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Short Communication

Belo Horizonte virus: a vaccinia-like virus lacking the A-type inclusion body gene isolated from infected mice

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Here is described the isolation of a naturally occurring A-type inclusion body (ATI)-negative vaccinia-like virus, *Belo Horizonte virus* (VBH), obtained from a mousepox-like outbreak in Brazil. The isolated virus was identified and characterized as an orthopoxvirus by conventional methods. Molecular characterization of the virus was done by DNA cross-hybridization using *Vaccinia virus* (VACV) DNA. In addition, conserved orthopoxvirus genes such as vaccinia growth factor, thymidine kinase and haemagglutinin were amplified by PCR and sequenced. All sequences presented high similarity to VACV genes. Based on the sequences, phenograms were constructed for comparison with other poxviruses; VBH clustered consistently with VACV strains. Attempts to amplify the ATI gene (*ati*) by PCR, currently used to identify orthopoxviruses, were unsuccessful. Results presented here suggest that most of the *ati* gene is deleted in the VBH genome.

The genus *Orthopoxvirus* comprises genetically and antigenically related viruses that replicate in the cytoplasm of vertebrate cells. The genus includes *Variola virus* (VARV), *Vaccinia virus* (VACV) and several pathogens of veterinary and human significance (Fenner *et al.*, 1989; Damaso *et al.*, 2000). VACV, the prototype of the *Poxviridae*, contains a large, linear, double-stranded DNA genome with a high level of nucleotide similarity with other members of the genus *Orthopoxvirus*. *Orthopoxvirus* genomes show a conserved restriction profile, and most differences occur in the larger fragments of *Hin*dIII-digested DNA near the genome termini (Esposito & Knight, 1985; Buller & Palumbo, 1991; Marques *et al.*, 2001; da Fonseca *et al.*, 2002).

Most strategies for fast taxonomic analysis of poxviruses involve rapid-screening techniques for identification of unknown isolates, based on sequencing or restriction fragment-length polymorphism (RFLP) of different genes (Roop *et al.*, 1995; Marques *et al.*, 2001; da Fonseca *et al.*, 2002). Meyer *et al.* (1994, 1997) proposed PCR amplification of the A-type inclusion body gene (*ati*) followed by restriction analysis as a rapid approach to differentiate orthopoxviruses. Indeed, characterization of genes such as haemagglutinin (*ha*) and *ati* has been used for identification of orthopoxvirus isolates including *Monkeypox virus* (MPXV), *Ectromelia virus* (ECTV), and VACV associated with cowpox-like outbreaks (Meyer *et al.*, 1997; Neubauer *et al.*, 1997; Damaso *et al.*, 2000; da Fonseca *et al.*, 2002; Trindade *et al.*, 2003).

Little is known about how orthopoxviruses are maintained in nature or about the role of wild reservoirs. It has been demonstrated that, despite its wide host range, different species of wild rodents are the natural reservoir hosts for *Cowpox virus* (CPXV) in Great Britain and probably in other parts of Europe, where CPXV is endemic (Chantrey *et al.*, 1999; Hazel *et al.*, 2000). Similarly to CPXV, and

The GenBank accession numbers of the sequences reported in this article are AF163843, AF163845, AF501620 and AY542799.

unlike VARV, VACV has a wide host range and is able to infect humans, cattle and rodents (Fenner *et al.*, 1989; da Fonseca *et al.*, 1999). Very little is known about the occurrence and circulation of orthopoxviruses in Brazil, but in recent years many isolates of vaccinia-like viruses have been reported (Damaso *et al.*, 2000; da Fonseca *et al.*, 2002; Trindade *et al.*, 2003). At a time when the possible recurrence of poxvirus diseases is being considered and discussed, the increasing number of isolations of vaccinia-like viruses in Brazil (some causing illness in humans) imply a genuine public health threat.

Here we report the molecular characterization of a naturally occurring A-type inclusion body (ATI)-negative vaccinia-like virus isolated from a mousepox-like outbreak that took place in the animal facility of the Biological Institute of the University of Minas Gerais, Brazil. Mice were obtained from the University of Campinas, State of São Paulo, Brazil, and were apparently healthy on arrival. After a few days, some animals died and others presented characteristic skin lesions, developing a generalized skin rash. A virus was isolated from clinical specimens after inoculation onto chorioallantoic membranes (CAMs) of chick embryonated eggs, and named *Belo Horizonte virus* (VBH) (Diniz *et al.*, 2001). After isolation, the virus was propagated and titrated in Vero cells as described by Campos & Kroon (1993), purified in sucrose gradients as described by Joklik (1962), and identified by conventional methods that included pock morphology in CAMs, electron microscopy and neutralization tests using anti-VACV polyclonal antibodies (Diniz *et al.*, 2001).

DNA was extracted from purified virus stocks by treatment with proteinase K, SDS and β-mercaptoethanol followed by phenol extraction, as described by Massung & Moyer (1991). Purified virus DNA (2 μg) was digested with *Hin*dIII enzyme (Promega), separated by electrophoresis on a 0·4 % (w/v) agarose gel and stained with ethidium bromide. Surprisingly, the digestion pattern obtained did not match that of ECTV (Esposito & Knight, 1985), a natural candidate due to the nature of the outbreak, but closely resembled digested patterns of VACV DNA (data not shown).

The digested DNA was transferred to a nylon membrane (Hybond-N; Amersham Pharmacia) using modified Southern-blot protocols (Sambrook *et al.*, 1989) and cross-hybridized with VACV strain Western Reserve (VACV WR) total genome as probe (Meyer *et al.*, 1997). The probe was labelled by nick translation (Nick Translation System; Promega) with [α-^{32}P]CTP according to the manufacturer's protocol. Samples were hybridized for 16 h at 65 °C and processed as described by Church & Gilbert (1984). After washing, membranes were exposed to X-Omat Kodak film. DNA from VACV WR (virus obtained from the National Institute for Medical Research, Mill Hill, London, UK) was also digested, transferred to membranes and hybridized with the same probe.

The *Hin*dIII restriction profile of VBH revealed a typical orthopoxvirus pattern (Fig. 1) and resembled that of VACV WR. Fragments shorter than 7 kb (Fig. 1i–m) derived from VBH DNA showed similarity in their migration pattern to VACV WR DNA, while small differences were detected in the migration of the larger fragments (Fig. 1a–h). Taken together, the similarity between the DNA-digested profiles from VACV and VBH and the occurrence of cross-hybridization suggested a close relationship between VBH and VACV.

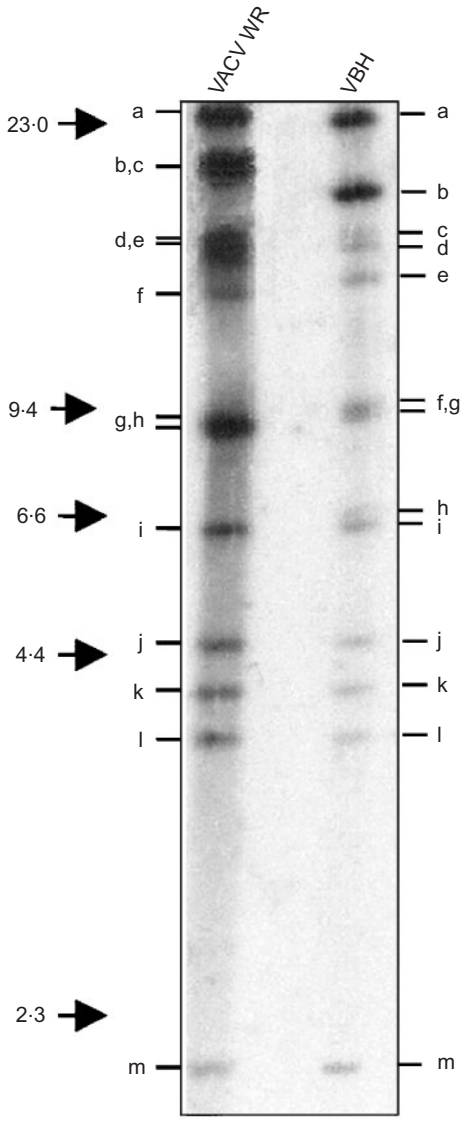


Fig. 1. Southern-blot analysis of digested VBH DNA using VACV WR DNA as probe. Purified VBH and VACV WR DNA were digested with *Hin*dIII, separated by electrophoresis in a 0·4 % agarose gel, blotted onto a nylon membrane and hybridized. Arrowheads indicate sizes of dsDNA markers in kbp.

In order to obtain more accurate phylogenetic information, we amplified orthopoxvirus-conserved genes including thymidine kinase (*tk*) and vaccinia growth factor (*vgf*)

from the VBH genome by PCR. The primers used to amplify the *vgf* gene were as described by da Fonseca *et al.* (1998). Oligonucleotides based on the *tk* nucleotide sequence of VACV WR were 5′-GCGAGGATCCAACGGCGGACATATTCAG-3′ and 5′-GCAGAAAGCTTTGAGTCGATGTAACAC-3′, and amplified a 530 bp DNA fragment. Restriction sites for *Hin*dIII and *Bam*HI were added (underlined). The purified VBH genome was used as a template and annealing was carried out at 45 °C. Amplified fragments were cloned into plasmid pUC18 (Sure-clone; Pharmacia). Alternatively, the *ha* coding sequence was amplified using primers EACP1 and EACP2, as described by Roop *et al.* (1995), and a 960 bp fragment was obtained and cloned into pGEM-T vector (pGEM-T Easy Vector Systems; Promega). The VBH PCR-amplified *tk*, *vgf* and *ha* genes were sequenced in both orientations by the dideoxy chain-termination method using M13 primers (Sanger *et al.*, 1977). DNA sequences of the VBH *tk*, *vgf* and *ha* genes were deposited in GenBank, under accession numbers AF163843, AF163845 and AY542799. Using BLAST (Altschul *et al.*, 1990) we searched GenBank for poxvirus sequences similar to these genes. The nucleotide and amino acid sequences of these genes were aligned using CLUSTAL W (Thompson *et al.*, 1994) and this alignment was edited and reformatted for phylogenetic analysis using BIOEDIT (Hall, 1999) and FORCON (Raes & Van der Peer, 1999). Neighbour-joining phenograms (Saitou & Nei, 1987) were constructed using TREECON (Van de Peer & de Wachter, 1997) based on Jukes & Cantor (1969) and Tajima & Nei (1984). To assess support for the inferred relationships, 500 bootstrap samples (Felsenstein, 1985) were generated. Distantly related *Leporipoxvirus* genes could be used as outgroup sequences, but this resulted in poorly supported phenograms. Therefore, phenograms were drawn unrooted (Fig. 2a, b). In addition, maximum-likelihood and Bayesian trees were constructed on the same data set using TREE-PUZZLE 5.0 (50 000 replicates, HKY substitution model, Hasegawa *et al.*, 1985; Strimmer & von Haeseler, 1996), PAUP* (500 bootstraps, GTR + G substitution model, Swofford, 1998), and MRBAYES (9 500 000 generations, four chains, Ronquist & Huelsenbeck, 2003), and resulted in similar phenograms.

Sequence analysis of *tk*, *vgf* and *ha* genes confirmed the cross-hybridization data, showing high similarity between VBH and VACV genes obtained from GenBank. The *tk* and *vgf* genes from VBH presented identities of up to 99 % with genes from VACV WR, the *ha* gene 95 % identity. Not surprisingly, the similarity between VBH and ECTV Moscow genes was lower, reaching 96, 90 and 93 % for *tk*, *vgf* and *ha*, respectively. Accordingly, VBH genes clustered with VACV genes in all phenograms including the concatenated, and are more distantly related to the VARV genes (Fig. 2a, b).

RFLP analysis using the *ati* gene has been used for poxvirus taxonomic characterization. Although the formation of typical A-type inclusions is restricted to cells infected with a few orthopoxviruses, such as CPXV, *Raccoonpox virus* and ECTV, the *ati* gene can be detected in the genome of other viruses (Meyer *et al.*, 1997; Funahashi *et al.*, 1988). However, PCR using VBH DNA as template and the primers ATI-up and ATI-low specified by Meyer *et al.* (1997) generated no products. The lack of DNA PCR amplification could be due to a deletion at the *ati* gene of VBH, a feature also found on the genome of certain VACV strains. To investigate this, we mapped the possible *ati* deletion through dot-blot hybridization employing oligonucleotides P4C1 (located within the *p4c1* gene) (5′-GGAGATCTAGACCACCGTTTCCCAGACATGAATATC-3′) and RNApol (located within the *rpo132* gene) (5′-GGAAGCTTTCTCTCTCCTCTCTTAACAAAAATTG-3′), designed based on CPXV Brighton Red (CPXV BR). Hybridization scored positive for both primers and indicated that the flanking regions of the *ati* gene are present in the VBH genome (data not shown).

In order to evaluate the extent of the VBH *ati* gene deletion, the oligonucleotides that scored positive on the dot-blot assay were used in PCRs (Funahashi *et al.*, 1988). Standard PCR mixtures contained 10 pmol of each primer (P4c1 and RNApol) plus 20 ng purified VBH or VACV WR DNA as templates. Annealing was performed at 58 °C. For VACV WR, as expected, a product of about 4·3 kb was obtained. However, for VBH a single DNA fragment of about 300 bp was detected (Fig. 3a), indicating that in the VBH genome a major portion is missing between the annealing positions of primers P4c1 and RNApol of the *ati* gene. The PCR DNA product was cloned using the pGEM-T Easy Vector Kit (Promega) and sequenced in both orientations. The sequence obtained was deposited in GenBank under accession number AF501620 and analysed using BLASTN and BLASTX programs (Altschul *et al.*, 1990). It showed high similarity to equivalent regions from other orthopoxviruses, especially to VACV (Fig. 3b). Alignment of the sequence revealed that only 112 nt of the *ati* gene are present, encoding only the C-terminal portion of the ATI protein (Fig. 3c). Other VACV strains also lack the *ati* gene (Goebel *et al.*, 1990; Johnson *et al.*, 1993; Osterrieder *et al.*, 1994).

For Brazil and South America there is little information about the occurrence of veterinary and wild poxviruses. However, many studies have been conducted in recent years regarding the circulation of poxviruses linked to cowpox-like outbreaks, and a growing number of vaccinia-like virus isolations have been reported (Damaso *et al.*, 2000; Trindade *et al.*, 2003). In this study we describe the isolation and characterization of a vaccinia-like virus obtained from a mousepox-like outbreak in an animal facility in Brazil. The virus, named VBH, is an ATI-negative virus that could not be identified using standard published PCR procedures. At this point it is difficult to speculate about the origin of VBH. The infected mice came from the Universidade de Campinas, where there was no ongoing research with VACV or any other poxvirus. The hypothesis that mice may have been infected by an endogenous source is improbable. However, the Institute's animal facility has

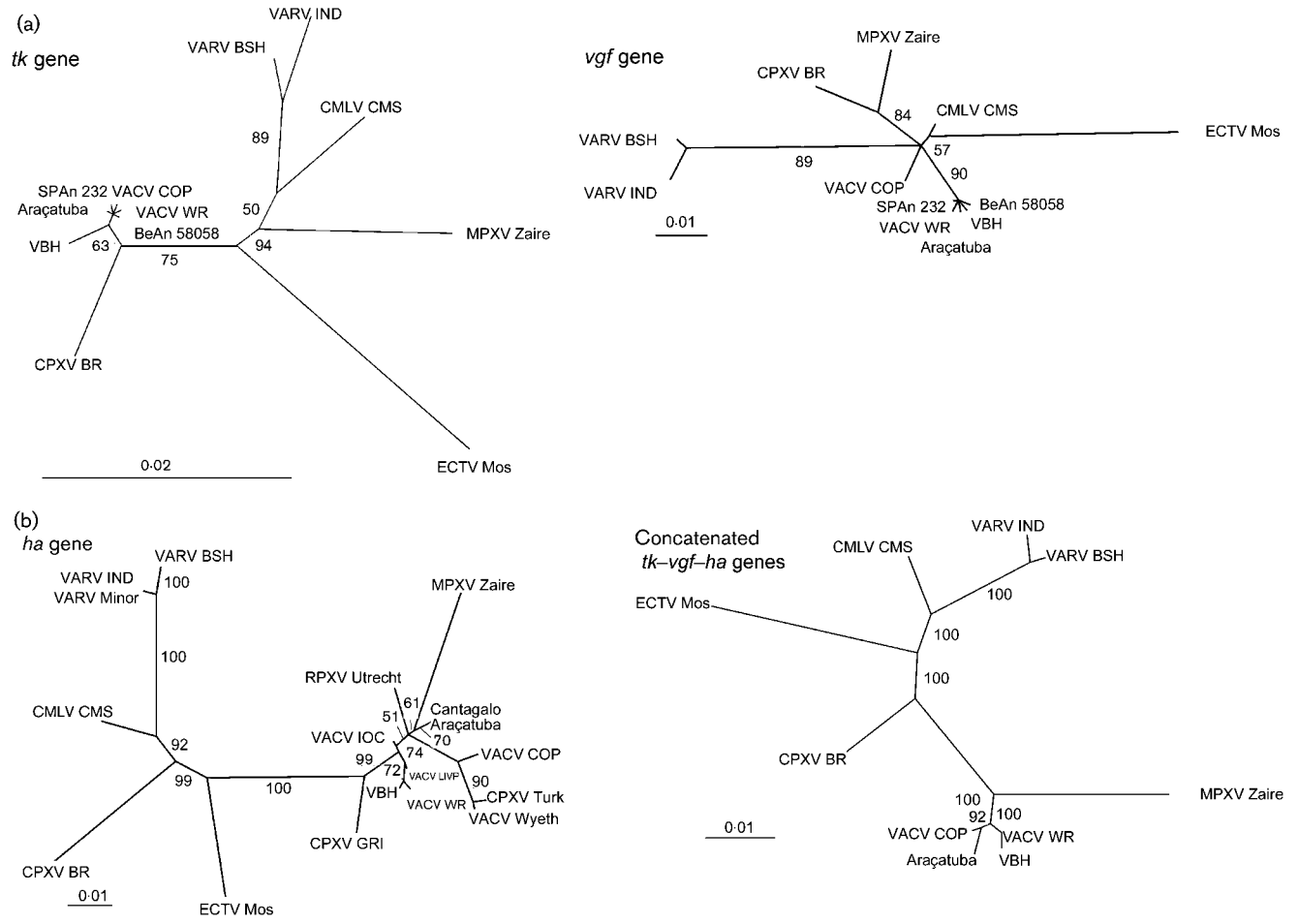
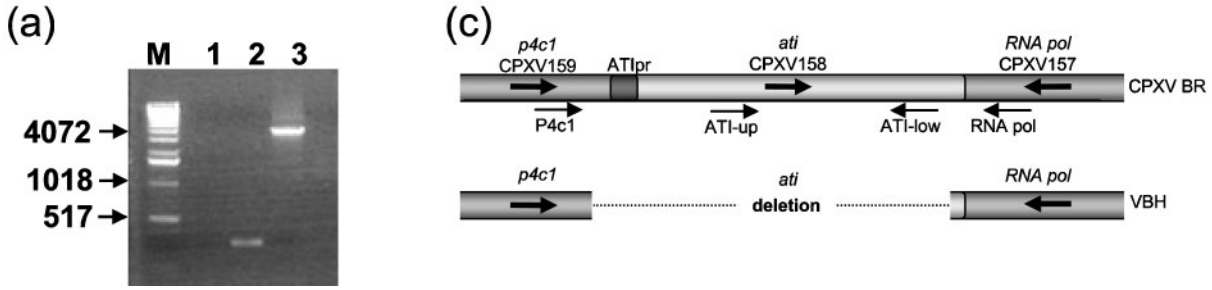


Fig. 2. Phenograms using (a) *tk* and *vgf* and (b) *ha* nucleotide sequences and the concatenated phenogram from viruses of the genus *Orthopoxvirus*. Five hundred bootstrap samples (Felsenstein, 1985) were generated; only bootstrap values above 50 % are shown. The scale indicates substitutions per site. VBH sequences obtained through nucleotide sequencing were compared to poxvirus sequences obtained from GenBank. *tk* gene: *Araçatuba virus* (Araçatuba; AF503169); BeAn 58058 virus (BeAn 58058; AF023448); VBH (AF163843); *Camelpox virus* CMS (CMLV CMS; AY009089); CPXV BR (AF482758); ECTV Moscow (ECTV Mos; AJ574815); MPXV Zaire (AF380138); SPAn 232 virus (SPAn 232; AF163842); VACV Copenhagen (VACV COP; M35027); VACV WR (AY243312); VARV India (VARV IND; X69198); VARV Bangladesh (VARV BSH; L22579). *vgf* gene: Araçatuba (AF503170); BeAn 58058 (U79140); VBH (AF163845); CMLV CMS (AY009089); CPXV BR (AF482758); ECTV Mos (AJ574815); MPXV Zaire (AF380138); SPAn 232 (AY523995); VACV COP (M35027); VACV WR (AY243312); VARV BSH (L22579); VARV IND (X69198). *ha* gene: Araçatuba (AY523994); VBH (AY542799); CMLV CMS (AY009089); *Cantagalo virus* (Cantagalo; AF229247); CPXV BR (AF482758); CPXV Gri-90 (CPXV GRI; Z99047); CPXV Turkmenia-1974 (CPXV Turk; Z99048); ECTV Mos (AF375092); MPXV Zaire (AF380138); Rabbitpox virus Utrecht (RPXV Utrecht; RVZ99049); VACV COP (M35027); VACV IOC (AF229248); VACV LIVP (Z99046); VACV WR (AY243312); VACV Wyeth (VVZ99051); VARV BSH (L22579); VARV IND (X69198); VARV minor (VARV Minor; Y16780).

Fig. 3. Analysis of the VBH *ati* gene. (a) PCR amplification using RNApol and P4c1 primers. PCR products were separated by electrophoresis on a 1·5 % agarose gel and stained with ethidium bromide. Lanes: M, molecular size markers; 1, negative control; 2, VBH DNA; 3, VACV WR DNA. Arrowheads indicate sizes of DNA markers in bp. (b) The PCR-amplified, 305 bp VBH DNA fragment was sequenced (AF501620) and compared with the nucleotide sequences of other poxviruses: CPXV BR (AF482758); VACV Copenhagen (VACV COP; NC001559); VACV WR (AY243312); ECTV Moscow (ECTV Mos; AF012825). Dashes, deletions; *, consensus sequence; //, sequence not shown. Sequences are shown in the minus strand, full-line arrows represent gene orientation and primers are in bold (positions: 155 725–155 698 and 151 372–151 347). Sequence co-ordinates are based on CPXV BR sequence. (c) Schematic comparison of sequenced regions of VBH and CPXV BR based on (b). Boxes represent genes; thin arrows, primer positions; bold arrows, orientation of genes; dashed lines, deleted area.



(b)

p4c1 / CPXV159

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                155725                                  155698
1 CPXV BR  155968//AGACCACCGT TTCCCAGACA TGAATATCAT AAGAGGCCGA AAGTTCTTCC
2 VBH            //********** ********** *********G *C*C------ ----------
3 VACV COP       //---------- ---------- ---------- ---------- ----------
4 VACV WR        //********** ********** ********** ********** **C*******
5 ECTV Mos       //********** ********** ********** ********** **C*******
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ati promoter — *ati* / CPXV158

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1 CPXV BR  155675//155390//155344//151639 ATCAGGAT-T ATAGCGAACT CTATTCTGCG
2 VBH      ------//------//------//------ ---------- ---------- *G*****ATA
3 VACV COP 138947//      //      //137909 *****ACG-* C--------- *G*****ATA
4 VACV WR  138746//      //      //134789 *****ACG-* C--------- *G*****ATA
5 ECTV Mos 147165//      //      //143629 ********C* CA**TACCA* ****A*CT*A

1 CPXV BR  AGTAACAATA CC-GAATCCA AAAATGTGTT TAGTGAATTA GCTTATCTTG ATGATCTTGA
2 VBH      GT*******C *---*CG**C *TTT*A*AA* *C*------- ---------- --*****C**
3 VACV COP GT*******C *---*CG**C *TTT*A*AA* *C*------- ---------- --*****C**
4 VACV WR  GT*******C *---*CG**C *TTT*A*AA* *C*------- ---------- --*****C**
5 ECTV Mos *CCTC*TCC* *AT*TTAT** *T***CCACC *CA***TCC* ATA*GGGGA* *ATTA*AG**

1 CPXV BR  TAAACTTGAT GATATTGATG AGTATCTACT A---AAC--- AATATTATGC CAGAAAAGAC
2 VBH      ---*T***** *********A T********* *CCA***TAG *******CA* **********
3 VACV COP ---*T***** *********A T********* *CCA***TAG *******CA* **********
4 VACV WR  ---*T***** *********A T********* *CCA***TAG *******CA* **********
5 ECTV Mos AG**GCAT** ***G**AG** CA*****T** *CCA*TA--- ---TC**CTT A********T

1 CPXV BR  GGTTTGAGAT CAACTTTATC TAATGGTTTA TAAAACGAAG GAGGTCTTCG TTCGAAATCT
2 VBH      **C******* ********** ********** ********** ****C***** **********
3 VACV COP **C******* ********** ********** ********** ****C***** **********
4 VACV WR  **C******* ********** ********** ********** ****C***** **********
5 ECTV Mos ********G* *T******** ****T****G ********** ****C****T **********
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rpo132 / CPXV157

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1 CPVX BR  AATTTGACTT TTACGCCTCT GGCATTCATT TGAGTAAGAA ATACTTTAGA TACGTGCGTG
2 VBH      ********** ********** ***G****** ********** ********** **********
3 VACV COP ********** ********** ***G****** ********** ********** **********
4 VACV WR  ********** ********** ***G****** ********** ********** **********
5 ECTV Mos ********** ****A***** T**G****** *****C**** ********** ******A***

              151372                          151347
1 CPVX BR  GTATCAATTT TTGTTAAGAG AGGAGAGAGA
2 VBH      ********** ********** **********
3 VACV COP ********** ********** **********
4 VACV WR  ********** ********** **********
5 ECTV Mos *****T**** ********** **********
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received colonies of mice from other facilities, and it is possible that infected animals were among these foreign colonies. However, this is virtually impossible to track. Nonetheless, the ubiquitous circulation of different VACV strains in Brazil, from wild and veterinary sources, suggests that epidemiological surveillance is needed.

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