nucleotide deletion signature showed previously at the *A56R* gene of those VACV strains. This 18-nucleotide deletion has been found in all Brazilian VACV strains isolated to date,

TABLE 1
Nucleotide and amino acid percentage of identities* among sequences of *Vaccinia virus* strains

	A56R gene sequences/hemagglutinin protein sequences†										
	WR	GP1V	GP2V	ARAV	PSTV	CTGV	VBH	IOC	COP	LST	Wyeth
WR		100	92.9	94.2	93.9	93.9	99.3	96.6	97.6	99.0	95.9
GP1V	100		92.9	94.2	93.9	93.9	99.3	96.6	97.6	99.0	95.9
GP2V	95.7	95.1		98.6	98.3	99.0	92.2	95.5	92.5	93.2	94.5
ARAV	96.3	96.3	99.2		99.7	99.7	93.5	96.9	93.9	94.6	95.5
PSTV	96.1	96.1	99.1	99.9		99.3	92.9	96.5	93.6	94.2	94.8
CTGV	96.1	96.1	99.3	99.9	99.7		93.2	96.5	93.6	94.2	95.5
VBH	99.4	99.4	95.1	95.7	95.5	96.1		95.9	96.9	98.3	95.2
IOC	97.1	97.1	97.9	98.7	98.5	98.6	96.5		96.9	96.9	95.8
COP	98.2	98.2	95.0	95.8	95.6	95.7	97.5	96.8		98.6	95.6
LST	98.9	98.9	95.5	96.3	96.0	96.2	98.3	97.1	99.1		95.6
Wyeth	96.7	96.7	96.8	97.6	97.5	97.5	96.1	97.5	96.4	96.3	

	E3L gene sequences/dsRNA-binding protein sequences‡										
	WR	GP1V	GP2V	ARAV	PSTV	CTGV	VBH	IOC	COP	LST	Wyeth¶
WR		100	96.8	96.8	100	96.8	100	97.9	96.8	98.4	
GP1V	100		96.8	96.8	100	96.8	100	97.9	96.8	98.4	
GP2V	98.6	98.6		100	96.8	100	96.8	96.8	98.9	97.4	
ARAV	98.8	98.8	98.8		96.8	100	96.8	96.8	98.9	97.4	
PSTV	100	100	98.6	98.8		96.8	100	97.9	96.8	98.4	
CTGV	98.4	98.4	99.5	99.6	98.4		96.8	96.8	98.9	97.4	
VBH	99.8	99.8	98.4	98.6	99.8	98.2		97.9	96.8	98.4	
IOC	98.8	98.8	98.2	98.4	98.8	98.8	98.8		96.8	97.4	
COP	98.2	98.2	98.6	98.8	98.2	99.1	98.1	98.6		98.4	
LST	98.8	98.8	98.1	98.2	98.8	98.2	98.6	98.4	99.1		

	B18R gene sequences/soluble alpha/beta inferferon receptor protein sequences§										
	WR	GP1V	GP2V	ARAV	PSTV	CTGV	VBH	IOC	COP	LST**	Wyeth¶
WR		100	99.4	99.7	99.7	99.7	100	99.0	99.0		
GP1V	99.8		99.4	99.7	99.7	99.7	100	99.0	99.0		
GP2V	99.2	99.2		99.7	99.7	99.7	99.4	99.0	99.0		
ARAV	99.4	99.4	99.9		100	100	99.7	99.4	99.4		
PSTV	99.4	99.4	99.9	100		100	99.7	99.4	99.4		
CTGV	99.4	99.4	99.9	100	100		99.7	99.4	99.4		
VBH	99.8	100	99.2	99.4	99.4	99.4		99.0	99.0		
IOC	99.1	99.1	99.0	99.1	99.1	99.1	99.1		98.7		
COP	99.1	99.1	99.2	99.4	99.4	99.4	99.1	98.9			

* Identities were calculated as the percentage of nucleotide or amino acid substitution on two by two comparison of strains sequences. Sequences alignments and the percentage of identity were calcuated by the software LALIGN. Nucleotide sequence identities are shown on the down left side of the table. Amino acid identities are presented in gray on the right side of the table. The compared nucleotide sequences correspond to positions †162207–163089, ‡47780–48349 and §179193–180124 of *A56R, E3L,* and *B18R* regions, respectively, according to the published sequence of VACV-WR genome (GenBank accession no. AY243312). *Vaccinia virus strains:* WR, Western Reserve; IOC, Instituto Oswaldo Cruz; COP, Copenhagen; LST, Lister. Brazilian *Vaccinia virus* strains: GP1V, Guarani P1; GP2V, Guarani P2; ARAV, Araçatuba; PSTV, Passatempo; CTGV, Cantagalo; VBH, Belo Horizonte.
E3L and *B18R* sequences of *Vaccinia virus* Wyeth are not available.
** The *B18R* gene is not present in the *Vaccinia virus* Lister genome.

and it has been proposed as a molecular signature of new world VACV strains. However, GP1V and VBH did not present this deletion. Additionally, the lack of this deletion in the GP1V and VBH sequences points out the necessity to review the 18-nucleotide deletion as a molecular signature of new world VACV strains.

To establish a phylogenetic relationship among GP1V, GP2V, and other VACV strains, alignments of the *A56R, B18R*, and *E3L* genes were used to construct a concatenated phylogenetic tree by the neighbor-joining method using the Tamura Nei model of nucleotide substitutions implemented in MEGA3.[21,22] The tree was midpoint-rooted, and 1,000 bootstrap replicates were performed (Figure 2A).

As expected, the concatenated phenogram tree grouped GP1V and GP2V within VACV strains. Although isolated from the same outbreak, at the same time, and from neighboring farms, GP1V and GP2V presented enough genetic divergence to place them in different branches of the phylogenetic tree. Whereas GP2V was clustered with Brazilian VACV strains isolated in previous bovine vaccinia outbreaks (ARAV, CTGV, and PSTV), GP1V was grouped together with VACV-WR and VBH. Neither GP1V nor GP2V were clustered with VACV strains LST and IOC that were used in Brazil during the smallpox vaccination campaign.[1,2]

Our findings show that there are genetically different populations of VACV circulating in the country and even in the same outbreak. It has been proposed that the Brazilian VACV isolates obtained from cowpox-like outbreaks form a genetically homogenous population that could have been originated from the spread of a smallpox vaccine strains, particularly the VACV IOC.[1] According to this theory, these strains could have escaped to wild and established circulation in some unknown host, becoming feral during this process.[1,2,6] However, the characterization of two different VACV strains isolated from a single outbreak, co-circulating in Brazil, points in a different direction. One hypothesis to explain this virus diversity is that different samples, from different sources, and under different circumstances, could have established circulation in nature. Thus, these viruses would have not one but multiple origins. This does not entirely exclude

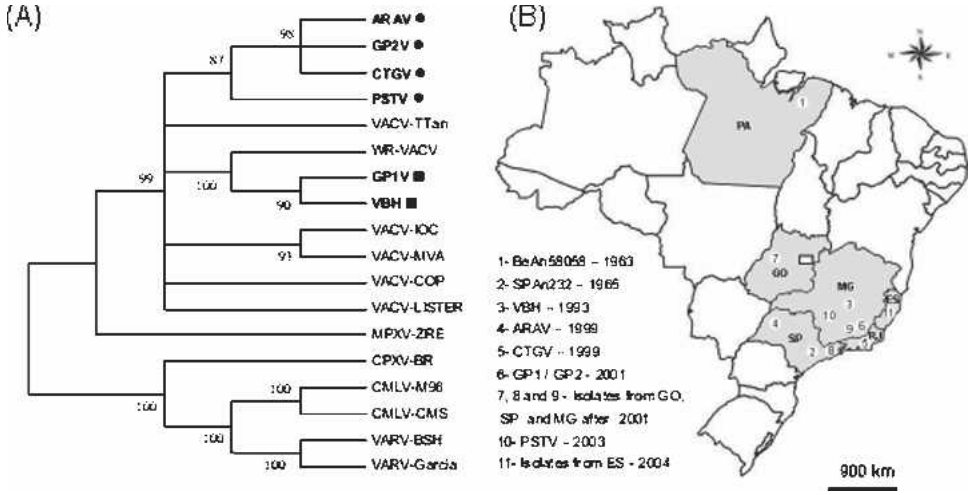

FIGURE 2. **A,** Consensus concatenated phylogenetic tree based on the nucleotide sequences of *Orthopoxvirus A56R, B18R*, and *E3L* genes. The tree was constructed by the neighbor-joining method using the Tamura Nei model of nucleotide substitutions implemented in MEGA3. The tree was midpoint-rooted, 1,000 bootstrap replicates were performed, and values > 70% are shown. Nucleotide sequences were obtained from GenBank. The GenBank accession numbers of *A56R*, *B18R*, and *E3L* sequences are GP1V (DQ206436, DQ194380, and DQ194385), GP2V (DQ206437, DQ194381, and DQ194386), ARAV (AY523994, DQ194382, and DQ194389), PSTV (DQ070848, DQ530239, and DQ530240), and VBH (DQ206435, DQ194383, and DQ194390), respectively. Brazilian VACV strains are shown in bold. ■, Brazilian strains that presented partial A26L gene deletion; ●, Brazilian strains that presented 18 nt deletion in the *A56R* gene. **B,** Brazilian states where and when Brazilian VACV strains were isolated. ES, Espírito Santo; GO, Goiás; MG, Minas Gerais; PA, Pará; RJ, Rio de Janeiro; SP, São Paulo. 1, BeAn 58058 virus in 1963; 2, SPAn232 virus in 1965; 3, Belo Horizonte virus in 1993; 4, ARAV in 1999; 5, CTGV in 1999; 6, GP1V and GP2V in 2001; 7, GO isolates after 2001; 8, SP isolates after 2001; 9, MG isolates after 2001; 10, PSTV in 2003; ES isolates in 2004 (unpublished data).

the possibility that the viruses could have evolved from a common origin, derived from a escapee vaccine strain, and differentiated by means of mutation and/or recombination events during circulation in nature. However, our results suggest that multi-factor events may be the best explanation for the VACV genetic diversity observed in Brazil, including the pre-existence and circulation of a possible autochthonous poxvirus.

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