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Brazilian Vaccinia virus strains show genetic polymorphism at the *ati* gene

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Abstract Nucleotide sequence comparison of the internal region of the *ati* gene of members of the *Orthopoxvirus* genera revealed that this gene is variable among different species, although within members of the same species it is considered to be well conserved. Previous studies indicated that there is genetic variability in the *ati* gene among some Brazilian Vaccinia virus strains. To further investigate this variability, we performed molecular analysis of the internal region of the *ati* gene of eight Brazilian Vaccinia virus strains. While the internal region of this gene in one strain was similar to the Western Reserve strain, four strains presented two blocks of deletions in the analyzed region, and the *ati* gene was almost entirely deleted from three other strains. These findings demonstrate that there is genetic polymorphism within the *ati* gene among different Brazilian Vaccinia virus strains.

Keywords Brazilian Vaccinia virus · Bovine vaccinia outbreak · *ati* gene · Genetic polymorphism · Poxvirus · *ha* gene

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Introduction

Several Vaccinia virus (VACV) outbreaks have occurred in Brazil since 1999, resulting in economic losses to farmers and affecting the health of humans and animals [1–3]. Since humans usually get infected after direct contact with infected cattle, these outbreaks are marked by zoonotic transmission [1]. Nonetheless, VACV is a prototype species of the *Orthopoxvirus* genus of the *Poxviridae* family [4].

The formation of basophilic cytoplasmatic inclusions, which constitute viroplasms or virus factories, is a common feature during poxvirus infections. However, prominent eosinophilic bodies referred to as A-type inclusions (ATI) are found in the cytoplasm of cells infected with only certain strains of Cowpox virus (CPXV), Ectromelia virus (ECTV), Racconpox virus, Fowlpox virus, Canarypox virus, and Volepox virus [4–11]. The protein that forms these inclusion bodies is approximately 160 kDa and is a late protein coded by the *ati* gene [10, 12–14]. Despite the absence of morphologically defined ATIs, ATI-related

proteins with relative molecular masses ranging from 92 kDa to 96 kDa have been described in Monkeypox virus (MPXV), Vaccinia virus (VACV), Variola virus (VARV), and Camelpox virus (CMLV) [15–17]. The deletion of two continuous adenosine residues within a consecutive AG in a tandem repeat region leads to a frame shift truncation of the *ati* gene, resulting in a premature stop codon, which culminates in the mass reduction of ATI protein [5, 10]. Although deletions, insertions, and base changes have been described in CMLV, CPXV, ECTV, MPXV, Rabbitpox virus (RPXV), VACV, and VARV *ati* genes, nucleotide sequence comparisons revealed that this gene is highly conserved among members of the same viral species [5, 10, 16, 18].

Due to variations in the *ati* gene, some methodologies for *Orthopoxvirus* species differentiation were developed using *ati* gene amplification by PCR and PCR-RFLP [16, 18]. Differentiation using the *ati* gene is based on different sizes of generated amplicons and unique profiles obtained after amplicon digestion with *Xba*I or *Bgl*II [16, 18]. These methodologies are similar to the ones used for *crmB* and *ha* genes, which have also been developed for *Orthopoxvirus* species differentiation [19, 20].

In this regard, PCR-RFLP [14] was applied for species determination of eight Brazilian VACV strains: SPAn232 (SAV), BeAn58058 (BAV), and Belo Horizonte virus (VBH), which were isolated from rodents, and five others, Araçatuba virus (ARAV), Guarani P1 virus (GP1V), Guarani P2 virus (GP2V), Passatempo virus (PSTV), and Muriaé virus (MURV), which were related to exanthemas outbreaks affecting cattle and dairy workers [1, 2, 21–25]. It was observed that SAV presented exactly the same restriction profile as the VACV strain Western Reserve (VACV-WR) and VACV strain Lister (VACV-LST). In contrast, ARAV, MURV, GP2V, and PSTV presented the same restriction profile which was not identical to those presented by VACV-WR, VACV-LST, and SAV. Moreover, attempts to amplify the *ati* gene of BAV, VBH, and GP1V using the same primers were unsuccessful [1, 2, 22, 24, 26]. Only when the primers designed for the *ati* gene flanking regions were used, fragments were obtained for these three virus strains. This fragment from VBH had been previously sequenced revealing that most of its *ati* gene is deleted [27]. It was reported that only the last 112 nucleotides at the 3′ end of the *ati* gene are found in the genome of VBH [27]. Considering the polymorphisms observed in the *ati* gene of the Brazilian VACV strains in relation to each other and to other VACV strains, we decided to sequence the internal region of the *ati* gene of ARAV, MURV, GP2V, PSTV, and SAV and the whole *ati* gene of BAV and GP1V. Finally, molecular and phylogenetic analyses were performed using other *Orthopoxvirus ati* sequences available in GenBank.

Materials and methods

Viruses and cells

The Brazilian VACV strains used on this study were ARAV, BAV, GP1V, GP2V, MURV, PSTV, and SAV, which were all previously described [1, 2, 22, 24–26]. All virus samples were first purified and seed stocks were maintained. Viral samples were propagated only twice since working stocks were produced using samples from the seed stocks. Viruses were propagated in Vero cells and purified on a sucrose gradient [28, 29].

Viral DNA isolation, *ati* gene amplification, and cloning

Viruses DNA was extracted from purified viral stocks [30] and used as template for *ati* gene amplification by PCR using the ATI up (5′-AATACAAGGAGGATCT-3′) and ATI low (5′-CTTAACTTTTTCTTTCT-3′) primers described by Meyer et al. [16] or the primers P4C1 (5′-GGAAGCTTT TCTTCTCCTCTCTTAACAAAAATTG-3′) and RNApol (5′-GGAGATCTAGACCACCGTTTCCCAGACATGAA TATC-3′), which were designed for the flanking regions of the *ati* gene [27]. All resulting amplicons were cloned into a pGEM-T vector (pGEM-T Easy Vector Systems, Promega Corp., Madison, WI).

Sequences, nucleotide sequencing, and molecular analysis

All *Orthopoxvirus ati* sequences, corresponding to the region flanked by ATI up and ATI low primers, and which were available in GenBank until 2006, were retrieved and used in the molecular analyses (Table 1). Vectors containing ARAV, BAV, GP1V, GP2V, MURV, PSTV, and SAV amplicons were sequenced at least three times in both orientations by the dideoxy method [31], using M13 universal and *ati* specific primers [16, 27] by ET Dynamic Terminator for MegaBACE (GE HEALTHCARE). Nucleotide sequences were assembled using the CAP3 Sequence Assembling Program (http://pbil.univ-lyon1.fr/cap3.php) [32] and deposited into GenBank (accession numbers are given on Table 1). The *ati* nucleotide sequences from Brazilian VACV strains were aligned with other *Orthopoxvirus* sequences using the CLUSTAL W software [33] and carefully hand edited. To estimate nucleotide identity

Table 1 GenBank accession number of the *Orthopoxvirus* sequences used in this study

Virus Species	Strains and abbreviations	GenBank accession numbers
Vaccinia virus	BeAn58058 (BAV)	*DQ*516062
	Belo Horizonte (VBH)	AF501620
	SPAn 232 (SAV)	*DQ*516056
	Guarani P1 (GP1V)	*DQ516061*
	Guarani P2 (GP2V)	*DQ516058*
	Araçatuba (ARAV)	*DQ516057*
	Muriaé (MURV)	*DQ516059*
	Passatempo (PSTV)	*DQ516060*
	3737 (VACV-3737)	DQ377945
	Acambis clone 1000 (VACV-A1000)	AY766183
	Acambis clone 2000 (VACV-A2000)	AY313847
	Acambis clone 3 (VACV-A3)	AY313848
	LC16m8 (VACV-LCm8)	AY678275
	LC16mO (VACV-LCmO)	AY678277
	Lister (VACV-LST)	AY678276
	Tian Tan (VACV-TT)	AF095689
	Western Reserve (VACV-WR)	AY243312
Rabbitpox virus	Utrecht (RPXV-UTR)	AY484669
Cowpox virus	Brighton Red (CPXV-BR)	AF482758
	CPR06 (CPXV-CPR06)	D00319
	GRI-90 (CPXV-GRI90)	X94355
Variola virus	Bangladesh-1975 (VARV-BSH)	L22579
	Garcia-1966 (VARV-GAR)	X76266
	India-1967 (VARV-IND)	X69198
Monkeypox virus	Congo_2003_358 (MPXV-CONG2003)	DQ011154
	COP-58 (MPXV-COP)	AY753185
	Gabun (MPXV-GABUN)	MVU84504
	Liberia_1970_184 (MPXV-LIB70)	DQ011156
	Sierra Leone (MPXV-SLEONE)	AY741551
	USA_2003_039 (MPXV-USA39)	DQ011157
	USA_2003_044 (MPXV-USA44)	DQ011153
	WRAIR7-61 (MPXV-WRAIR7-61)	AY603973
	Zaire_1979-005 (MPXV-ZAIRE79)	DQ011155
	Zaire-96-I-16 (MPXV-ZAIRE96)	AF380138
Ectromelia virus	Moscow (ECTV-MOS)	AF012825
Camelpox virus	CMS (CMLV-CMS)	AY009089
	CP-1 (CMLV-CP1)	X69774
	M-96 (CMLV-M96)	AF438165

(%) among *ati* sequences of some *Orthopoxvirus*, analyses were performed using the LALIGN software (William R. Pearson, University of Virginia, http://www.ch.embnet.org/software/LALIGN_form.html) *In silico* analyses of the *Orthopoxvirus ati* sequences were also performed using the WebCutter software (version 2.0, Max Heiman, http://rna.lundberg.gu.se/cutter2/) in order to find and compare the localization of *Xba*I restriction sites.

Phylogenetic analyses

Phylogenetic trees were constructed by Neighbor-joining and Maximum Parsimony methods implemented in MEGA3 [34]. The Neighbor-joining tree was constructed using the Tamura Nei nucleotide substitution model [35]. The Maximum Parsimony tree was constructed using 100 replications of random addition trees. For both trees,

multiple indel events were considered as a single event and bootstrap analyses were done with 1,000 replicates.

Results and discussion

Amplification of the internal region of the *ati* gene

The *ati* gene amplification of the Brazilian VACVs strains ARAV, GP2V, MURV, PSTV, and SAV was performed using the ATI up and ATI low primers [16] resulting in amplicons of approximately 1,600 bp (data not shown). For BAV and GP1V whose *ati* gene amplifications could not be performed with those primers, amplification was done using primers P4C1 and RNApol [27], resulting in the production of fragments of approximately 290 bp (data not shown). All of these amplicons were cloned and three clones for each virus were sequenced.

Nucleotide sequence analysis

The sequencing of the BAV and GP1V amplicons revealed that both were 289 bp long. These sequences were identical to each other and when compared to VBH, VACV-WR and CPXV strain Brighton Red (CPXV-BR) sequences, they presented 100%, 99.6%, and 89.9% identity, respectively (data not shown). Indeed, BAV and GP1V *ati* gene sequences presented the same deletion that was found in VBH sequence. Only the last 112 nucleotides at the 3′end of the gene that overlaps with the terminal region of the RNA polymerase gene remained. Since the RNA polymerase is an essential gene for virus multiplication, it can be presumed that these 112 nucleotides actually belong to the 3′ end of the RNA polymerase gene and that the *ati* gene is deleted in those strains. Also, expression of a smaller ATI protein is not possible since the *ati* gene promoter is also deleted in these viruses. Large deletions of the *ati* gene have previously been characterized in the VACV Copenhagen (VACV-COP) strain, where only 1,131 nucleotides of this gene are present [36]. However, in the VACV-COP, the *ati* gene promoter is present, unlike BAV, GP1V, and VBH.

The ARAV, GP2V, MURV, and PSTV *ati*-PCR amplicons were 1,573 bp in length while the SAV amplicon was 1,606 bp long. The sequences were compared to each other and to other *Orthopoxvirus* sequences to determine nucleotide identity (%) between them (Table 2). When the Brazilian VACV strains were compared to each other, ARAV, GP2V, MURV, and PSTV sequences presented the highest identities with values ranging from 99.1% to 99.3%. Lower values of identity, ranging from 95.6% to 95.8%, were obtained when those sequences were compared to the SAV sequence. When the Brazilian VACV strains sequences were compared to other VACV strain *ati* sequences, including the RPXV strain Utrecht (RPXV-UTR), which is considered a VACV subspecies, the highest values of identity were those belonging to SAV, ranging from 98.2% to 99.3%. ARAV, GP2V, MURV, PSTV sequences presented the highest identity values when compared to each other and to the VACV-LST sequence, which was one of the vaccine strains used in Brazil during the smallpox eradication campaign [37]. Yet, SAV *ati* sequence was more divergent from the other Brazilian VACV strain sequences and more similar to VACV-WR strains. When all those sequences were compared to other *Orthopoxvirus* sequences, the highest degrees of similarity were found in relation to CPXV-BR than to other *Orthopoxvirus* sequences.

Comparison of the internal region of Orthopoxvirus *ati* gene

When analyzing the alignment of the *ati* sequences, two blocks of deletions, each one containing 12 and 21 nucleotides, were observed in the ARAV, GP2V, MURV, and PSTV sequences (Fig. 1A). However, these deletions were not observed in the SAV sequence. These deletions, totaling 33 nucleotides, correspond exactly to the difference in size observed between the SAV (1,606 bp) and ARAV, GP2V, MURV, and PSTV sequences (1,573 bp). These findings suggest the existence of a signature deletion that is exclusive to Brazilian VACVs isolated from bovine vaccinia outbreaks. However, this deletion is not an obligatory pattern as seen for GP1V, which has a complete deletion of the *ati* gene. Similar deletion events had been previously observed when 18 nucleotides were deleted in *ha* sequences of Brazilian VACV strains isolated from bovine vaccinia outbreaks [1–3, 24]. Besides these two blocks of deletions, nucleotide substitutions were observed between Brazilian and other VACV strains sequences. A schematic diagram of ARAV, GP2V, MURV, PSTV, and SAV *ati* sequences compared with other *Orthopoxvirus* sequences is shown in Fig. 1B. The deletions present in the VACV sequences when compared to CPXV sequences were also observed in ARAV, GP2V, MURV, PSTV, and SAV sequences. Furthermore, regarding the CPXVs, ARAV, GP2V, MURV, PSTV, SAV, VACVs and VARVs sequences, MPXV sequences presented one deletion of 18 nucleotides. Moreover, the MPXV strains Congo_2003_358 (MPXV-CONG2003), Gabun (MPXV-GABUN), Zaire_1979-005 (MPXV-ZAIRE79) and Zaire-96-I-16 (MPXV-ZAIRE96) presented another deletion of 456 nucleotides when compared to CPXVs, ARAV, GP2V, MURV, PSTV, SAV, VACVs and VARVs sequences.

Table 2 Nucleotide identities of *ati* gene internal region sequences of Araçatuba virus (ARAV), Guarani P2 virus (GP2V), Muriaé virus (MURV), Passatempo virus (PSTV), SPAn232 virus (SAV) and other *Orthopoxvirus* available in GenBank

Virus	Nucleotide identity (%)				
	ARAV	GP2V	MURV	PSTV	SAV
ARAV					
GP2V	99.3				
MURV	99.4	99.2			
PSTV	99.3	99.1	99.6		
SAV	95.8	95.6	95.9	95.7	
VACV-WR	96.5	96.4	96.5	96.4	99.3
VACV-LST	97.1	96.9	96.9	97.2	98.9
VACV-LCmO	96.9	96.7	96.7	97.0	98.7
VACV-LCm8	96.9	96.7	96.7	97.0	98.7
VACV-TT	96.6	96.4	96.5	96.7	98.2
RPXV-UTR	96.6	96.4	96.4	96.7	98.5
CPXV-BR	90.6	90.5	90.5	90.7	92.1
CPXV-GRI90	90.6	90.6	90.6	90.7	92.1
VARV-GAR	85.4	85.3	85.3	85.5	87.2
VARV-IND	85.3	85.2	85.2	85.4	87.1
VARV-BSH	84.8	84.8	84.8	84.9	86.6
MPXV-USA39	80.7	80.6	80.7	80.8	82.2
MPXV-USA44	80.7	80.6	80.7	80.8	82.2
MPXV-W7-61	80.6	80.6	80.6	80.8	82.1
MPXV-COP	80.6	80.6	80.6	80.8	82.1
MPXV-ZAIRE79	69.8	70.0	70.0	69.9	69.9
MPXV-CONG_2003	69.4	69.4	69.5	69.5	69.8
MPXV-ZAI_96	69.4	69.4	69.4	69.5	69.7
ECTV-MOS	66.0	66.1	65.4	66.1	66.7

Vaccinia virus: Western Reserve (VACV-WR), Lister (VACV-LST), LC16mO (VACV-LCmO), LC16m8 (VACV-LCm8), Tian Tan (VACV-TT), Rabbitpox virus Utrecht (RPXV-UTR), Cowpox virus: Brighton Red (CPXV-BR), GRI-90 (CPXV-GRI90), Variola virus: Garcia-1966 (VARV-GAR), India-1967 (VARV-IND), Bangladesh-1975 (VARV-BSH), Monkeypox virus: USA_2003_039 (MPXV-USA39), MPXV-USA_2003_044 (MPXV-USA44), WRAIR7-61 (MPXV-W7-61), COP-58 (MPXV-COP), Zaire-1979 (MPXV-ZAIRE79), Congo_2003_358 (MPXV-CONG2003), Zaire-96-I-16 (MPXV-ZAIRE96) and Ectromelia virus Moscow (ECTV-MOS)

In silico restriction analysis of the internal region of the *ati* gene

Since ARAV, GP2V, MURV, and PSTV had different restriction profiles for the *ati* amplicon after digest with *Xba*I, we decided to investigate how the blocks of deletions and nucleotide substitutions are associated with this distinct restriction profile exhibited by these viruses. We also compared these profiles with those obtained for other VACV strains. All VACV *ati* sequences were submitted to an in silico restriction analysis for the enzyme *Xba*I. When the ARAV, GP2V, MURV, and PSTV *ati* amplicon sequences were digested with that restriction enzyme, six fragments were obtained, four of them being identical in size to those observed for SAV, VACV-WR, VACV-LCmO, VACV-LCm8, and VACV-LST. These fragments consisted of 323, 216, 100, and 69 bp (Fig. 2). Additionally, two other fragments of 568 and 297 bp were observed (Fig. 2). These two fragments are the result of the digestion of the 898 bp fragment, where the 33 nucleotide deletion is present, in two smaller blocks. This digestion was due to the replacement of a cytosine (position 136 031 of VACV-WR complete genome) for an adenine that led to the appearance of a new *Xba*I restriction site in the sequences of ARAV, GP2V, MURV, and PSTV (Fig. 1A). These facts explain why the ARAV, GPV2, MURV, and PSTV PCR-RFLP profiles were not identical to the VACV-WR, VACV-LST, and SAV profiles [1, 2, 22, 24]. Interestingly, other VACV strains also exhibited different digestion patterns, as observed for VACV strains Tian Tan (VACV-TT), 3737 (VACV-3737), Acambis clone 3 (VACV-A3), and Acambis clone 2000 (VACV-A2000) (Fig. 2).

Since this variability in the *Xba*I digestion profile was observed in both the Brazilian VACV sequences and other VACV sequences, we decided to investigate if the *Xba*I digestion profile of the *ati* internal region presents such

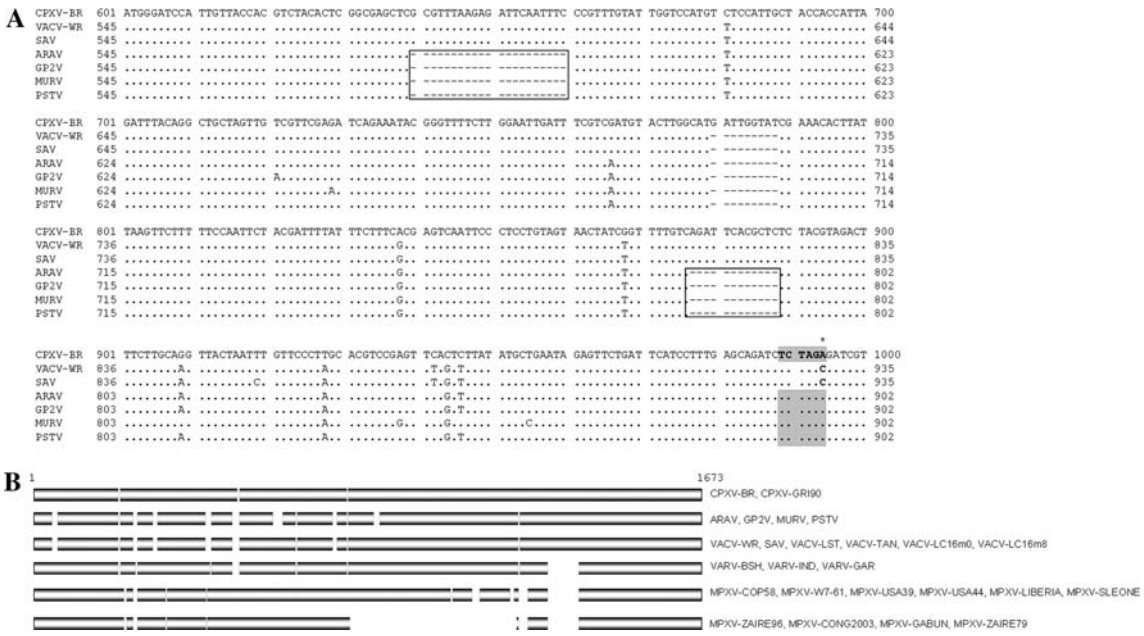

Fig. 1 Analysis of *ati* gene internal region alignment. (**A**) Partial alignment of the ARAV, GP2V, MURV, PSTV, and SAV *ati* gene internal region. Amplicons were amplified using ATI up and ATI low primers as described by Meyer and colleagues [16], cloned and sequenced. Sequences were aligned with other similar poxviruses sequences available in the GenBank database. Deletion regions among compared sequences are shown. (-) represent deletions; (.) represent nucleotide identities; (†) indicates the nucleotide substitution sites that lead to a new *Xba*I restriction sites in *ati* sequences of ARAV, MURV, GP2V, and PSTV shown in grey. Boxes show the 12 and 21 nucleotide deletions. (**B**) Schematic illustration of the ARAV, GP2V, MURV, PSTV, and SAV *ati* gene internal region alignment. Amplicons were amplified using ATI up and ATI low primers as described by Meyer et al. [16], cloned and sequenced. Sequences were aligned with other similar poxviruses sequences available in the GenBank database. Deletion regions among compared sequences are shown

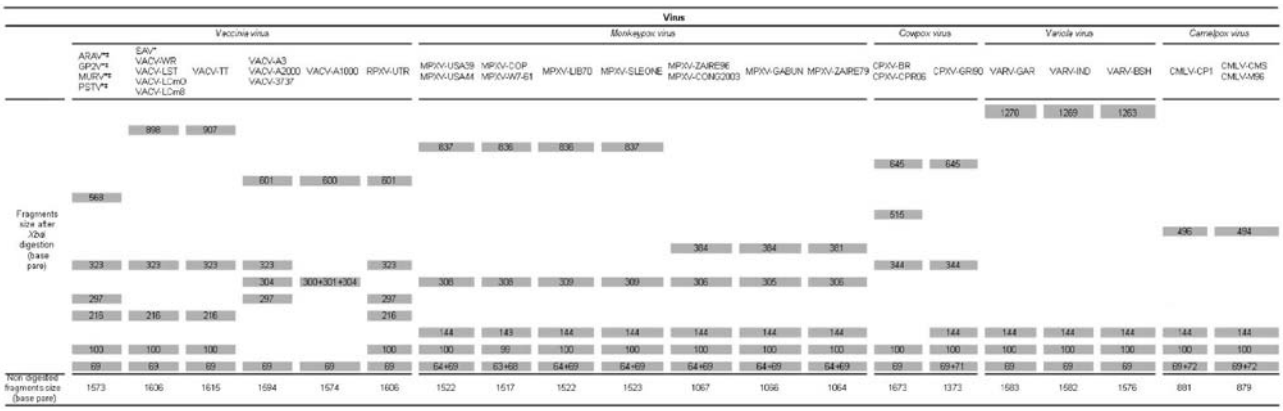

Fig. 2 Expected *Orthopoxvirus ati* PCR-RFLP fragments after in silico analysis with *Xba*I. Note that the diagram resembles the pattern observed after agarose gel electrophoresis

variability in other *Orthopoxvirus*. After these in silico analyses, it was observed that the VARV and CMLV strains exhibited well-conserved digestion patterns for each species, but CPXV and MPXV strains showed different patterns for each species (Fig. 2). It is known that *Orthopoxvirus* species differentiation based on the *ati* gene sequence using the previously described PCR-RFLP [16] is an efficient method. Indeed, it was previously used by our group as a diagnostic tool for Brazilian VACVs [1, 2, 22–24]. However, the occurrence of the *Orthopoxvirus* species, including MPXV and VACV strains, with different *ati* deletion patterns indicates that *ati* PCR-RFLP should not be used alone for *Orthopoxvirus* differentiation, since the variability observed on *ati* gene of these viruses could result in profiles different from the expected ones. This is especially relevant for the case of Brazilian VACV strains, for

which the existence of polymorphisms in the *ati* gene, and even the entire gene deletions have been confirmed. Thus, it is important to use other genes for *Orthopoxvirus* differentiation. In fact, some methodologies based on the *ha* gene have been developed for that purpose [20, 38]. Recently, Damaso and colleagues [38] proposed two strategies to identify Brazilian VACV isolates based on the amplification of the *ha* gene by PCR. However, the *ha* gene of the Brazilian VACV strains also present polymorphisms that could result in problems or even cause misdetection and misidentification of these viruses. It has been established that the majority of the VACV strains isolated in Brazil until now posses an 18 nt deletion in the *ha* sequence [1–3, 24]. Besides single nucleotide polymorphisms found in the *ha* gene, it has already been demonstrated that some Brazilian VACV strains do not posses this 18 nt deletion in the *ha* sequence. Examples of those are VBH [27] and GP1V, with the latter isolated during a vaccinia outbreak in Brazil [24]. In this regard, the usage of more than one methodology, such as the ones based on more conserved genes such as *tk* and *vgf*, for *Orthopoxvirus* detection and differentiation must always be kept in mind. Finally, the methodologies based on the analysis of *ati* and *ha* genes could be used as additional tools to identify and detect possible polymorphisms among viruses.

Phylogenetic inferences

Phylogenetic trees were constructed using *Orthopoxvirus* *ati* sequences. Both trees constructed using Neighbor-joining (Fig. 3) or Maximum Parsimony (data not shown) methods presented similar topologies supported by high bootstrap values. All VACVs were grouped in a cluster apart from the other *Orthopoxvirus*. SAV clustered with VACV-WR while ARAV, GP2V, MURV, and PSTV were more closely related to each other. Similar clustering of these Brazilian VACVs, isolated during bovine vaccinia outbreaks, has also been observed during phylogenetic analysis based on the *ha* gene. Phylogenetic analyses based on the *ha* gene sequence showed that ARAV, GP2V, MURV, PSTV and the Cantagalo virus (CTGV), another Brazilian VACV isolated during a vaccinia outbreak, are closely related to each other while VBH, GP1V, and VACV-WR exhibited a close relationship [1, 3, 24, 25]. No correlation between the original host of the virus and the *ati* gene sequence was observed in this study, or in previous phylogenetic studies based on the *ha* sequence. It was observed that MURV, isolated from a milker, is closely related to ARAV, GP2V, PSTV, and CTGV, all isolated from cattle. GP1V, isolated from cattle, clustered with BAV and VBH that were isolated from rodents [1, 3, 24, 25]. Regarding the strains isolated from rodents, it was shown that SAV did not cluster with BAV and VBH, when the *ati* gene was analyzed. Moreover, in phylogenetic analyses based on *ati* and *ha* sequences, none of the Brazilian VACV presented a close relationship with vaccine strains, such as VACV-LST, which was used during the Smallpox Eradication Campaign in Brazil [1, 24, 25].

The fact that the *ati* gene is not an essential gene could have lead to less selective pressures on this gene during the

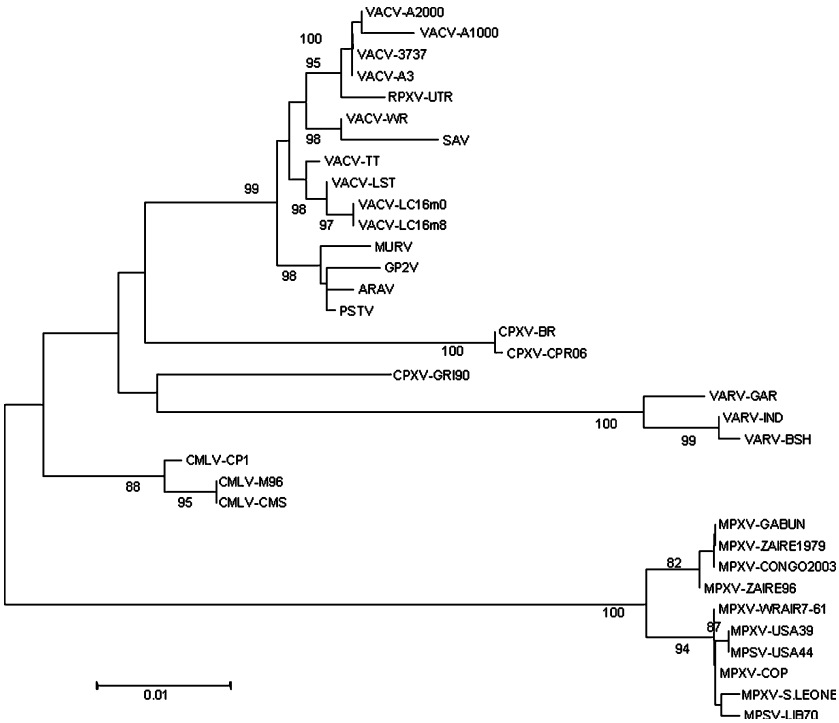

Fig. 3 *Orthopoxvirus* phylogenetic analysis based on *ati* gene. The midpoint-rooted tree was constructed based on the internal region of the *ati* gene (region corresponding from nucleotides 1,35,104 to 1,36,708 of Vaccinia virus, strain Western Reserve). The tree was constructed by Neighbor-joining method using the Tamura Nei model of nucleotide substitutions implemented in MEGA3. Multiple indel events were considered as a single indel event and bootstrap analyses were done with 1,000 replicates. Bootstrap values above 80% are shown. Strain names are presented according to abbreviations shown in Table 1

evolution of *Orthopoxvirus*. This could have enabled the appearance of deletions in this gene that would have been maintained in the virus population, resulting in different *ati* gene patterns seen among *Orthopoxvirus*, including the Brazilian VACV strains. At this point, the origin of *ati* gene deletions including minor deletions of 21 and 12 nucleotides or deletion of the entire gene, and even the origin of these viruses must be questioned. If they do not have the same ancestral lineage, these different *ati* patterns could have been originated from different strains with distinct *ati* genes. Therefore, these different strains could had been introduced in Brazil during the Smallpox Eradication Campaign, when different VACV strains were used for vaccine production [37]. Moreover, these strains could have endemically circulated through the country previous to the smallpox eradication campaign. However, if they do have the same ancestor, the internal region of the *ati* gene similar to SAV probably originated with the currently circulating strain. A virus with the same *ati* structure of SAV could have suffered mutations giving rise to two distinct groups.

Our results reinforce the fact that more than one VACV strain is circulating in Brazil. Therefore, based on the structure of the *ati* gene, we suggest that there are three different groups of Brazilian VACV strains. The first group is represented by SAV, which does not have any deletion in the studied region of the *ati* gene. The second one is represented by ARAV, GP2V, MURV, and PSTV, which have two blocks of deletions. The third group is represented by GP1V, BAV, and VBH, which have the entire *ati* gene deleted.

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