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Original article

Brazilian Vaccinia virus strains are genetically divergent and differ from the Lister vaccine strain

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Abstract

Vaccinia virus is responsible for an important zoonotic disease affecting dairy cattle and humans in Brazil, but little is known about the origin, epidemiology and evolution of these Brazilian Vaccinia virus strains. In this work, seven Brazilian Vaccinia virus strains and the Lister-derived Brazilian vaccine strain, named Lister-Butantan, were compared based on the sequences of ten host range and virulence related genes. Comparison of Brazilian Vaccinia virus strains with Lister-Butantan revealed several differences. Phylogenetic analyses confirmed the existence of genetically distinct Brazilian Vaccinia virus groups and has not thus far demonstrated a close relationship between Brazilian strains and Lister-Butantan. In this study, the BeAn58058 and SPAn232 strains were grouped together with the Belo Horizonte and Guarani P1 strains. Additionally, genetic polymorphisms in host range and virulence genes as well as differences in the deduced amino acid sequences were detected among Brazilian Vaccinia virus. This genetic diversity may result in a plethora of different biological properties presented by Brazilian Vaccinia virus, including differences in adaptation to the host as well as pathogenic properties. Furthermore, co-circulation of these divergent strains could increase the possibility of recombination events in nature, leading to the formation of new variants with unpredictable pathogenic potential.

Keywords: Poxvirus; Vaccinia virus; Smallpox; Vaccine; Poxvirus infections; Phylogeny, Genetic diversity; Genetic polymorphisms

1. Introduction

Members of the Poxvirus family, which includes the Variola virus (VARV) and Vaccinia virus (VACV), have a large double-stranded DNA genome and replicate in the cell cytoplasm of infected vertebrate or invertebrate hosts [1]. Despite the eradication of smallpox, poxviruses are still a source of concern due to the possible accidental release of VARV or its use as a biological weapon, as well as the emergence of zoonotic poxvirus infections around the world, including Monkeypox virus, Cowpox virus (CPXV) and Vaccinia virus [2].

In Brazil, several poxviruses have been isolated since the 1960s and characterized as VACV strains. In fact, VACV is the agent of an emerging zoonotic disease that has been recognized over the last decade and affects bovine dairy cattle and their handlers in Brazil, with a sizeable impact on the local public health system [3–10]. The origin and natural host of VACV remain unknown, but current knowledge indicates that VACV is an independent *Orthopoxvirus* lineage, not directly derived from CPXV or VARV [11]. One hypothesis to explain VACV origin is that a naturally occurring (but now rare or even extinct) VACV-like virus was introduced centuries ago as a vaccine during early smallpox vaccination attempts and gave rise to the VACV. In fact, Horsepox virus (HSPV)

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has been considered as a possible intermediate in the transition from CPXV to VACV [12]. Likewise, the origin of Brazilian Vaccinia virus strains (BR-VACVs) is also unknown. Hypotheses to explain the existence of these VACVs in nature include the following: these viruses could be naturally occurring VACVs or they could have been derived from vaccine strains that escaped into the wild and established a stable circulation in some reservoir during the Smallpox Eradication Campaign [4–6,8,12].

It is difficult to precisely establish which, and how many, different vaccine strains were used during the Smallpox Eradication Campaign in Brazil, as it is complicated to determine the origin of these strains. In 1968, vaccine strains Paris, NYCBOH and Lister were used for production of freeze-dried vaccine and, after 1971, NYCBOH and Lister were used for vaccine production in Brazil [13]. The Instituto Butantan (São Paulo) and the Instituto Oswaldo Cruz (Rio de Janeiro) were in charge of vaccine production for systematic smallpox vaccination in Brazil [14]. In the Institute Butantan, the Lister strain (Lister-Butantan) was used and in the Institute Oswaldo Cruz, a vaccine strain, currently known as IOC, was produced [4,13,14]. Thus, if the vaccine origin hypothesis of BR-VACVs circulation is true, we should be able to identify a genetic resemblance between Brazilian isolates and the Lister-Butantan strain, as this last virus was the most widespread smallpox vaccine in Brazil. Moreover, there is no apparent documentation of plaque purification before the distribution of the Lister strain around the world [15]. One can speculate that different virus populations were favored or evolved in different ways in response to different growing conditions in many laboratories during the Smallpox Eradication Campaign. In this way, the comparative study of BR-VACVs with Lister-Butantan (LTBUT) is of great importance. To do this, we have analyzed seven BR-VACV isolates and the vaccine strain LTBUT based on ten sequences (Table 1), including both conserved and non-conserved open reading frames (ORFs).

2. Materials and methods

2.1. Viruses and DNA isolation

Strains BeAn58058 (BAV), SPAn232 (SAV) and Belo Horizonte (VBH) were isolated from rodents in North or Southeast states of Brazil in 1963, 1979 and 1993, respectively [3,5,7]. Strains Araçatuba (ARAV), Passatempo (PSTV), Guarani P1 (GP1V) and Guarani P2 (GP2V) were isolated from cattle in the Southeast region of the country from 1999 to 2003 [6,8,9]. The Lister-derived Brazilian vaccine strain LTBUT was obtained from the Instituto Butantan (São Paulo, Brazil). All strains were plaque-purified, propagated in Vero cells, purified by sucrose gradient centrifugation and used for viral DNA extraction [3].

2.2. Polymerase chain reaction (PCR) and nucleotide sequencing

The chosen ORFs were as indicated in Table 1, and were amplified from BR-VACVs and LTBUT using specific primers (Table 2) [9,16,17]. After amplification, DNA fragments were purified and cloned into pGEM-T vector (Promega). Three clones for each ORF were sequenced in both orientations at least three times. Sequencing reactions were performed using ET Dynamic Terminator for MegaBACE (GE Healthcare) and sequences were automatically edited.

2.3. Sequence analyses

BR-VACVs and LTBUT sequences were compared to each other and also to published *Orthopoxvirus* sequences available in GenBank, including IOC and Cantagalo virus (CTGV). CTGV is another Brazilian Vaccinia virus strain isolated during a bovine Vaccinia outbreak in 1999 in the Southeast region [4]. Nucleotide (nt) and deduced amino acid (aa) sequences of each ORF were aligned separately on the basis of codon positions using CLUSTAL W implemented in the software Mega 3 [18], and alignments were checked and manually edited. Distances and nucleotide sequence identities among BR-VACVs and LTBUT as well the GC content (%) of each ORF were estimated using Mega 3 [18]. Multiple alignments of each ORF containing only VACV sequences were submitted to codon positive selection test using the HyPhy package [19]. Deduced aa sequences were analyzed both for existing changes and to predict whether substitutions could affect protein function using the software SIFT [20].

Phylogenetic analyses were carried out with different concatenated alignments using the Neighbor-joining (NJ) method implemented in Mega3 [19] and the Maximum Likelihood (ML) method implemented in PAUP*4.0b10 using the nt substitution model GTR + G + I. ORFs were concatenated in the same order that they occur in the Vaccinia virus strain Western Reserve (VACV-WR) genome. The reliability of branching patterns was tested through bootstrap sampling. Analyses to detect possible recombination events among different BR-VACVs were performed using software implemented in the Recombination Detection Program 2 package [21].

3. Results

3.1. PCR and sequencing

Amplicons of all ORFs were obtained from all BR-VACVs and LTBUT, with the exception of B19R (previously referred to as B18R), which was not amplified from LTBUT. A consensus sequence for each ORF from each strain was determined and sixty-one new sequences from BR-VACVs and LTBUT were generated and deposited in GenBank (Table 1).

3.2. Nucleotide sequence analyses

The GC content ratios of BR-VACVs and LTBUT genes ranged from 32.8% to 40.2%, consistent with the ratios of other known VACV sequences. Sequence analyses revealed that BR-VACVs nt sequences shared from 97.5% to 100% identity when compared to each other. Among BR-VACVs, ARAV, GP2V and PSTV sequences presented higher nt similarity

Table 1
Open reading frames and GenBank accession numbers from sequences analyzed in the study

ORF and its function/product	ORF size (bp)[a]	Number of analyzed bases (bp)	Vaccinia strains	GenBank accession numbers
C6L	456	456	ARAV	EF051269
Unknown function			BAV	EF051270
			GP1V	EF051271
			GP2V	EF051272
			PSTV	EF051274
			SAV	SPAn232
			VBH	EF051276
			LTBUT	EF175981
C7L	453	453	ARAV	EF051277
Host range, viral determinants for multiplication in human cells [24]			BAV	EF051278
			GP1V	EF051279
			GP2V	EF051280
			PSTV	EF051282
			SAV	EF051283
			VBH	EF051284
			LTBUT	EF175982
K1L	855	855	ARAV	EF051285
Host range, viral determinants for multiplication in human cells [24]			BAV	EF051286
			GP1V	EF051287
			GP2V	EF051288
			PSTV	EF051290
			SAV	EF051291
			VBH	EF051292
			LTBUT	EF175983
K2L	1110	1110	ARAV[c]	EF175987
Codes for a protein homolog to the Cowpox virus serine proteinase inhibitor [26]			BAV	EF175990
			GP1V	EF175991
			GP2V[c]	EF175988
			PSTV[c]	EF175989
			SAV	EF175992
			VBH	EF175993
			LTBUT	EF175994
K3L	267	267	ARAV	EF175965
Host range, codes for a competitive inhibitor of the double-stranded RNA-dependent protein kinase [23]			BAV	EF175968
			GP1V	EF175969
			GP2V	EF175966
			PSTV	EF175967
			SAV	EF175970
			VBH	EF175971
			LTBUT	EF175972
E3L	573	570 (partial sequence, base 4 to 573)	ARAV[b]	DQ194389
Host range, codes for dsRNA binding-protein [23]			BAV	DQ194388
			GP1V[b]	DQ194385
			GP2V[b]	DQ194386
			PSTV[b]	DQ530240
			SAV	DQ194387
			VBH[b]	DQ194390
			LTBUT	EF175984
A56R	942	816 (partial sequence, base 61 to 876)	ARAV[b,d]	AY523994
Hemagglutinin, found in the EEV [26]			BAV	DQ206442
			GP1V[b]	DQ206436
			GP2V[b,d]	DQ206437
			PSTV[b,d]	DQ070848
			SAV	DQ222922
			VBH[b]	DQ206435
			LTBUT[d]	EF175985

(continued on next page)

Table 1 (*continued*)

ORF and its function/product	ORF size (bp)[a]	Number of analyzed bases (bp)	Vaccinia strains	GenBank accession numbers
B5R	954	954	ARAV	EF051261
Codes for EEV protein,			BAV	EF051265
with role in the			GP1V	EF051266
EEV-neutralization [25]			GP2V	EF051262
			PSTV	EF051263
			SAV	EF051267
			VBH	EF051268
			LTBUT	EF175986
B8R	819	819	ARAV	EF175973
Interferon-gamma			BAV	EF175976
soluble receptor [23]			GP1V	EF175977
			GP2V	EF175974
			PSTV	EF175975
			SAV	EF175978
			VBH	EF175979
			LTBUT	EF175980
B19R[e]	1053	991(partial sequence, base 61 to 1050)	ARAV[b]	DQ194382
Interferon-alpha/beta			BAV[b]	AF261890
soluble receptors [23]			GP1V[b]	DQ194380
			GP2V[b]	DQ194381
			PSTV[b]	DQ530239
			SAV	DQ194384
			VBH[b]	DQ194383
			LTBUT[f]	–

ORF, open reading frame; ARAV, Araçatuba virus; BAV, BeAn58058 virus; GP1V, Guarani P1 virus; GP2V, Guarani P2 virus; PSTV, Passatempo virus; SAV, SPAn232 virus; VBH, Belo Horizonte virus; LTBUT, Lister-Butantan.

[a] Based on Vaccinia virus Western Reserve sequence.

[b] Previously published sequences.

[c] The complete sequences of K2L from ARAV, PSTV and GP2V were 1095 bp long, due to a 15 bp deletion.

[d] LTBUT partial sequence of A56R is 804 bp long while ARAV, GP2V and PSTV sequences are 798 bp long.

[e] B19R has been previously referred to as B18R.

[f] LTBUT does not posses this ORF.

values when compared to each other, with the exception of K2L, K3L and E3L sequences. BAV, SAV, GP1V and VBH sequences presented higher nt similarity values when compared to each other, and were identical regarding ORFs C7L, K2L, and K3L. Similarity values from 98.6% to 100% were observed when LTBUT sequences were compared to the sequences of Lister and of Lister-derived strains, as LC16m0 and LC16m8 [15]. When all BR-VACVs' nt sequences were compared to LTBUT sequences, identity values ranging from 97.5% to 99.9% were observed.

Single nucleotide polymorphisms (SNPs) were observed between each of the correlated ORFs analyzed for the BR-VACVs. Regarding ORF A56R two consecutive indels were observed. LTBUT presented a 15 bp deletion when compared to BR-VACV sequences, resulting in the loss of 5 amino acids (aa) (DADLY) (Fig. 1). The second indel was represented by one deletion of 18 bp in ARAV, GP2V, PSTV, CTGV and IOC sequences when compared to BAV, SAV, GP1V and VBH, leading to the loss of 6 aa (DTYNDN) (Fig. 1). Concerning the ORF K2L, ARAV, PSTV and GP2V sequences showed one 15 bp deletion, leading to a 5 aa deletion (ASTIM) in the deduced aa sequence when compared to all VACV sequences (Fig. 1). Multiple alignments of each ORF from VACV strains were submitted to positive selection analysis using the HyPhy software. Considering Bayes factor over 20 as an indicator of positive selection, all analyzed ORFs presented at least one codon that evolved under positive selection (Fig. 1). The ORF B8R was the only exception to this result (Fig. 1).

Multiple alignments of all deduced aa sequences showed substitutions differentiating each of the BR-VACVs. Moreover, unique aa substitutions were observed at 21 sites (8 located at sites evolving under positive selection) when the deduced aa sequences of ARAV, GP2V and PSTV were compared to VBH, GP1V, BAV and SAV sequences all together. These aa substitutions were observed in C6L, C7L, B19R, K1L, A56R, and B5R deduced protein sequences (Fig. 1). Additionally, 8 aa changes occurring at positive selection sites differentiated GP2V and ARAV from PSTV, VBH, GP1V, BAV and SAV. Two were observed in the K2L deduced protein and the other six were located in the E3L predicted protein sequence (Fig. 1).

For the following analyses, aa positions are given according to the VACV-WR sequences. The deduced aa sequence of

Table 2
Primers used to amplify open reading frames (ORFs) C6L, C7L, K1L, K2L, K3L, B5R, B8R, B19R, E3L and A56R from Vaccinia virus strains

Primer	Sequence 5′–3′	Target ORFs	Amplicon size[a]
C6L F	GTACTAGATCCTCATAAGTG	C6L and C7L	1538
C7L R	ATACAGACAATGCTTACAGG		
K1L F	ATAGTACGATGCAATGAG	K1L and K2L	1408
K2L R	AACATATGACTAGGGACC		
K2L F	CATAATAGTAGATGCCTC	K2L and K3L	1338
K3L R	TTATCCCAATTTACGAGC		
B5R F	TTTTAGTGCTGCACAGTG	B5R	1135
B6R R	AGTAAAAATGCTCTAACG		
B7R F	ATGATGGTGATGAGCGAC	B8R	1102
B8R R	CCACTGTATAATATGCAG		
B18R5 [17]	CGCAGGATCCACAGTTACGCCATAGAC	B19R[b]	1015
B18R3[17]	CCAGAAGCTTCTCCAATACTACTGTAGT		
E3L F [9]	AGGCGGATCCATGTCTAAAATCTATATC	E3L	589
E3L R [9]	TCGCAAGCTTTCAGAATCTAATGATGAC		
EACP1 [16]	ATGACACGATTGCCAATAC	A56R	960
EACP2 [16]	CTAGACTTTGTTTTCTG		

[a] Expected size in relation to VACV-WR whole genome sequence (GenBank accession number AY243312).
[b] B19R has been previously referred to as B18R.

proteins involved with interferon resistance had well-conserved sequences. Regarding K3L and B8R, all BR-VACV aa sequences were identical, with the exception of PSTV. The PSTV K3L sequence presented replacement of a K with an N (K22N) at position 22, while a F29L substitution was observed in the PSTV B8R aa sequence (Fig. 1). E3L protein sequences of ARAV, GP2V and CTGV were identical to each other and exhibited six aa differences when compared to PSTV, VBH, SAV, BAV and GP1V sequences (Fig. 1). Finally, aa substitutions were observed in sequences belonging to GP2V (P75L), BAV (S237P), ARAV, GP2V, PSTV and CTGV (R148K) of the B19R protein. Another conservative aa alteration of T346S was observed in all BR-VACV sequences, with the exception of CTGV (Fig. 1).

Regarding the A56R predicted protein sequence, ARAV, GP2V, PSTV and CTGV showed seven aa differences when compared to other BR-VACVs (Fig. 1). Five aa substitutions (D28N, R36Q, A59G, A244P and G262A) observed in A56R sequences of PSTV, GP2V, VBH and SAV were predicted as intolerable by SIFT software, indicating aa changes that could potentially alter protein conformation and biological function. In the K2L predicted protein sequence, two tolerable aa substitutions were observed in ARAV and GP2V sequences when compared to other BR-VAVCs (Fig. 1).

Analysis of the K1L aa sequence revealed that GP1V presented two unique non-conservative aa alterations. ARAV, GP2V and PSTV sequences presented six aa differences when compared to other BR-VACVs (Fig. 1). When the B5R predicted aa sequences from ARAV, GP2V and PSTV were compared to other BR-VACVs, six tolerant aa alterations were observed (Fig. 1). Two other aa changes were observed in the ARAV (S83F) and in GP1V and SAV sequences (K2T) (Fig. 1), the latter of which was predicted to be not tolerated.

Except for ARAV, GP2V and PSTV, which showed one conservative aa substitution (K41Q), the inferred aa sequences of ORF C7L from all VACV strains were identical (Fig. 1). The K41Q substitution was also observed in CPXV-BR and HSPV (strain MNR-76) sequences. Finally, the deduced C6L aa sequence of SAV presented three aa substitutions (A3V, I25T and V53G) and two conservative aa changes were observed among BR-VACVs sequences (E62D and G74S) (Fig. 1).

When all BR-VACV aa sequences (except B19R) were compared to LTBUT sequences, aa dissimilarities were observed. In the same way, when sequences of BR-VACV (A56R, E3L, K1L, K3L and B19R) were compared to IOC sequences, aa dissimilarities were also observed in each analyzed protein sequence (Fig. 1). When all BR-VACV aa sequences were compared to other VACVs, 19 aa were observed to be unique to at least one of the BR-VACVs. The aa dissimilarities were also observed when LTBUT sequences were compared to Lister, LC16m0 and LC16m8 sequences (Fig. 1).

3.3. Phylogenetic analysis

Initial analyses were performed using the data set containing ORFs C6L, C7L, K1L, K2L, K3L, E3L, A56R, B5R and B8R. BR-VACVs were grouped in a cluster of VACV that also contained Rabbitpox virus (a close relative of VACV) [22] and HSPV (Fig. 2A). The BR-VACVs were subdivided into two main groups; the first included GP1V, VBH, BAV, SAV and VACV-WR and the second included PSTV, ARAV and GP2V. These two groups were observed in all trees and supported by bootstrap values $\geq$98% (Fig. 2A,B). It was also demonstrated that ARAV and GP2V are more closely related to each other than to PSTV. BAV, GP1V and VBH were also more closely related to each other than to SAV (Fig. 2B). None of the BR-VACVs shared a significantly broad relationship with vaccine strains, including LTBUT, Lister or derived vaccine strains such as LC16m0 and LC16m8. By NJ analysis,

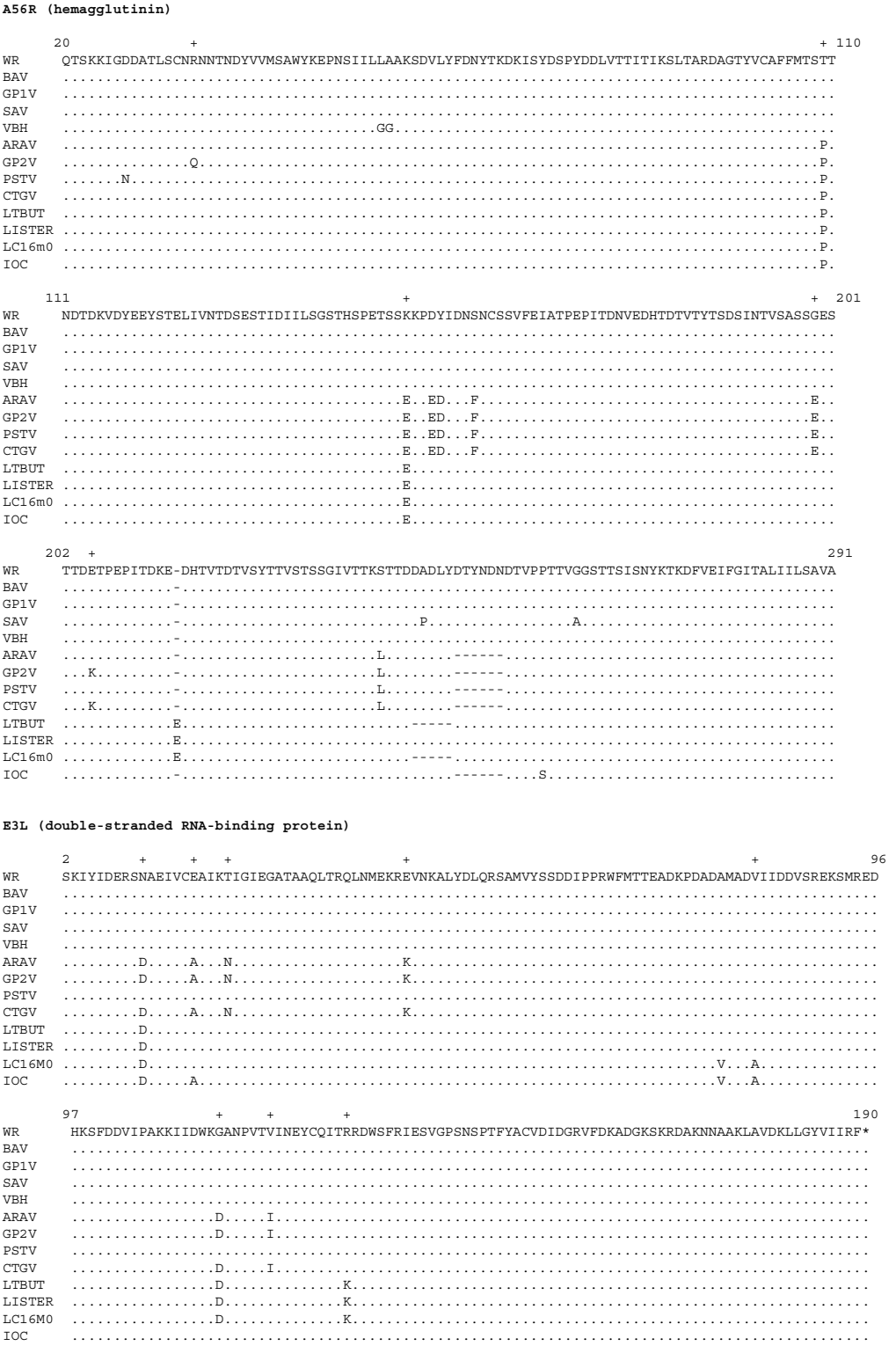

Fig. 1. Multiple alignments of the deduced amino acid (aa) sequences of ORFS A56R, E3L, K1L, B5R, K2L, K3L, B8R, B19R, C7L and C6L from Brazilian Vaccinia virus strains and other Vaccinia virus sequences. Positions are given according to Vaccinia virus strain Western Reserve aa sequences. (+) represents codons under positive selection, (.) represents amino acid identity with WR and the difference in aa sequence is represented by a single letter aa code, (−) represents deletions and (*) represents stop codons. In K1L aa sequences, ankyrin regions (ANK1−6) and the C-terminal non-ANK are underlined. In the B5R aa sequence, short consensus repeats (SCR1−4) and the ''stalk region'' are underlined and in italic. Vaccinia virus strains: Western Reserve (WR), Araçatuba (ARAV), BeAn58058 (BAV), Guarani P1 (GP1V), Guarani P2 (GP2V), SPAn232 (SAV), Belo Horizonte (VBH), Cantagalo (CTGV); vaccine strains: Lister-Butantan (LTBUT) and Instituto Oswaldo Cruz (IOC).

K1L (host range protein)

```
       1                   +                ANK1                            ANK2                  92
WR     MDLSRINTWKSKQLKSFLSSKDAFKADV HGHSALYYAIADNNVRLVCTLLNAGALKNL L ENEFPLHQAATLEDTKIVKILLFSGLDDSQFD D
BAV    ............................. ............................. . ............................... .
GP1V   ........................G.. ..............L.............. . ............................... .
SAV    ............................. ............................. . ............................... .
VBH    ............................. ............................. . ............................... .
ARAV   ............................. ............................. . .........................M...... .
GP2V   ............................. ............................. . .........................M...... .
PSTV   ............................. ............................. . .........................M...... .
CTGV   ............................. ............................. . .........................M...... .
LTBUT  ......................T..... ............................. . ............................... .
LISTER ............................I N........................... . ............................... .
LC16m0 ............................I N........................... . ............................... .
IOC    ............................I N........................... . .........................M...... .

      93        ANK3                              ANK4                              ANK5                    190
WR     KGNTALYYAVDSGNMQTVKLFVKKNWRLMFYG KT GWKTSFYHAVMLNDVSIVSYFLSEIPSTFDLA I LLSCIHITIKNGHVDMMILLLDYMTSTNTNN
BAV    ................................ .. ............................. . ............................
GP1V   ................................ .. ............................. . ............................
SAV    ................................ .. ............................. . ............................
VBH    ................................ .. ............................. . ............................
ARAV   ................................ .. .........................L..... . ......T.................AF.....
GP2V   ................................ .. .........................L..... . ......T.................AF.....
PSTV   ................................ .. .........................L..... . ......T.................AF.....
CTGV   ................................ .. .........................L..... . ......T.................AF.....
LTBUT  ........P....................... .. ............................. . ............................
LISTER ................................ .. ............................. . ............................
LC16m0 ................................ .. ............................. . ............................
IOC    ................................ .. ............................. . .........................AF.....

     191      ANK6                                C-terminal non-ANK                                    284
WR     SL LFIPDIKLAIDNKDIEMLQALFKYDINIYS ANLENVLLDDAEIAKMIIEKHVEYKSDSYTKDLDIVKNNKLDEIISKNKELRLMYVNCVKKN*
BAV    .. ................................ ..................................................................
GP1V   .. ................................ ..................................................................
SAV    .. ................................ ..................................................................
VBH    .. ................................ ..................................................................
ARAV   .. ................................ .........................F........................................
GP2V   .. ................................ .........................F........................................
PSTV   .. ................................ .........................F........................................
CTGV   .. ................................ .........................F........................................
LTBUT  .. ................................ ....................K...........................................
LISTER .. ................................ ..................................................K...............
LC16m0 .. ................................ ..................................................K...............
IOC    .. ................................ ..................................................................
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B5R (EEV type-I membrane glycoprotein)

```
       1+                          SCR1        ++              +                                ++    +  SCR2          105
WR     MKTISVVTLLCVLPAVVYS TCTVPTMNNAKLTSTETSFNDKQKVTFTCDQGYHSSDPNAVCETDKWKYENPC KKM CTVSDYISELYNKPLYEVNSTMTLSCNGET
BAV    ..................... ..................................................... ... ..........................
GP1V   .T................... ..................................................... ... ..........................
SAV    .T................... ..................................................... ... ..........................
VBH    ..................... ..................................................... ... ..........................
ARAV   ..................... ......................NN............................. ... .......F..................
GP2V   ..................... ......................NN............................. ... ..........................
PSTV   ..................... ......................NN............................. ... ..........................
LTBUT  ..................... ..................................................... ... ..........................
LISTER ..................... ..................................................... ... ......V...................
LC16m0 ..................... ...................................L.................. ... ......V....N..............

     106      SCR2                              SCR3                                                SCR4          209
WR     KYFRCEEKNGNTSWNDTVTC PNAE CQPLQLEHGSCQPVKEKYSFGEYMTINCDVGYEVIGASYISCTANSWNVIPSC QQK CDMPSLSNGLISGSTFSIGGVIHL
BAV    ..................... .... ..................................................... ... ..........................
GP1V   ..................... .... ..................................................... ... ..........................
SAV    ..................... .... ..................................................... ... ..........................
VBH    ..................... .... ..................................................... ... ..........................
ARAV   ..................... .... ..................................................... ... ..........................
GP2V   ..................... .... ..................................................... ... ..........................
PSTV   ..................... .... ..................................................... ... ..........................
LTBUT  ..................... .... ..................................................... ... ..........................
LISTER ..................... .... .........................I........................... ... ..........................
LC16m0 ..................... .... ..................................................... ... ..........................

     210    SCR4                +       "stalk region"                                                    317
WR     SCKSGFTLTGSPSSTCIDGKWNPVLPIC VRTNEEFDPVDDGPDDETDLSKLSKDVVQYEQEIESLEATYHIIIVALTIMGVIFLISVIVLVCSCDKNNDQYKFHKLLP*
BAV    ..................... ..................................................................................
GP1V   ..................... ..................................................................................
SAV    ..................... ..................................................................................
VBH    ..................... ..................................................................................
ARAV   ....................I..T. ..S..K..........................................................................
GP2V   ....................I..T. ..S..K..........................................................................
PSTV   ....................I..T. ..S..K..........................................................................
LTBUT  ....................T. ..S..K..........................................................................
LISTER ....................T. ..S..K..........................................................................
LC16m0 ....................I..T. ..S..K..........................................................................
```

Fig. 1. (*continued*).

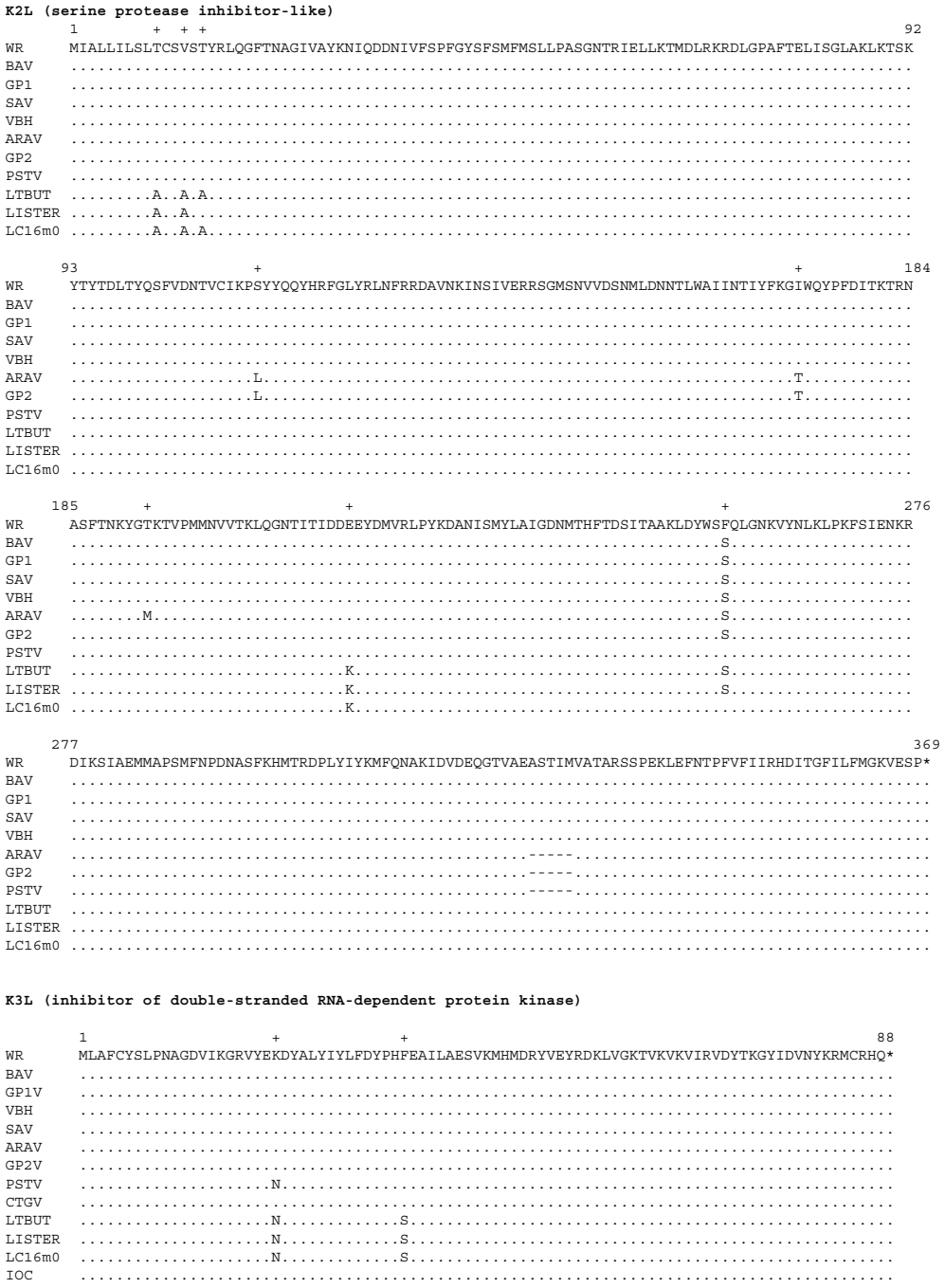

Fig. 1. (*continued*).

LTBUT was grouped with Lister-derived strains LC16m0 and LC16m8 (data not shown). Using the same dataset, recombination detection analyses did not detect any recombination events among BR-VACVs (data not shown). In order to include Brazilian strain CTGV and vaccine strain IOC, phylogenetic analyses were performed using a smaller dataset containing C7L, K1L, K3L, E3L and A56R sequences from VACV strains, Cowpox virus, Horsepox virus and Rabbitpox virus. All trees suggested that CTGV is closely related to ARAV and GP2V. Moreover, IOC did not cluster with any BR-VACVs, including CTGV, after phylogenetic analyses using this dataset (Fig. 2C).

4. Discussion

Taken together, our results indicate that there are different and genetically diverse groups of BR-VACVs circulating in Brazil. This genetic diversity is represented by SNPs and indels that result in differences in the deduced aa sequences of the analyzed ORFs. Moreover, some aa changes observed in

B8R (interferon gamma soluble receptor)

```
      1                                                                                92
WR    MRYIIILAVLFINSIHAKITSYKFESVNFDSKIEWTGDGLYNISLKNYGIKTWQTMYTNVPEGTYDISAFPKNDFVSFWVKFEQGDYKVEEY
BAV   ................................................................................
GP1V  ................................................................................
SAV   ................................................................................
VBH   ................................................................................
ARAV  ................................................................................
GP2V  ................................................................................
PSTV  ..........................L.....................................................
LTBUT ...............................................................................
LISTER ..............................................................................
LC16m0 .............................................................................

      93                                                                              184
WR    CTGLCVEVKIGPPTVTLTEYDDHINLYIEHPYATRGSKKIPIYKRGDMCDIYLLYTANFTFGDSKEPVPYDIDDYDCTSTGCSIDFVTTEKV
BAV   ................................................................................
GP1V  ................................................................................
SAV   ................................................................................
VBH   ................................................................................
GP2V  ................................................................................
ARAV  ................................................................................
PSTV  ................................................................................
LTBUT ...............................................................................
LISTER ..............................................................................
LC16m0 .............................................................................

     185                                                                            272
WR    CVTAQGATEGFLEKITPWSSKVCLTPKKSVYTCAIRSKEDVPNFKDKMARVIKRKFNKQSQSYLTKFLGSTSNDVTTFLSMLNLTKYS*
ARAV  ................................................................................
BAV   ................................................................................
GP1V  ................................................................................
GP2V  ................................................................................
PSTV  ................................................................................
SAV   ................................................................................
VBH   ................................................................................
LTBUT ..........................................................T.....................
LISTER ..........................................................T.....................
LC16MO ..........................................................T.....................
```

B19R (interferon alpha/beta soluble receptor)

```
     20                                        +                                      130
WR    SYAIDIENEITEFFNKMRDTLPAKDSKWLNPACMFGGTMNDIAALGEPFSAKCPPIEDSLLSHRYKDYVVKWERLEKNRRRQVSNKRVKHGDLWIANYTSKFSNRRYLCTV
BAV   ................................................................................
GP1V  ................................................................................
SAV   ................................................................................
VBH   ................................................................................
ARAV  ................................................................................
GP2V  ..........................................................L.....................
PSTV  ................................................................................
CTGV  ................................................................................
IOC   ................................................................................

    131             +                                                  +             +               +   240
WR    TTKNGDCVQGIVRSHIRKPPSCIPKTYELGTHDKYGIDLYCGILYAKHYNNITWYKDNKEINIDDIKYSQTGKELIIHNPELEDSGRYDCYVHYDDVRIKNDIVVSRCKI
BAV   ................................................................................P....
GP1V  ................................................................................
SAV   ................................................................................
VBH   ................................................................................
ARAV  ................K...............................................................
GP2V  ................K...............................................................
PSTV  ................K...............................................................
CTGV  ................K...............................................................
IOC   ................K.....................................................K.............N.....................

    241                                                                                +   349
WR    LTVIPSQDHRFKLILDPKINVTIGEPANITCTAVSTSLLIDDVLIEWENPSGWLIGFDFDVYSVLTSRGGITEATLYFENVTEEYIGNTYKCRGHNYYFEKTLTTTVVL
BAV   ................................................................................S....
SAV   ................................................................................S....
GP1V  ................................................................................S....
VBH   ................................................................................S....
ARAV  ................................................................................S....
GP2V  ................................................................................S....
PSTV  ................................................................................S....
CTGV  ................................................................................
IOC   ................................................................................
```

Fig. 1. (*continued*).

BR-VACVs' sequences were associated with codons under positive selection, which could correlate with adaptation to different hosts in nature.

Conservative and non-conservative aa substitutions were observed in BR-VACVs' sequences, most of which were predicted to be tolerated, possibly not affecting protein function. Nonetheless, these aa substitutions could alter the binding affinity of proteins for their ligands, resulting in different biological properties. Genes involved with interferon (IFN) resistance; including K3L, E3L, B8R and B19R [23] were well conserved among BR-VACVs, which reflects the importance of this immune evasion strategy in the Poxvirus family. However,

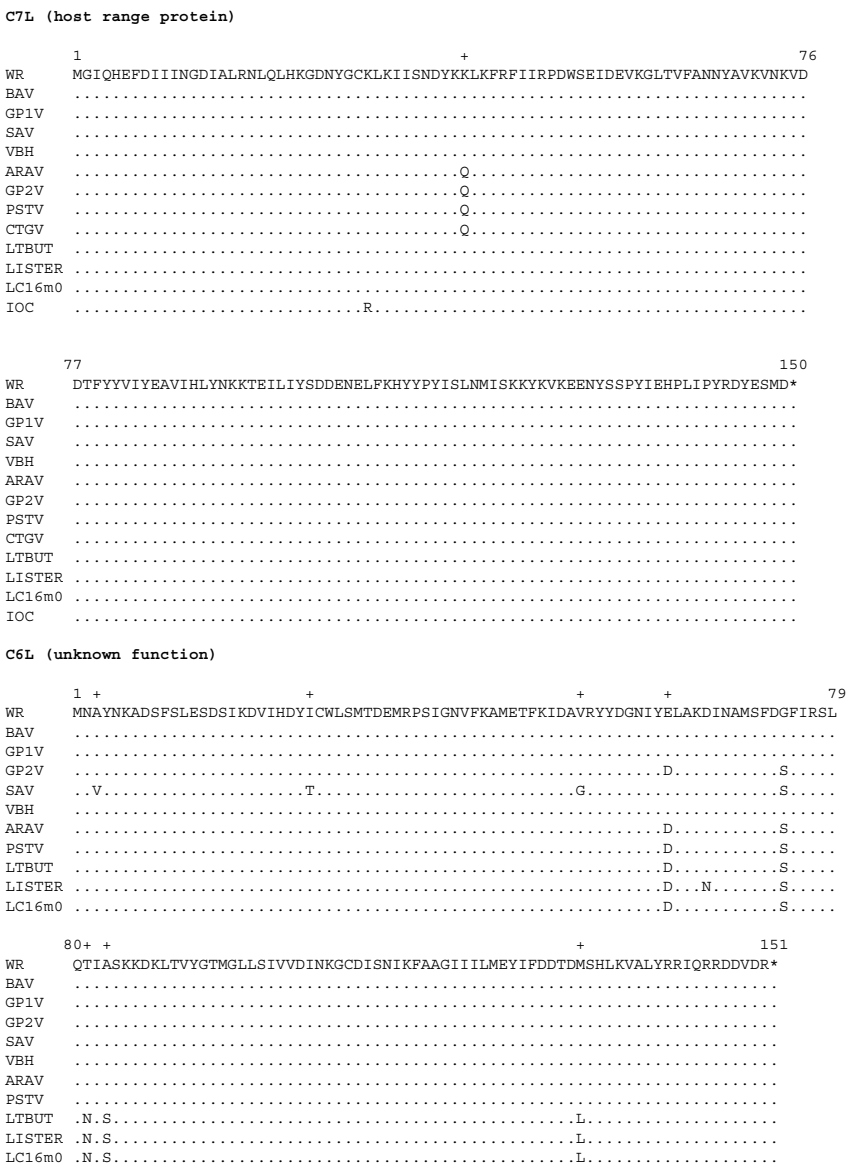

Fig. 1. (*continued*).

a changes were observed in some of the IFN related BR-VACV predicted proteins, mainly in the E3L aa sequence, which may result in variable IFN susceptibility among these strains.

Both C7L and K1L genes are necessary for virus multiplication in mammalian cells [24]. While the C7L aa sequence presented only one aa change in the ARAV, GP2V and PSTV sequences, K1L presented more variable sites. The K1L protein sequence contains multiple ankyrin repeats (ANKs 1 to 6). In this study, predicted aa changes were observed at ANK1, ANK2, ANK4, ANK5 and at the C-terminal non-ANK region of the K1L inferred protein sequence from BR-VACVs [24]. In the same way, aa changes were also observed in proteins determined to be in the envelope of extracellular enveloped virus (EEV). For the B5R deduced aa sequence, ARAV, GP2V and PSTV shared aa substitutions at the SCR1, SCR4 and ''stalk'' regions and ARAV presented another non-conservative aa substitution at the SCR2 region of the protein. The SCR1–4 and ''stalk'' regions are found in the ectodomain of B5R, and it has been shown that at least SCR1, SCR2 and/or the stalk regions are involved in EEV neutralization by antibodies [25].

Finally, the serpin SPI-3 (homolog to K2L product) of CPXV is found to be associated with hemagglutinin (coded by A56R) in EEV particles and deletion of aa 330 to 373 in SPI-3 blocked its association with hemagglutinin [26]. This region corresponds to aa 326 to 369 of the VACV-WR protein and, in ARAV, GP2V and PSTV K2L deduced aa sequences, residues 227 to 331 are deleted. This deletion, together with aa changes in the A56R deduced aa sequence, could alter the association of the K2L product with hemagglutinin in EEV particles.

Although recombination events among BR-VACVs were not detected by the programs and datasets we used, recombination remains a possible explanation for the fact that K2L, K3L and E3L sequences of PSTV are more similar to those belonging to SAV, VBH, GP1V, BAV and VACV-WR. Phylogenetic inferences demonstrated that different BR-VACVs analyzed here cluster into two major groups; one formed by

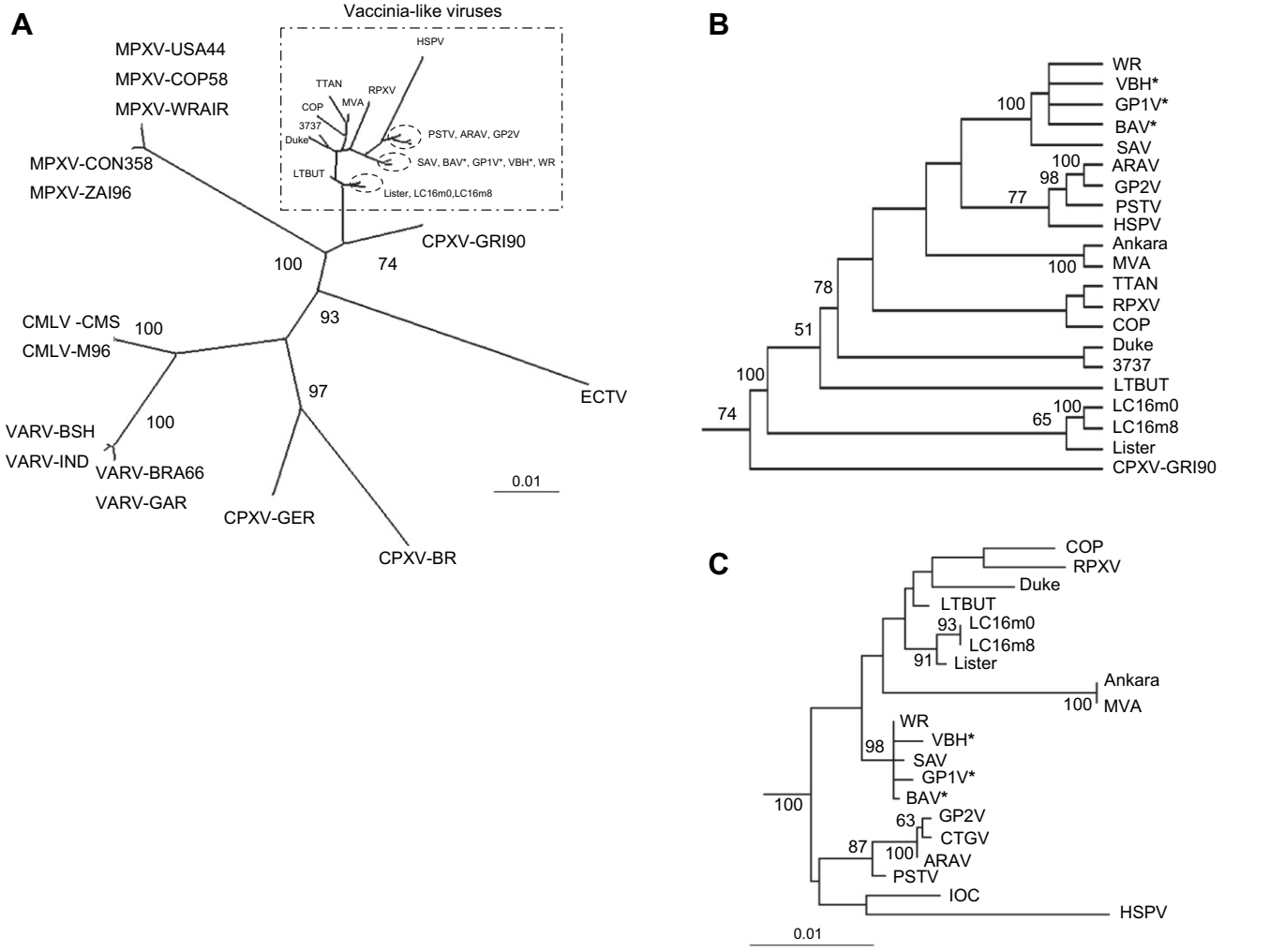

Fig. 2. Phylogenetic trees of Brazilian Vaccinia virus strains and *Orthopoxvirus*. All trees were reconstructed by the Maximum Likelihood method implemented in PAUP*4.0b10. (A) Unrooted phylogenetic tree constructed on the basis of concatenated alignment of nucleotide sequences of ORFs C6L, C7L, K1L, K2L, K3L, E3L, A56R, B5R and B8R. (B) Subtree of (A) after considering CPXV-BR as root. Bootstrap values from 1000 replicates are shown. Likelihood settings from best-fit model selected (GTR + G + I): Lset Base = (0.3497 0.1639 0.1847) Nst = 6 Rmat = (1.0000 3.1209 0.4615 0.4615 4.5230) Rates = gamma Shape = 0.7161 Pinvar = 0.6285. (C) Subtree of a phylogenetic tree constructed based on the concatenated alignment of nucleotide sequences of ORFs C7L, K1L, K3L, E3L and A56R, with root at CPXV-BR. Bootstrap values from 100 replicates are shown. Likelihood settings from best-fit model selected (GTR + G + I): Lset Base = (0.3571 0.1616 0.1838) Nst = 6 Rmat = (1.0000 2.4403 0.4031 0.4031 4.1233) Rates = gamma Shape = 0.7130 Pinvar = 0.6277. (*) indicates BR-VACVs that have the ati gene deleted. Abbreviations of each strain and the GenBank accession numbers are as follows. Vaccinia virus: Western Reserve (WR) (AY243312), Lister (AY678276), LC16m0 (AY678277), LC16m8 (AY678275), Duke (DQ439815), 3737 (DQ377945), Ankara (U94848), Modified virus Ankara (MVA) (AY603355), Copenhagen (COP) (M35027); Cowpox virus: Brigthon Red (CPXV-BR) (AF482758), Germany 91-3 (CPXV-GER) (DQ437593), GRI-90 (CPXV-GRI90) (X94355); Horsepox virus MNR76 (HSPV) (DQ792504); Rabbitpox virus (RPXV) (AY484669); Ectromelia virus Moscow (ECTV) (AF012825); Camelpox virus: CMS (CMLV-CMS) (AY009089), M-96 (CMLV-M96) (AF438165); Variola virus: India-1967 (VARV-IND) (X69198), Brazil 1966 (VARV-BRA66) (DQ441419), Garcia-1966 (VARV-GAR) (U18338), Bangladesh-1975 (VARV-BSH) (L22579); Monkeypox virus: Congo_2003_358 (MPXV-CON358) (DQ011154), COP-58 (MPXV-COP58) (AY753185), WRAIR7-61 (MPXV-WRAIR) (AY603973), Zaire-96-I-16 (MPXV-ZAI96) (AF380138), USA_2003_044 (MPXV-USA44) (DQ011153).

strains which possess the 18 bp deletion in the ORF A56R (ARAV, GP2V and PSTV) and the other containing those strains that do not (BAV, SAV, GP1V and VBH). It is also interesting to note that each group contains subgroups. In one group, ARAV and GP2V were more closely related to each other than to PSTV. In the other group, BAV, GP1V and VBH were more closely related to each other than to SAV (Fig. 2). This could indicate the existence of a wider number of viral populations circulating in Brazil with different origins. In fact, previous studies demonstrated that BAV, GP1V and VBH do not possess the A-type inclusion body (ati) gene (A26L), while SAV does [5,7,9,17,27]. This difference regarding the presence of the ati gene reinforces the existence of two viral populations in the second BR-VACV group. Based on our results and previous results regarding the ati gene, there are at least three different viral populations circulating in Brazil. Recent studies demonstrated that there are two distinct Monkeypox virus clades in Africa, consistent with differences in epidemiological, virulence and clinical features of human monkeypox disease [28,29]. In the same way, 45 Variola virus

strains isolated in many parts of the world were subdivided into three phylogenetic groups coincident with their geographical origin and case-fatality rate [30]. Thus, the clustering of BR-VACVs into different groups may also reflect differences in the virulence and geographic range of those strains.

Analyzing the nucleotide and amino acid sequences, similarities and dissimilarities were observed among BR-VACVs and IOC. In contrast to BAV, SAV, GP1V and VBH, strains ARAV, GP2V, PSTV and CTGV had a deletion comprising six aa in the A56R protein as well as IOC. This unique deletion could suggest that some BR-VACVs (ARAV, GP2V, PSTV and CTGV) could have originated from IOC. On the other hand, some aa differences were found between IOC and ARAV, GP2V, PSTV and CTGV: six aa differences in the deduced aa sequences of A56R, six aa dissimilarities in E3L, five others in K1L and two differences in the protein sequences of B19R and C7L (Fig. 1). Using the dataset containing ORFs C7L, K1L, K3L, E3L and A56R, it was not possible to establish a phylogenetic relationship between BR-VACVs and the vaccine strain IOC. Although the hypothesis of a vaccine origin for BR-VACVs cannot be completely excluded, our analyses indicate that the BR-VACVs analyzed here are probably not derived from VACV-LTBUT or other Lister-derived vaccine strains. A number of facts support this idea. LTBUT sequences presented some SNPs and a few aa changes when they were compared to LC16m0 and LC16m8 sequences. This was not unexpected since it was previously demonstrated that the Lister strain, and probably many other vaccine strains, consists of diverse virus populations [15]. However, similar to Lister and Lister-derived strains (LC16m0 and LC16m8) [15], LTBUT does not contain a copy of the B19R ORF (although in the last case we looked for ORF presence by PCR only), in contrast to BAV, SAV, GP1V, VBH, ARAV, GP2V, PSTV and CTGV. Moreover, phylogenetic analysis (using the dataset containing genes C6L, C7L, K1L, K2L, K3L, E3L, A56R, B5R and B8R) showed that LTBUT, Lister, LC16m0 and LC16m8 did not exhibit a close ancestral relationship with any of the BR-VACVs analyzed in this study.

Interestingly, the results also indicated that SAV, BAV, GP1V and VBH are closely related to VACV-WR. Although VACV-WR has never been used for vaccination, it is derived from the New York City Board of Health strain (NYCBOH), which was in fact used for vaccine production in Brazil [13]. Therefore, we cannot rule out the possibility that a closely WR-related strain might have been introduced and became established in nature. Additionally, given the genetic diversity found between the different BR-VACVs groups, we can speculate that they could have had different origins. On the other hand, if they had a common origin, the different virus groups could have originated by different selective pressures that they might have faced while circulating in different natural reservoirs and environments.

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