Characterization of a vaccinia-like virus isolated in a Brazilian forest

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The SPAn232 virus (SPAnv) was isolated from sentinel mice in the forest of Cotia, São Paulo, Brazil. It was grouped originally as a Cotia virus (CV) sample due to serological cross-reaction with the latter. However, SPAnv presented genetic characteristics that differed from CV and indicated that SPAnv is a member of the vaccinia virus (VV) subgroup. SPAnv showed a HindIII-digested DNA pattern similar to those of the WR and Lister strains of VV. Also, SPAnv presented genes homologous to the vaccinia growth factor, thymidine kinase and A-type inclusion (ATI) genes from VV. RFLP analysis of the SPAnv ATI homologous gene indicated that the virus belongs to the VV group. Nucleotide sequences from SPAnv genes showed up to 99% similarity with the same genes from VV. Such a relationship was confirmed visually through the drawing of phylogenetic trees. The results point out the occurrence of a VV strain that is possibly in active circulation in the forests of Southeast Brazil.

Few poxviruses are medically significant or are involved in human diseases. The most important poxvirus disease, smallpox, which is caused by variola virus, was eradicated worldwide during the 1970 and 1980 decades (Fenner et al., 1989). A number of poxviruses of veterinary importance have been studied also and include viruses that infect laboratory animals, pets, zoo animals and cattle. Many outbreaks come from emerging, naturally occurring poxviruses and some of them, such as monkeypox virus, may cause disease in humans (Jezek et al., 1986; Heymann et al., 1998). Moreover, the use of live vaccines in uncontrolled populations involves the risk of recombination between wild poxviruses and these newly introduced viruses. Although this may be suitable for vaccine propagation, deleterious effects caused by the appearance of recombinants are also possible. To foresee the impact of such campaigns, the surveillance of naturally occurring poxviruses is essential and has been conducted in places like west and central Europe, following vaccination with the vaccinia virus (VV)—rabies vaccine (Boulanger et al., 1996; Sandvik et al., 1998).

Very little is known about the occurrence and ecology of veterinary poxviruses in Brazil. Many occurrences are related to mousepox virus outbreaks in animal facilities, but most cases remain unpublished. Outbreaks of parapoxviruses in goats and sheep herds have been documented also (Mazur & Machado, 1998). Moreover, the use of orthopoxvirus related to VV, the BeAn 58058 virus (BAV), obtained from a wild rodent captured on the border of the Amazon rain forest (da Fonseca et al., 1998). Similarly, the isolation and characterization of a VV-like virus, named Cantagalo virus, was reported recently. This virus was isolated from cattle and, eventually, from humans in the state of Rio de Janeiro, Southeast Brazil (Damaso et al., 2000). Another poxvirus, the Cotia virus (CV), was isolated initially from sentinel mice in 1961, in Cotia county, São Paulo, an area of forest in the southeastern region of the country (Lopes et al., 1965). The virus has been re-isolated consistently and it was proposed to be a recombinant between leporipoxviruses and orthopoxviruses (Ueda et al., 1978, 1995; Esposito et al., 1980).

The sample of SPAn232 virus (SPAnv) was obtained during a Brazilian government effort to survey rural regions with reported circulation of unknown viruses. The sample was isolated from sentinel mice that had been exposed in the Cotia forest. It was considered initially to be another CV isolate, as the virus presented serological cross reaction with the viruses isolated previously (unpublished results). We received SPAnv in our laboratory as a CV sample and intended to use the virus as a comparison tool to characterize BAV (da Fonseca et al., 1998). The serological relationship between these two viruses had been described more than two decades ago (Woodall, 1967; Ueda et al., 1978). However, after initial experiments, we

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realized that the sample we were working with presented remarkable differences from the CV isolate described originally. Moreover, the virus presented extensive similarity with members of the VV subgroup.

In order to characterize the SPAnv isolate, we performed a number of analyses at the genomic level. To avoid misinterpretation due to laboratory cross contamination, two plaque-purified and independently maintained SPAnv samples were analysed throughout the study. VV samples used for comparison were the WR and Lister strains of VV. BAV was included also. All viruses were plaque purified by standard methods to assure genetic homogeneity. Viruses were grown in Vero cells supplemented with 1 or 5% foetal calf serum. When necessary, viruses were purified on sucrose gradients, as described previously (Joklik, 1962).

For the generation of restriction patterns and DNA cross hybridization of total genomes, DNA from purified suspensions of VV WR, VV Lister, BAV and SPAnv was extracted, as described by Massung & Moyer (1991), and digested with HindIII (Promega). The resolved fragments were transferred onto nylon membranes and hybridized with total (non-digested) VV Lister DNA, labelled with $^{32}$PCTP by nick translation (Nick Translation system, Promega) under stringent conditions (Sambrook et al., 1989). The same blots were also used to detect the presence of genes homologous to the thymidine kinase (TK), vaccinia growth factor (VGF) and A-type inclusion (ATI) genes of VV using the PCR-amplified coding regions of these genes as probes (Meyer et al., 1997; da Fonseca et al., 1998).

The coding regions of the SPAnv TK and VGF homologous genes, cloned into pUC19 plasmids, were sequenced in both orientations (Sanger et al., 1977) using M13 universal primers. Several independent clones were sequenced to prevent mistakes due to Taq polymerase. Sequences were analysed by
Characterization of a vaccinia-like virus

Fig. 2. Nucleotide analyses of the SPAnv VGF homologous gene. (A) The coding region of the SPAnv VGF homologous gene was amplified by PCR, cloned and sequenced in both directions. The sequence was aligned and compared with sequences from BAV, VV strains Copenhagen and WR and Myxoma virus. Three nucleotide deletions in the SPAnv sequence, compared to the VV and BAV sequences, are indicated (*). (B) Nucleotide sequences of the VGF homologous genes from a number of poxviruses, including SPAnv, were used to construct the tree based on a dissimilarity matrix method. Optimal bootstraps of 1000 replicates were done.

search and alignment with similar sequences from GenBank using BLAST programs (Altschul et al., 1990). Amino acid sequences of the SPAnv TK and VGF proteins were inferred from nucleotide sequences. Phylogenetic tree analyses were performed using the TREECON program (Van de Peer & De Wachter, 1994). Bootstrap analyses of 1000 replicates were done.

The SPAnv genome presented a profile closely similar to that obtained for VV HindIII-digested DNA (compared with both WR and Lister strains) (Fig. 1A). The main differences are
located in the large upper fragments of the digested VV and SPAnv DNA. The migration patterns of the fragments labelled A and B (Fig. 1A) from the SPAnv DNA HindIII restriction profile resemble the analogous fragments from the VV Lister DNA profile, but not those of VV WR which are differently placed. The migration pattern of fragment C from SPAnv DNA is similar to fragment C from VV WR DNA, but there is no correlated fragment in the VV Lister restriction profile. Furthermore, the SPAnv D fragment has no analogous fragment in either the Lister or the WR VV DNA profile. Smaller fragments retain the same migration pattern for each of the viruses, with little variation. The restriction pattern of SPAnv is similar also to the BAV profile and, again, differed on the larger DNA fragments. Fig. 1(A) also illustrates the result of the cross-hybridization between the SPAnv, VV Lister, VV WR and BAV genomes. Labelled VV Lister DNA hybridizes extensively with VV WR, BAV and SPAnv DNA.

Characterization of the TK gene (Weir & Moss, 1983) and the VGF gene, a growth factor encoded by some poxviruses (McFadden et al., 1995), has been used before as a taxonomic tool to study ungrouped poxviruses (Fenner et al., 1989; Ueda et al., 1995; da Fonseca et al., 1998). We searched the SPAnv genome for the presence of these genes, conserved typically in the genome of many orthopoxviruses. As expected, we found a homologous TK gene in the SPAnv genome when filters described in Fig. 1 were hybridized with the VV WR TK gene probe. The homologous SPAnv TK gene is localized on fragment K, which co-migrates with the J fragment of VV WR (Fig. 1B). The TK gene of VV WR is located in the central portion of the linear genome at the left end of the J fragment (Weir & Moss, 1983; Fenner et al., 1989; Johnson et al., 1993). The BAV TK gene is localized also in the same analogous HindIII restriction fragment of its digested DNA (da Fonseca et al., 1998). To look for the SPAnv VGF homologous gene, the same filters were re-probed with the VV WR VGF probe. Surprisingly, the VGF probe hybridizes within three different fragments of the SPAnv HindIII restriction profile (Fig. 1B), suggesting possibly that the virus encodes three copies of the VGF homologous gene in its genome, localized in the B, C and D fragments, respectively. Due to the possibility that the D fragment could be a subproduct of incomplete digestion, we tried to detect genes from the B and C fragments in the D fragment. We probed the same filters from Fig. 1 with labelled C8L and BSR genes. Such genes were not detected in the D fragment (data not shown), suggesting that the extra DNA fragment is not a submolar product of digestion but is a product of genetic rearrangement in the SPAnv genome. Such polymorphism is not unprecedented for poxviruses and this peculiarity is found also in the genome of cowpox virus (da Fonseca et al., 1999). Indeed, spontaneous deletions and duplications in the variable external portions of the linear DNA seem to be a common feature of the evolution of poxviruses (Pickup et al., 1984). An amplified ATI gene fragment from VV WR was used as a probe in Southern-blot hybridizations of total virus genomes as described. The probe hybridized with homologous fragments in SPAnv, VV Lister- and VV WR-digested genomes (Fig. 1B).

After sequencing, both the TK and VGF homologous genes from SPAnv presented nucleotide similarity of more than 99% with the correlated VV WR genes. In the SPAnv TK gene, only two silent nucleotide substitutions were detected when genes from VV WR and SPAnv were compared (data not shown). When we compared the VGF homologous gene from SPAnv with the VGF gene from VV WR, we detected three nucleotide deletions, leading to alterations in two amino acids, Ser → Gly and Glu → Lys, and the loss of one Asn residue. These deletions are indicated in Fig. 2(A). All six codons for cysteine amino acid residues, which are known to be essential for protein conformation and biological activity of this family of proteins (James & Bradshaw, 1984), were found to be intact on the SPAnv VGF homologous gene. The nucleotide sequences were also used to draw phylogenetic trees. In both cases, when sequences from the TK or VGF genes were used, SPAnv was placed in the same branch as VV WR and BAV (Fig. 2B). The trees drawn from the sequences of the TK and VGF genes were very similar in both shape and evolutionary distance among the viruses. Only the VGF-derived tree is shown (Fig. 2B).

A PCR-based method for rapid screening and taxonomic differentiation is being used currently in orthopoxvirus taxonomy. The assay utilizes primers designed from the ATI gene sequence from cowpox virus to amplify a variable portion of this gene. The specificity of this assay is enhanced by the use of restriction enzymes, Xbal or BglII (Meyer et al., 1994, 1997). We amplified the ATI gene from SPAnv and also from VV WR for comparison. The profile obtained after digestion of the SPAnv ATI amplicon with Xbal was identical to the VV WR pattern, as shown in Fig. 3.

Taken together, our results indicate that SPAnv is a VV sample isolated from the wild in the region of Cotia county, Brazil. The virus was isolated from sentinel mice exposed to
airborne vectors, which is a solid indication that the virus was circulating actively in that environment. The natural host of SPAnv is not known, although the isolation of the virus from exposed suckling mice suggests circulation on arthropod vectors. Circulation of vaccine orthopoxviruses in domestic or wild animals is a rare phenomenon, but one which has been reported previously (Lum et al., 1967; Baxby et al., 1986). The most notorious case is the history of buffalopoxvirus in India (Dumbell & Richardson, 1993). Recently, the Cantagalo virus was isolated from outbreaks in Southeast Brazil and it seemed to be another case of established VV in the wild (Damaso et al., 2000). However, its host remains unknown. In Europe, orthopoxvirus antibodies have been found in a wide range of wild animals, such as rodents, shrews, cats and other carnivores (Nowotny, 1994; Sandvik et al., 1998; Tryland et al., 1998a, b). Very little information about poxvirus reservoirs in Brazil is available.

Concerning the possible origin of SPAnv, it is tempting to associate the virus with vaccine samples that may have escaped to the wild during smallpox vaccination. This seems to be the case for Cantagalo virus, which is genetically related to the IOC strain of VV (Damaso et al., 2000). However, it is impossible to track back to the original vaccine strain from which SPAnv originated because many different samples were used at the same time in the region. They include the Lister, WR (Brazilian Health Ministry, personal communication) and IOC (Damaso et al., 2000) strains of VV and even pools made of different strains. However, it is important to note that SPAnv was isolated in 1979 and the smallpox vaccination in Brazil was terminated in 1973. This goes beyond short-term infection of VV, described as the occurrence of contact between vaccinated humans and domestic animals (Lum et al., 1967), and points to another case of VV establishment in the wild. This finding is strengthened by the detection of new VV-like outbreaks in the São Paulo State (unpublished results). Therefore, the establishment of VV in nature is not as rare as stated before, but it is possibly more complex and frequent than once thought.

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