Recent isolates of parapoxvirus of Finnish reindeer (Rangifer tarandus tarandus) are closely related to bovine pseudocowpox virus

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Cases of papular stomatitis in Finnish reindeer have been reported for many years. The causative agent was thought to be Orf virus (ORFV), one of the Parapoxviridae, although this assumption was based mainly on clinical symptoms, pathology and electron microscopy. Here sequence analyses of the viral DNA isolated from a recent outbreak of disease in 1999–2000 are presented in comparison to that isolated from earlier outbreaks in 1992–1994. The results show that the virus isolated from the 1999–2000 outbreak is most closely related to Pseudocowpox virus, whereas those from previous years grouped with ORFV. The present study describes a method for genetic characterization and classification of parapoxviruses (PPVs) and provides for the first time an extended phylogenetic analysis of PPVs isolated from Finland, established members of the genus Parapoxvirus and selected members of the subfamily Chordopoxvirinae.

A contagious disease causing erosions, papules, pustules and ulcers in the mouth has been recognized in Finnish reindeer for many years, particularly during winter. Although the most severe outbreak occurred in the winter of 1992–1993, when approximately 400 reindeer died and about 2800 showed clinical signs of disease, further outbreaks have been reported sporadically ever since. Electron microscope studies, and later PCR studies (Büttner et al., 1995), demonstrated that the outbreak was caused by a parapoxvirus (PPV). PPVs, members of the Poxviridae, are highly contagious, being transmitted by direct contact between animals or indirectly via environmental contamination (Haig & Mercer, 1998). Worldwide they are the cause of contagious pustular dermatitis in sheep and goats (Orf virus, ORFV) and in cattle (Pseudocowpoxvirus, PCPV, and Bovine papular stomatitis virus, BPSV; Moyer et al., 2000), while a less severe disease is found in Red deer in New Zealand (Parapoxvirus of red deer in New Zealand, PVNZ).

The aetiological agent of the disease in reindeer is not known, although based on clinical symptoms and pathology, ORFV was thought to have been the main cause of the 1992–1993 outbreak. Here we report the findings of an investigation into the cause of an outbreak of disease in 1999–2000, and compare the results with those obtained from the earlier outbreak. PCR was used to amplify specific regions of PPV genomes direct from clinical specimens, and phylogenetic analysis of the resulting PCR products was used to determine the most likely cause of disease. Since Rangiferine herpesvirus 1 (RanHV-1) has been shown to be prevalent in Finnish reindeer (Ek-Kommonen et al., 1982, 1986), and a bovine herpesvirus 1-like virus has been isolated from clinically similar cases of disease in reindeer in Sweden (Rockborn et al., 1990), the samples were also analysed with PCR specific for these ruminant alphaherpesviruses (Ros & Belák, 1999).

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Eighty-one clinical samples, from 57 reindeer showing symptoms of papular stomatitis during the winter of 1999–2000, were collected for PCR analysis. The samples, both scabs and vesicle swabs, originated from different parts of northern Finland. DNA was purified from the samples using the QIAamp DNA Mini kit (Qiagen). Similarly, DNA was obtained from several PPV reference strains (Fig. 1b) grown in vitro. DNA was also extracted from a further four clinical specimens: two reindeer scab samples taken in northern Finland in 1992 and 1994; a paraffin wax-embedded tissue block obtained from clinically ill sheep in 1997 from northern Finland; and a paraffin-block sample taken from a cow during a severe outbreak in Parainen (south-west Finland) in 1999. The DNA was purified from paraffin blocks essentially as described by Jackson et al. (1990). Finally, two PPV DNA samples from Germany (PPV strain BO29 and PCPV strain BO35) were included in the studies. BO29 was isolated from a person who had had close contact with sheep and had suffered typical orf lesions (isolate BO15; Büttner & Rziha, 2002), and BO35 is a PPV strain isolated in 2000 from a cow’s teat in Germany.

Initially, PCR amplifications were performed using three primer sets, C1/C2, C3/C4 and F1/F2. The two PPV-specific primer sets C1 (5′-AGG AGC TCA TGT CTG TGA TG-3′) and C2 (5′-CAC CAG CAC CTG GTA GTC-3′), and C3 (5′-TGC TTC ACG AAC ATG CGG CC-3′) and C4 (5′-TCT CGC GGT CCA GCA CTT TA-3′) were designed to amplify 458 and 745 bp products, respectively, of the ORFV orthologue of the Vaccinia virus (VACV) gene A3L encoding the major core protein P4b (Rosel & Moss, 1985; Goebel et al., 1990). The ORFV-specific primer set F1 (5′-TCA ATA TGG ATG AAA ATG AC-3′) and F2 (5′-ACA GAC GGC AAC ACA GCG GT-3′) was designed to amplify a 301 bp product of the ORFV orthologue of the VACV fusion protein gene A27L (Rodriguez & Esteban, 1987; Goebel et al., 1990). After initial denaturation at 99°C for 8 min, AmpliTaq polymerase (Perkin Elmer) was added during an 80°C incubation period of 10 min, and the amplifications were performed for 35 cycles of denaturation (95°C, 1 min), annealing (58°C, 1 min for the C1/C2 and C3/C4 primers; 47°C, 1 min for the F1/F2 primers) and extension (72°C, 1 min). The RanHV-1-specific PCR was performed as described by Ros & Belák (1999). Positive

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**Fig. 1.** (a) Alignment of deduced amino acid sequences of the coding region of the envelope protein 554 bp gene segment (184 aa) of PPVs studied in this work. Dots indicate amino acid identity with ORFV strain Orf 11. (b) Reference virus strains used in this study and nomenclature of PPV-positive isolates. Reindeer isolates from 1999–2000 are designated F99.***R where F = Finland; the following two numbers = year of sampling; symbols after the full point = sample identification number; and R = reindeer. The clinical reindeer scab samples taken in northern Finland in 1992 and 1994 are designated F92.849R and F94.848R, respectively. The paraffin wax-embedded tissue block obtained from clinically ill sheep in 1997 is designated F97.391S, and the paraffin-block sample taken from a clinically ill cow in 1999 is designated F99.177C.
samples from the PPV-specific PCRs were subjected to the semi-nested PCR described by Inoshima et al. (2000), which is designed to amplify 594 and 235 bp products of the PPV orthologue of the VACV gene F13L encoding the major envelope antigen p37K.

Amplification products were obtained from all the PPV reference strains with at least one of the PPV-specific primer pairs. Samples from ten reindeer (18%) from the 1999–2000 outbreak were found to be positive in the PPV-specific core protein PCRs, with the remaining being negative in all the PCR assays. Each of the ten positives was also positive in the semi-nested PCR (Inoshima et al., 2000), although only three gave amplicons of 594 bp after the first round of PCR. None of the ten positive reindeer samples was positive in either the ORFV- or RanHV-1-specific PCRs, suggesting that at least a proportion of the popular stomatis observed in the 1999–2000 outbreak in Finnish reindeer could be attributed to a PPV other than ORFV. The results demonstrated that the conserved core protein, in particular, is an acceptable target for diagnostic PCR since the combination of primers used detects all recognized PPVs.

To characterize the viral DNA isolated from these reindeer in more detail, the PCR products were sequenced and subjected to phylogenetic analyses. Sequencing was performed at the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki. The sequences of both strands of the PCR products were determined using PCR primers, although the primer sequences were subsequently excluded from the analyses. The conceptual amino acid sequences of each of the PCR products were obtained using the program Transeq of the EMBOSs software package version 2.6.0.

Pairwise comparisons of both DNA and amino acid sequences corresponding to both regions of the major core protein and the major envelope protein revealed that reindeer PPV isolates from 1999–2000 showed 96–100% nucleotide and amino acid identity with PCPV strains BO35 and VR634 and an isolate from a Finnish cow (F99.177C), whereas the reindeer PPV isolates from earlier years (F92.849R and F94.848R) showed the highest nucleotide and amino acid identity with ORFV reference strains. Fig. 1(a) shows the alignment of the predicted envelope protein sequences of PPVs displaying the most variable sites among the protein regions studied.

To analyse further the genetic relationships between Finnish PPVs, reference PPVs and other members of the Chordopoxvirinae, phylogenetic trees were constructed from alignments of the conceptual core and envelope protein sequences. These analyses were performed with the PHYLIP package version 3.6b (Felsenstein, 2003). Phylogenetic trees were inferred using distance, parsimony and maximum-likelihood methods. The model of amino acid substitution chosen for both distance and maximum-likelihood methods was the Jones–Taylor–Thornton model (Jones et al., 1992) with four gamma rates. The gamma distribution parameter alpha and the relative rate for gamma rate categories were calculated using the program TREE-PUZZLE version 5.1 (Schmidt et al., 2002). The phylogenetic trees were inferred from the distance matrices using the neighbour-joining method. Maximum parsimony analyses were carried out using the program PROTPARS, and maximum-likelihood analyses (with randomized input order and global rearrangements) were performed using the program PROML. The reliability of the trees was determined by 1000 data-set bootstrap resampling with the programs SEQBOOT and CONSENSE.

Because the trees did not differ significantly, only the maximum-likelihood trees are presented (Fig. 2a–c). The results show that PPVs form three or four phylogenetic lineages depending on the virus species included in the analyses. The lineages are in accordance with the established PPV genera. PPVs were always clearly separated from other chordopoxviruses (ChPVs), which is consistent with the fact that PPVs differ from the other ChPVs in their morphology and mostly also in their genome size (Moyer et al., 2000). The results obtained with the different methods were consistent in that ChPVs were always seen in five main groupings: Parapoxvirus; Orthopoxvirus; Capripoxvirus; Suipoxvirus/Leporipoxvirus/Yatapoxvirus; Avipoxvirus; and Molluscipoxvirus. Trees calculated from all gene regions with all methods revealed that F99/00.R strains were clustered in the same lineage with the PCPV strains VR634 and BO35 and the isolate from a cow (F99.177C), while F92.R, F92.849R and F94.848R were grouped with the ORFV strains and an isolate from a sheep (F97.391S), all with high bootstrap support.

It has been suggested that phylogenetic analyses based on single genes/proteins can give rise to ambiguous tree topologies. Therefore in order to gather further support for the branching order observed above, the amino acid alignments from the core and envelope protein gene were concatenated and subjected to maximum-likelihood analysis with 100 data-set bootstrap replicates. This method is thought to resolve more accurately the branching order of species because it minimizes the effect of sampling variation (Huelsenbeck et al., 1996). Most PPV samples with missing data were excluded from the analysis in order to improve the likelihood of finding a fully resolved tree topology. The tree obtained from concatenated data is presented in Fig. 3. As expected, reindeer PPV strains from 1992–1994 grouped together with ORFV, and the reindeer PPV strains from the recent outbreak grouped with PCPV, with high bootstrap support. The tree also verifies the results obtained from the single gene analyses, displaying the same five main groupings of ChPVs. These results are in accordance with recent data published by Gubser et al. (2004): their results from the combined phylogenetic analysis of 17 conserved proteins from all ChPV genera except the PPVs showed the same main grouping of ChPVs, minus the PPPVs, that we found in our study.
Fig. 2. Phylogenetic trees of chordopoxviruses (ChPVs) calculated from the deduced amino acid sequences of three different gene regions studied. Accession numbers for sequences used in the analyses are: AB044797 (BPSV strain Aomori); AB044795 (ORFV strain Iwate); AF414182 (SPV, sealpox virus); U60315 (MOCV, Molluscum contagiosum virus subtype 1); AJ293568 (YLDV, Yaba-like disease virus); AF170722 (SFV, Rabbit fibroma virus ‘Shope fibroma virus’); AF325528 (LSDV, Lumpy skin disease virus isolate Neethling); AF198100 (FWPV, Fowlpox virus); AF380138 (MPXV, Monkeypox virus strain Zaire-96-I-16); AF410153 (SWPV, Swinepox virus isolate 17077-99); L22579 (VARV, Variola virus strain Bangladesh 1975); M35027 (VACV, Vaccinia virus strain Copenhagen); AF438165 (CMLV, Camelpox virus); AF482758 (CPXV, Cowpox virus strain Brighton Red); AY077833 (SPPV, Sheeppox virus); AY077835 (GTPV, Goatpox virus); and AF012825 (ECTV, Ectromelia virus strain Moscow). The amino acid sequences of ChPVs were edited to correspond to the amino acid sequences of PPVs, and the alignments were created using CLUSTAL_X (Thompson et al., 1997). Trees were generated using the maximum-likelihood method, based on (a) 139 and (b) 163 aa sequences of the core protein gene, and (c) 184 aa sequences of the major envelope protein gene of PPVs. Numbers on the trees show percentage of bootstrap support calculated for 1000 replicates; scale bar represents 10 aa substitutions per site. The graphical output was created using TREEVIEW (Page, 1996).
The results presented here are similar to those from a recent analysis of the *Fil* gene of ORFV (Scagliarini et al., 2002), which suggests there is little variation between PPVs originating from different animal species and from different geographical areas. However, further analysis of the reindeer PPV 1999–2000 isolates will be required before it can be determined for certain whether the limited differences found between reindeer PPV and virus isolated from a cow (F99.177C) represent adaptation of the same virus to different host species, or indicate separate virus species. Transmission of a PPV from cow to reindeer, or vice versa, cannot be excluded at this time even though there are no obvious connections between the various outbreaks of disease in reindeer and in cattle. Indeed, PPV infection in cattle is not considered a serious problem in Finland, despite the recent epidemic in Parainen in 1999 and two previous outbreaks, one in south-west Finland in 1971 (Estola & Neuvonen, 1974) and the other in Hame in 1974 (Kuokkanen & Launis, 1975), all of which were geographically remote from where the affected reindeer were found. Recently, Tryland et al. (2001) also showed that infection of semi-domesticated reindeer in Norway was caused by PPV. Further characterization of the Norwegian virus will show whether the symptoms in Finland and Norway are caused by the same virus.

This is the first time sequence analysis has shown that the outbreaks of papular stomatitis in Finnish reindeer may result from infection with different species of parapoxvirus. Further work is required to determine whether the outbreaks of disease in 1992–1994 and 1999–2000 were due to infection with ORFV and PCPV, respectively, or whether the disease is caused by a separate virus species closely related to both ORFV and PCPV. If reindeer are susceptible to both ORFV and PCPV this will have implications for animal husbandry in terms of taking care to minimize contact between reindeer and potential sources of infection.

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**References**


