REINDEER PARAPOXVIRUS:

MOLECULAR BIOLOGY AND DETECTION

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ACADEMIC DISSERTATION

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ABSTRACT

Parapoxviruses (PPVs) are zoonotic viruses which cause contagious pustular skin infections of sheep, goats and cattle worldwide. In addition, they have more recently been shown to infect other animals such as red deer, seals, camels and reindeer. Cases of contagious pustular stomatitis in Finnish reindeer have been reported for many years. This economically important disease occurs typically during winter and is more common in the southern parts of the reindeer herding districts than in the north. The first severe outbreak occurred in the winter 1992-1993, and during the winter of 1999-2000 and in the late winter 2007 outbreaks of the disease were again observed. Usual symptoms include diminished appetite, drooling, fever, and later erosions and ulcerative lesions in the mouth. The aims of this study were to establish specific and rapid detection methods for the causative agent of the disease and characterize the viruses circulating in Finland.

The causative agent of reindeer pustular stomatitis was originally considered to be *Orf virus* (ORFV) of the genus *Parapoxvirus*. PCR methods amplifying different regions of the PPV genomes were developed to analyse clinical samples obtained from outbreaks of the disease in reindeer and later from viruses isolated from the disease of sheep and cattle in Finland. Subsequent phylogenetic analyses of the Finnish PPVs, known members of the genus *Parapoxvirus* and selected members of the subfamily *Chordopoxvirinae* were conducted to identify the virus species isolated from reindeer. The results showed that the reindeer PPV from 1999-2000 is most closely related to the cattle PPV *Pseudocowpox virus* (PCPV) whereas the PPV strains from the winter of 1992-1993 outbreak grouped with sheep ORFV strains. Reindeer samples from the 2007 outbreak were identified as both PCPV and ORFV. Analysis of the similarity between genes of reindeer PCPV and ORFV isolates, Finnish sheep ORFV and cattle PCPV isolates indicated that these viruses have been circulating among Finnish reindeer, cattle and sheep at least ten years.

Since the initial classification of the viruses causing pustular stomatitis in Finnish reindeer relied solely on the partial sequence analysis of virion core- and EEV envelope phospholipase protein sequences, the genome of PCPV-like reindeer isolate (F00.120R) was sequenced by shotgun sequencing of plasmid sublibraries of cosmids covering the central region of the genome, and by sequencing transposon random insertion libraries of plasmids derived from each end of the genome. The F00.120R and the genomic sequence of a reference strain of PCPV (VR634) were annotated and analyzed in this study. This first characterization of PCPV genomes revealed that F00.120R and VR634 are 135 and 145 kb in length and contain 131 and 134 putative genes, respectively. The organization of their genomes was found

to be similar to that of other PPVs and both included 88 predicted genes that are conserved across all sequenced poxviruses. F00.120R was found to have four, possibly fragmented, genes at the left terminus and another near the central region of the genome that are not present in ORFV or *Bovine papular stomatitis virus* (BPSV; another PPV) genomes. In addition, the F00.120R genome was found to lack six genes seen near the right genome terminus of other PPVs. Comparing the PPV proteomes and whole genome phylogenetic analyses confirmed the classification of PCPV as a separate species within the PPV genus and verified that the virus causing pustular stomatitis in reindeer in 1999-2000 can be classified as PCPV.

The observed six gene deletion at the right terminus of the F00.120R genome was further investigated in an attempt to use it in differentiating PCPV and ORFV causing pustular stomatitis in reindeer. The preliminary PCR analyses of wild type virus and early passages of F00.120R implied that the deletion of genes may have arisen during cell culture of the virus. The sequence around the deleted region was determined by sequencing two cloned overlapping PCR fragments from F00.120R wt virus isolated from lesion material. The same region was sequenced from an Italian PCPV field isolate (It1303). Further PCR analyses together with sequence determination showed that a 5431 bp sequence containing genes 116-121 was likely to have been deleted from the F00.120R genome prior to the 7th passage in cell culture. In addition, genes 116-121 were present in It1303 and in other isolates of reindeer and bovine PCPV isolated in Finland during the years 2005-2010. These results indicate that the genome of reindeer PCPV is about 140 kbp in length and has 137 genes instead of previously estimated length of 135 kbp and 131 genes; it contains homologues of all known ORFV genes and this analysis further reinforces the close genetic relationship between PCPV and ORFV.

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LIST OF ORIGINAL PUBLICATIONS

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I Tikkanen MK, McInnes CJ, Mercer AA, Buttner M, Tuimala J, Hirvela-Koski V, Neuvonen, E., Huovilainen, A. Recent isolates of parapoxvirus of Finnish reindeer (rangifer tarandus tarandus) are closely related to bovine pseudocowpox virus. J Gen Virol. 2004 Jun;85(Pt 6):1413-8.

II Hautaniemi M, Ueda N, Tuimala J, Mercer AA, Lahdenperä J, McInnes CJ. The genome of pseudocowpoxvirus: Comparison of a reindeer isolate and a reference strain. J Gen Virol. 2010 Jun;91(Pt 6):1560-76.

III Hautaniemi M, Vaccari F, Scagliarini A, Laaksonen S, Huovilainen A, McInnes CJ. Analysis of deletion within the reindeer pseudocowpoxvirus genome. Virus Res. 2011 Sep;160(1-2):326-32.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

aa	amino acid
AGID	agar gel immunodiffusion test
ANK	ankyrin-like repeat
Asn	asparagine
ATI	A-type inclusion body
Bcl-2	B cell leukemia/lymphoma 2
BPSV	*Bovine papular stomatitis virus*
CBP	chemokine binding protein
CBP-II	type II CC-chemokine-binding protein
CCEV	Camel contagious ecthyma virus
CEV	cell-associated enveloped virion
ChPV	chordopoxvirus
CMLV	*Camelpox virus*
CPE	cytopathic effect
CPVX	*Cowpox virus*
Cys	cysteine
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
ECTV	*Ectromelia virus*
EEV	external enveloped virion
ELISA	enzyme-linked immunosorbent assays
EM	electron microscopy
EV	extracellular enveloped virion
FWPV	*Fowlpox virus*
G+C	guanine and cytosine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTPV	*Goatpox virus*
IFA	immunofluorescence assay
IFN	interferon
IFN-γ	interferon gamma
IL-10	interleukin 10
IL-2	interleukin 2
ITR	inverted terminal repeat
IV	immature virion
LSDV	*Lumpy skin disease virus*
MDBK	Madin–Darby bovine kidney
MDOK	Madin–Darby ovine kidney

MOCV	*Molluscum contagiosum virus*
MPXV	*Monkey poxvirus*
MV	intracellular mature virions
NF-kB	nuclear factor kappa B
NK	natural killer (cell)
ORF	open reading frame
ORFV	*Orf virus*
p.i.	post infection
PCPV	*Pseudocowpox virus*
PCR	polymerase chain reaction
PKR	double-stranded RNA dependent protein kinase, protein kinase R
PPV	parapoxvirus
PVNZ	*Parapoxvirus of red deer in New Zealand*
RanHV-I	rangiferine herpes virus 1
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rtPCR	real-time PCR
SFV	Shope fibroma virus
SPPV	*Sheeppox virus*
SPV	Sealpoxvirus
SWPV	*Swinepox virus*
TNF-α	tumor necrosis factor alpha
VACV	*Vaccinia virus*
VACV_Cop	*Vaccinia virus* strain Copenhagen
VARV	*Variola virus*
VEGF	vascular endothelial growth factor
VHD	VEGF homology domain
vIL-10	viral interleukin 10
vVEGF	viral vascular endothelial growth factor
wt	wild type
WV	wrapped virion
YLDV	*Yaba-like disease virus*

1 INTRODUCTION

1.1 GENERAL INTRODUCTION TO PARAPOXVIRUSES

Parapoxviruses (PPV) belong to the family *Poxviridae*, which consists of over 80 known or tentative species that infect both animal (subfamily *Chordopoxvirinae*) and insect (subfamily *Entomopoxvirinae*) hosts. The viruses within the family have large, brick-shaped (220-450 nm x 140-260 nm) or ovoid (250-300 nm x 160-190 nm) particles with genomes of linear double-stranded DNA (dsDNA) varying in size from 130 kbp to 375 kbp. The subfamily *Chordopoxvirinae* consists of nine genera on the basis of their natural host-range, morphology and their genetic and antigenic relatedness. In addition, the *Chordopoxvirinae* includes several unclassified poxviruses, which might form additional genera once their phylogenetic relationships have been established. The classified genera are *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, *Suipoxvirus* and most recently *Cervidpoxvirus* (Skinner et al., 2012). All chordopoxviruses cause disease characterised by cutaneous lesions, but the severity of the disease varies from localized self-limiting infection (for example contagious ecthyma caused by PPV *Orf virus*) to systemic disease with high mortality (for example smallpox caused by *Variola virus* of the genus *Orthopoxvirus*). Differences in the ability to infect various host species are common to Chordopoxviruses, for example human is the only host for *Molluscum contagiosum virus* whereas several Orthopoxviruses have broad host-ranges with multiple rodent reservoir hosts (Barthold et al., 2011; Kinnunen et al., 2011; Skinner et al., 2012).

1.1.1 TAXONOMY AND HOST RANGE

The International Committee on Taxonomy of Viruses (ICTV) currently recognizes four species in the genus *Parapoxvirus*. These are *Orf virus* (ORFV) (synonyms contagious pustular dermatitis virus and contagious ecthyma virus), *Bovine papular stomatitis virus* (BPSV), *Pseudocowpox virus* (PCPV) (synonyms Milker's nodule virus and Paravaccinia virus) and *Parapoxvirus of red deer in New Zealand* (PVNZ). ORFV, the prototype member of the genus, infects mainly sheep and goats, whereas BPSV and PCPV infect cattle. Tentative species of the genus include Auzduk disease virus, Camel contagious ecthyma virus, Chamois contagious ecthyma virus and Sealpox virus (Barthold et al., 2011; Skinner et al., 2012). PPVs can also infect

humans (Haig and Mercer, 1998). In addition, PPVs have been shown to infect several species of terrestrial and marine wildlife worldwide such as Japanese serows (*Capricornis crispus*) (Inoshima et al., 2001), reindeer (Falk, 1978; Kummeneje and Krogsrud, 1979), musk ox (Kummeneje and Krogsrud, 1978), Sichuan takin (Guo et al., 2004), domestic cat (Fairley et al., 2008), Californian sea lions (*Zalophus californianus*) (Wilson et al., 1969), harbor seal (*Phoca vitulina*) (Becher et al., 2002) and grey seal (*Halichoerus grypus*) (Nettleton et al., 1995).

The major criteria for classifying parapoxvirus species are the distinctive virion morphology and affected host, combined with restriction fragment length polymorphism (RFLP) and cross-hybridization analyses of the genome. Nowadays, these are becoming less important as taxonomic groupings can in many cases be concluded from phylogenetic analyses of PCR amplified genomic fragments (Barthold et al., 2011; Skinner et al., 2012).

1.1.2 STRUCTURE AND REPLICATION

Parapoxviruses are ovoid particles of 220–300 x 140–170 nm in size, with a single thread-like surface tubule surrounding the particle in a spiral fashion (Naginton and Horne, 1962). Inside the surface membrane is the enveloped core containing genomic DNA together with several proteins, and lateral bodies between the core and the surface membrane (Skinner et al., 2012). Cryoelectron microscopy of ORFV-infected cells has shown that ORFV produces two forms of virions: intracellular mature virions (MVs) and wrapped virions (WVs), which differ in that the WV is a MV particle surrounded by two additional membranes derived from the trans-Golgi network (Spehner et al., 2004). During virus egress, the WV loses its outermost membrane and becomes an extracellular enveloped virion (EV). In *Vaccinia virus* (VACV), MV and EV are the two main infectious forms that are produced during the virus replication cycle (Moss, 2006). The demonstration of ORFV MV, WV and EV (Spehner et al., 2004; Tan et al., 2009), in addition to the fact that ORFV has homologues of most VACV genes encoding proteins associated with structure and morphogenesis, as well as genes involved in replication and transcription of the genome (Delhon et al., 2004; Mercer et al., 2006), suggests that the morphogenesis and replication cycle of ORFV and VACV are likely to be similar. A schematic representation of the poxvirus replication cycle, based primarily on that of VACV, is presented in Fig. 1.

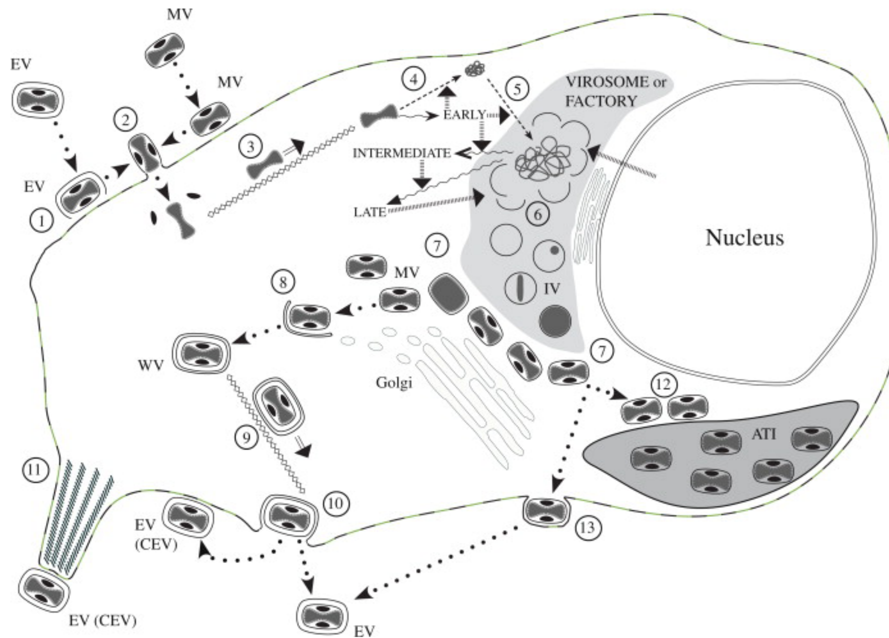

Fig.1. *The infectious cycle of poxviruses, based primarily on that of vaccinia virus (VACV): ATI, A-type inclusion body; IV, immature virion; MV, mature virion; WV, wrapped virion; EV, enveloped virion; CEV, cell-associated enveloped virion. (1) Disruption of envelope of EV upon binding to cell surface receptors, essentially revealing MV, which like naked MV can (2) fuse directly with the cell membrane (mediated by the fusion complex) to release the naked core (and lateral bodies). The core is (3) transported to the perinuclear region along microtubules. Early genes are expressed (wavy arrows) directly from the intact core; early gene products mediate: (4) uncoating of the core, (5) DNA replication and intermediate gene expression. Intermediate gene products (with involvement of some host proteins derived from the nucleus) mediate late gene expression. Late gene products include structural proteins (including polymerase required for early gene expression) and proteins required for morphogenesis. Single membrane crescents are assembled (6) to enclose viral core proteins and genomic DNA (the latter is cleaved from concatameric intermediates), forming IV. These mature (7) to MV that, in VACV and many other mammalian poxviruses, are transported to the trans-Golgi/endosomal compartment for (8) wrapping with a double membrane to produce WV. These are (9) transported to the cell surface along microtubules, where they (10) exocytose, losing the outer of the two additional membranes, to form EV. The EV can remain on the cell surface as CEV or become free in the medium. CEV can (11) be propelled away from the cell on the tips of actin-driven projections. MV of some poxviruses can (12) alternatively be transported to and incorporated into ATI. The avipoxviruses do not appear to form WV to any significant extent, rather production of EV involves MV transport to the plasma membrane where they undergo budding to exit the cell (13). Reprinted from Skinner et al. 2012, with permission.*

The replication of PPVs, as well as the replication of the other poxviruses, occurs in the cytoplasm of infected cells (Fig. 1) (Balassu and Robinson, 1987; Thomas et al., 1980). This is enabled by the presence of a complete transcriptional system within the poxvirus core, which can initiate mRNA synthesis immediately after infection and without host protein synthesis. There are three distinct phases of gene expression: early, intermediate and late. The sequential expression of these gene classes is under the regulation of phase-specific promoters and transcription factors, and the expression of each class is dependent on prior expression of proteins of the preceding class (Moss, 2001). Studies with ORFV and PCPV have shown that PPV DNA replication begins 4-8 h post infection (p.i.) and continues to 25-36 h p.i., and that the first virus-induced polypeptides can be detected starting at 10 h p.i. Both ORFV and PCPV viral particles appear at 12-18 h p.i. and they are produced until at least 48 h p.i. (Balassu and Robinson, 1987; Thomas et al., 1980).

1.2 PARAPOXVIRUS GENOMIC PROPERTIES

The PPV genome is a linear dsDNA molecule of 130-140 kbp with inverted terminal repeats (ITR) and cross-linked ends (Fraser et al., 1990; Mercer et al., 1987). PPV ITRs, which are identical but oppositely oriented sequences at the two ends of the genome, are between 1.2 and 4.0 kbp in length (Delhon et al., 2004; Fraser et al., 1990; Mercer et al., 1987; Mercer et al., 2006). Studies with ORFV strain NZ2 have revealed that the two strands of DNA are covalently closed with hairpin loops of about 100 bp (Mercer et al., 1987), and the regions adjacent to the hairpin loops contain a conserved motif that is required for the resolution of replicating concatemeric forms of poxvirus DNA (Mercer et al., 2006).

The nucleotide composition of PPV genomes are unusually guanine and cytosine G+C rich (on average 63-64%) (Delhon et al., 2004; Mercer et al., 2006; Wittek et al., 1979) compared to other poxviruses (30-40%) with the exception of *Molluscum contagiosum virus* (MOCV) (64%) (Skinner et al., 2012). However, analysis of three isolates of ORFV and one isolate of BPSV have shown that their G+C content differs from the average in the terminal regions of the genome, and is especially low (approximately 42-47%) in parts of the right end of the genome. The pattern of genome G+C variation is so similar among ORFV and BPSV and distinct from that of the other poxviruses including MOCV as to form a signature. This signature might represent a genus-specific feature of the PPVs (Mercer et al., 2005).

The ORFV and BPSV genomes have only recently been sequenced and are predicted to contain 132 and 133 genes, respectively (Delhon et al., 2004; Mercer et al., 2006). The majority of the genes are non-overlapping but closely spaced, there are no introns, and the transcription is directed generally towards the nearest end. The central parts of the sequenced PPV genomes contain genes that predominantly

have orthologues to VACV and other poxvirus genes essential for virus replication, transcription and morphogenesis. This central core region lacks two (D9R and F15R in VACV strain Copenhagen; VACV_Cop) of the 90 conserved genes present in all other vertebrate poxviruses, and thus reduce the minimum essential chordopoxvirus genome to 88 genes (Delhon et al., 2004; Mercer et al., 2006; Upton et al., 2003) . In contrast, the genes located at the ends of the genome have important roles in virulence and virus-host interaction. The terminal regions of PPV genomes, primarily the right end of the genome, contain genes that are not found in other poxviruses (Delhon et al., 2004; Mercer et al., 2006).

The terminal regions of PPV genomes vary considerably, and do not cross-hybridize between species (Gassmann et al., 1985; Robinson and Mercer, 1995). Comparison of the sequenced ORFV and BPSV genomes have shown an average 94% amino acid sequence identity between ORFV strains SA00 and IA82. Even the 20 most variable genes of three strains of ORFV share on average 80% identity. In contrast there is only an average of 71 % identity between SA00 and BPSV (Delhon et al., 2004; Mercer et al., 2006).

1.2.1 GENOMIC REARRANGEMENTS

Genomic rearrangements involving the genome termini have been reported for ORFV (Cottone et al., 1998; Fleming et al., 1995; McInnes et al., 2001; Rziha et al., 2000). These rearrangements are associated with serial passage of the virus in cell culture, and include deletion, duplication and translocation of blocks of sequences. Similar rearrangements have been described for other poxviruses, and are often found to result in virus attenuation in comparison to their parental strains (Cottone et al., 1998; Fleming et al., 1995; Kotwal and Moss, 1988; McInnes et al., 2001; Meyer et al., 1991).

The majority of the genomic rearrangement events described in ORFV are surprisingly similar involving duplication of a region in the right end of the genome, translocation of the duplicated region to the left end of the genome, which in turn results in the loss of sequence from that end. Characterization of the serially cell culture-passaged ORFV strain NZ2 revealed that 19.3 kbp from the right end of the genome had been duplicated and replaced 6.6 kbp of sequence at the left end. The net result of the transposition event was a deletion of 3.3 kbp of unique DNA encoding three genes designated previously as E2L, E3L and G1L (Fleming et al., 1995) [ORFs 005, 007 and 008, respectively (Delhon et al., 2004)]. Similar rearrangements have occurred within ORFV strains D1701 and Orf-11, both of which have been serially passaged in cell culture. In D1701, appoximately 4.3 kbp of the left end of the genome was replaced by a duplicated right end sequence of approximately 18 kbp, resulting in the loss of ORF005 (Cottone et al., 1998). In Orf-11, duplication and translocation

events resulted in a loss of approximately 7.7 kbp sequence containing genes 005, 007, 008 and 80% of the gene previously designated as G2L [ORF 009; (Delhon et al., 2004)] (McInnes et al., 2001).

The variant ORFV strains lacking ORFs 005, 007, 008 and the majority of ORF009 produced smaller lesions and resolved more rapidly, which implies that the deleted genes may be associated with virulence and that they are non-essential for virus replication *in vivo* (Fleming et al., 1995; McInnes et al., 2001). The ORF005 gene is predicted to encode a 6 kDa protein with an unknown function, which has some sequence homology with a DNA binding protein of African swine fever virus and is also found in *Cowpox virus*, *Ectromelia virus* and VACV Western reserve strain (Mercer et al., 1996). The ORFV ORF007 gene, a homologue of the VACV_Cop F2L gene, encodes dUTPase. dUTPase reduces the incorporation of dUTP into the DNA by catalysing the hydrolysis of dUTP to dUMP, which may aid virus replication (Cottone et al., 2002; Fleming et al., 1995). The ORF008 gene has been shown to encode a functionally active ankyrin-like repeat (ANK)/F-box protein, which is suggested to direct the degradation of host proteins, including antiviral factors, in order to favour viral replication (Sonnberg et al., 2008). The ORFV ORF009 gene encodes a protein that is a homologue of the VACV_Cop F11L, which has been reported to inhibit RhoA signaling required for VACV infection-induced cell motility and morphogenesis (Delhon et al., 2004; Valderrama et al., 2006). Although the variant NZ2 and Orf-11 strains are less virulent than their ancestral wild type strains, so is the strain D1701, which lacks only ORF005. It is thus unclear whether the rearrangements and the loss of genes alone can explain the reduced virulence. However, it is likely that all virulence and immuno-modulatory factors of PPVs act in concert, and removal of one particular gene might be expected to have only a partially reduced effect on virulence (Cottone et al., 1998; McInnes et al., 2001).

1.2.2 GENES ASSOCIATED WITH VIRULENCE AND HOST IMMUNOMODULATION

Many poxviruses encode virulence factors that can subvert their host‘s defense mechanisms by inhibiting or modifying the early stages of the host response during viral replication. The genes involved in PPV virulence and immunomodulation include the ankyrin-like repeat (ANK)/F-box proteins (ORFs 008, 123, 126, 128, and 129 in ORFV and in addition ORFs 003 and 004 in BPSV), the interferon (IFN) resistance protein (ORF020), the chemokine-binding protein (ORF112), viral vascular endothelial growth factor (vVEGF; ORF132), viral interleukin 10 (vIL-10; ORF127), the GM-CSF inhibitory factor (ORF117), an inhibitor of apoptosis (ORF125) and an NF-κB inhibitor (ORF121).

The poxviral ANK/F-box proteins consist of two domains: one containing ankyrin repeat motifs and one an F-box like domain. Almost all chordopoxviruses have several genes encoding either ANK repeat or ANK/F-box proteins implying their significance for poxviruses (Mercer et al., 2005). Both domains have well-known functions in eukaryotic cells; F-box proteins facilitate protein destruction by promoting ubiquitination of specific proteins in the ubiquitin-proteasome pathway (Ho et al., 2008; Zhang et al., 2009) whereas ANK repeat domains are one of the most common protein–protein interaction domains found in nature (Mosavi et al., 2004). ORFV ANK/F-box proteins have recently been shown to be functionally active by interacting with one of the cellular E3 ubiquiting ligase complex proteins through their F-box-like domain (Sonnberg et al., 2008). This implies that poxviruses could use their ANK/F-box proteins to remove unwanted host cell proteins in order to favour their replication in some way. Inhibitory roles in ubiquitination pathways are also possible (Mercer et al., 2005; Sonnberg et al., 2008; Zhang et al., 2009). Some of the other poxvirus ANK/F-box proteins have been shown to inhibit virus induced apoptosis (Ink et al., 1995; Mossman et al., 1996), to influence virus virulence (Mossman et al., 1996; Wang et al., 2006), host-range and tissue tropism (Ink et al., 1995; Johnston et al., 2005; Shchelkunov et al., 1993). Whether the PPV ANK/F-box proteins have any of these functions is currently not known.

The vVEGF gene (ORF 132) from ORFV has been shown to influence the nature of the lesion and virulence of the virus in infected animals (Savory et al., 2000; Wise et al., 2007). The vVEGF genes of PPVs are orthologues of the mammalian VEGF gene family members, which stimulate proliferation and permeability of vascular endothelial cells and induce angiogenesis by binding to a set of mammalian tyrosine kinase receptors (Ferrara, 2004). It has been demonstrated that all vVEGFs of established PPV species possess the ability to induce endothelial proliferation and angiogenesis (Inder et al., 2007; Ueda et al., 2003; Ueda et al., 2007; Wise et al., 2003), and that the vVEGF gene is responsible for the highly vascularized and proliferative nature of ORFV lesions (Savory et al., 2000; Wise et al., 2003). Sequence variation between vVEGF proteins is remarkably high, varying from 35 - 63% between PPVspecies with isolates of the same species even showing as little as 38 % amino acid sequence identity (Delhon et al., 2004; Inder et al., 2007; Lyttle et al., 1994; Mercer et al., 2002; Mercer et al., 2006; Ueda et al., 2003; Ueda et al., 2007; Wise et al., 2003). The functional similarity and amino acid identity between cellular and viral VEGFs suggests that vVEGF genes have likely been acquired from their host(s), although it is difficult to predict with certainty the genetic origin of vVEGFs due to the hypervariability of the sequence (Ueda et al., 2007).

Parapoxvirus vIL-10 is an ortholog of cellular interleukin (IL)-10, which is a multifunctional cytokine capable of both immunosupressive and immunostimulatory functions in many cell types. The N-terminal region of vIL-10s show little sequence similarity with the cellular IL-10s, whereas the C-terminal two thirds of the protein

is highly conserved between vIL-10 and IL-10, possibly due to this region containing the residues which interact with the IL-10 receptor (Imlach et al., 2002; Rziha et al., 2003). The functional similarity, high amino acid identity and phylogenetic analyses of vIL-10 orthologs from ORFV, BPSV, *Epstein-Barr virus*, *Equid herpesvirus 2* with the corresponding sequences from their vertebrate hosts have suggested that these viruses have acquired the IL-10 gene from their hosts independently at different times during evolution (Delhon et al., 2004; Fleming et al., 2007; Hughes, 2002; Rziha et al., 2003). The primary function of vIL-10 appears to be the suppression of early host responses to virus infection (Haig et al., 2002). Both IL-10 and vIL-10 have been shown to inhibit the production of IL-8 and tumor necrosis factor (TNF)-α from macrophages and keratinocytes and the production of IFN-γ and GM-CSF from lymphocytes (Haig et al., 2002). In addtion, ORFV vIL-10 has been reported to be a virulence factor when the virus lacking the vIL-10 gene was shown to induce smaller less severe lesions compared to the lesions caused by the virus with an intact vIL-10 gene (Fleming et al., 2007).

The interferon (IFN) resistance gene ORF020 is a homologue of VACV_Cop E3L, a double stranded RNA (dsRNA)-binding protein. It inhibits, amongst other activities, a protein kinase R (PKR), and is essential for the broad host-range of VACV *in vitro* and affects virulence *in vivo* (Brandt and Jacobs, 2001; Vijaysri et al., 2003; Vijaysri et al., 2008). IFN induced activation of PKR by viral dsRNA inactivates translation initiation factor eIF-2, which in turn inhibit virus (and host) protein translation and thus effectively blocks virus replication. Both ORFV ORF020 and E3L proteins have two domains: a C-terminal domain, which binds to dsRNA and is responsible for inhibition of the host IFN system, virulence and the ability of the virus to replicate in a wide variety of cells *in vitro*; and the N-terminal domain, which is not essential for virus replication in cell culture (Haig and Mercer, 1998; Jacobs and Langland, 1996; Vijaysri et al., 2003), but has been shown to be required for viral pathogenesis in a mouse model (Brandt and Jacobs, 2001). This has led to a suggestion that the differences between the N-terminal domain of ORFV and BPSV ORF020 protein might be important for host range and pathogenesis of these viruses (Delhon et al., 2004).

The ORFV ORF112 gene encodes a protein that shares some amino acid and functional identity with the poxvirus type II CC-chemokine-binding (CBP-II) proteins of the *Orthopox* and *Leporipox* genera (Lalani et al., 1998; Seet et al., 2003a) and VACV_Cop A41L chemokine binding virulence factor (Clark et al., 2006). CBP-II proteins can prevent chemokine signaling by binding and inhibiting CC chemokine interactions with their receptors and thus prevent chemokines from recruiting and activating immune cells during the processes of inflammation and infection. Although most poxviruses have been shown to encode proteins that modulate host chemokine pathways in some way, there is not a single class of chemokine modulator that is present in all poxviruses. This has been suggested to

reflect differences in host and tissue tropism and as a consequence helps to explain clinical differences in diverse poxviral diseases (Seet et al., 2003a; Seet et al., 2003b).

ORFV has been shown to encode a protein (GIF) that is capable of binding and inhibiting the ovine cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2) (Deane et al., 2000; McInnes et al., 2005). Homologues of GIF have been found in PCPV and BPSV, but not from other poxviruses. GM-CSF stimulates haematopoietic progenitor cells to develop into neutrophils, monocytes/macrophages and eosinophils. In non-haematopoietic tissues, GM-CSF is involved in activation and recruitment of these cells into the sites of inflammation. In addition, GM-CSF is also involved in the recruitment and development of antigen-presenting dendritic cells, whereas IL-2 is a multifunctional cytokine which promotes proliferation and activation of $CD4^+$ helper and $CD8^+$ cytotoxic T cells and natural killer (NK) cells (Haig, 2001; Haig and McInnes, 2002). The structural features required for functional ORFV GIF include a WSXWS-like motif, six cysteine (Cys) residues linked by disulfide bonds and asparagine (Asn) -linked glycosylation sites (McInnes et al., 2005). The GIF protein is conserved in ORFV strains (94-99% amino acid sequence identity) and the ORFV and PCPV GIFs share 88% amino acid sequence identity. There is, however, only 37-40% amino acid sequence identity between ORFV and BPSV GIFs, although the BPSV GIF, like the PCPV GIF does contain the six Cys residues, the Asn-linked glycosylation sites and the WSXWS-like motif, all of which have been shown critical for the biological activity of ORFV GIF (Deane et al., 2009; Delhon et al., 2004; McInnes et al., 2005). However, only PCPV and ORFV GIFs have been shown to bind GM-CSF and IL-2. The reason for the inability of BPSV GIF to bind GM-CSF and IL-2 is not known, but it has been suggested that the slight differences in the BPSV WSXWS-like motif alter its binding specificity towards an activity that is better suited for its biological niche. The role of GIF in PPV pathogenesis is currently not known (Deane et al., 2009).

Apoptosis, a regulated form of cell death, is an important host defence mechanism against virus infection. Poxviruses have been shown to encode several anti-apoptotic proteins that function to prevent apoptosis in an effort to prolong their own survival (Taylor and Barry, 2006), but so far only one has been identified in ORFV. The ORFV ORF125 encodes a Bcl-2-like protein, that localizes to mitochondria and blocks UV-induced apoptosis (Westphal et al., 2007). Members of the Bcl-2 protein family are regulators that either inhibit or promote mitochondrial apoptosis. The ORFV Bcl-2-like protein has been shown to bind to pro-apoptotic Bcl-2 family members and prevent them for triggering apoptosis when irradiated with UV-light (Westphal et al., 2007; Westphal et al., 2009). Orthologs of ORFV125 are present in PCPV, BPSV and PVNZ and they display similar features to the ORFV Bcl-2-like protein (Westphal et al., 2009).

The most recently identified virulence factor in ORFV is encoded by ORF121, which has been reported to inhibit the nuclear factor kB (NF-kB) signaling pathway. The members of NF-kB family are transcription factors that mediate expression of large number of different cellular genes by binding to specific promoter DNA sequences. The genes regulated by NF-kB transcription factors are involved in innate immunity, inflammation and apoptosis, all of which are critical for early antiviral responses (Diel et al., 2011b). ORF121 has been shown to bind to NF-kB p65 protein preventing its phosphorylation and nuclear translocation, which as a consequence blocks the transcription activity of p65. The infection of sheep with ORFV from which ORF121 had been deleted, resulted in an attenuated disease phenotype indicating that ORF121 is a virulence factor (Diel et al., 2011b). Other ORFV NF-kB inhibitors appear to include ORFs 002 (Diel et al., 2011a) and 024 (Diel et al., 2010), but neither of them have shown significant effects on ORFV pathogenesis in sheep implying that these genes are not essential for virus virulence in the natural host.

1.3 EPIDEMIOLOGY AND PATHOGENESIS OF PARAPOXVIRUS INFECTIONS

ORFV, BPSV and PCPV infections are common in sheep-, goat and cattle producing countries worldwide. The disease caused by PPVs is generally characterised by a contagious pustular dermatitis in the mucous membrane of the oral cavity, tongue, lips and teats of infected animals. Mild and non-erosive inflammatory lesions can also be observed on the muzzle of calves. The virus usually enters the host through abrasions on the skin, and pustules leading to scabs are typically observed at the site of infection. ORFV infects mainly lambs and kids but infection can occur in older animals as well. Morbidy in a flock can be very high, but mortality in uncomplicated cases rarely exceeds 1%. If the disease is complicated with secondary infections of bacteria, fungi or infestations of larva, the mortality rate can be as high as 50% (Haig and Mercer, 1998; Robinson and Balassu, 1981). The disease is spread either by direct contact or indirectly via environmental contamination. The indirect transmission is common because PPVs are resistant viruses which can survive in scabs, especially in a dry environment, or in other contaminated material for extensive periods of time (Klein and Tryland, 2005; McKeever and Reid, 1986).

PCPV and BPSV are cattle viruses, which like ORFV can also infect humans (Haig and Mercer, 1998; MacNeil et al., 2010). PCPV infection is commonly transmitted by cross-suckling of calves or improperly disinfected milking equipment. Disease caused by BPSV has generally little clinical importance and it can occur in cattle of all ages, although the incidence in calves is higher than in adult animals (Barthold et al., 2011). Both PCPV and BPSV usually cause relative mild disease, but severe cases of BPSV infection have also been reported (Inoshima et al., 2009; Jeckel et

al., 2011; Leonard et al., 2009). PVNZ, the most recently classified member of the genus *Parapoxvirus*, infects red deer (*Cervus elaphus*). The lesions are reported to be found variably on the muzzle, lips, face, ears and neck, but they have also been found on the growing antlers of stags (Horner et al., 1987). Initially, PVNZ was found in farmed red deer on eight farms in New Zealand during the winter of 1985-1986 (Horner et al., 1987), and until recently, it had only been found in New Zealand. The first PVNZ infection outside New Zealand was reported in red deer in Stelvio Park in the Italian Alps in the winter 2008-2009. Clinical signs in these red deer included proliferative lesions, erosions, and ulcers on the lips and hard palate (Scagliarini et al., 2011). PVNZ infection in humans has not been reported.

Epidemiological studies of PPV infections in Japanese serows (*Capricornis crispus*) have shown that PPV infections are widespread in Japan with 28% seroprevalence during the years 1984-1999 (Inoshima et al., 1999; Inoshima et al., 2001; Suzuki et al., 1993). Both BPSV and ORFV have been reported to infect Japanese serows (Inoshima et al., 2001), but recent genetic analyses have shown that the disease is mainly caused by ORFV (Inoshima et al., 2010). Other free-ranging ruminants that have been shown to be susceptible to ORFV when experimentally exposed to virus include mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), pronghorn (*Antilocapra americana*) and wapiti (*Cervus elaphus nelsoni*) (Lance et al., 1983). White-tailed deer have also been associated with PCPV infection; PPV lesions in two deer hunters, who had handled deer carcasses, were shown to be caused by PCPV (Roess et al., 2010).

Parapoxvirus disease of camels is widespread in camel-rearing regions of the world and is characterised by localised lesions mainly on the lips and nares of infected animals although other sites may also become affected. The disease is caused by Camel contagious ecthyma virus (CCEV), a tentative member of the parapoxvirus genus, which has been suggested to be ORFV on the basis of serological analysis (Azwai et al., 1995). However, recent phylogenetic analyses imply that the causative agent of CCEV cases in the Arabian Peninsula (Abubakr et al., 2007) and in India (Nagarajan et al., 2010; Nagarajan et al., 2011) is more closely related to PCPV than to ORFV.

PPVs replicate in epidermal keratinocytes and localized lesions progress through stages of papule, vesicle, pustule and scab to resolution. The disease is self-limiting in uncomplicated cases, and the scabs peel off the skin usually leaving no scar. Primary lesions can be severe and proliferative, and sometimes continued proliferation of the epithelium leads to dense wart-like outgrowth. Secondary complications cause lesions to become ulcerative and necrotic without scab formation, which delays healing (Haig and Mercer, 1998; Robinson and Balassu, 1981). Usually scabs develop within one week and resolve in 4-6 weeks, but there have been cases of persistent long lasting ORFV infections in goat kids that lasted three (Guo et al., 2003) and six months (Abu Elzein and Housawi, 1997).

1.3.1 DISEASE IN REINDEER

The first PPV infections in reindeer were reported in experimental animals at the National Reindeer Research Station in Lødingen, Norway (Kummeneje and Krogsrud, 1979). At the same time four human cases were associated with the reindeer PPV infections (Falk, 1978). Since then, there has been two more reported PPV oubreaks in Norway, the first occurred in Troms County in 1999 and the second in Nordland County in 2000 (Tryland et al., 2001). The clinical signs included small papillomas in the lips, gums and the palate but also cauliflower-like papillomas were observed in the lips of some reindeer. Phylogenetic analyses of partial B2L gene sequences, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis suggested that both of these outbreaks were caused by ORFV (Klein and Tryland, 2005). Because ORFV is enzootic in sheep and goats in Norway it is likely that the source of infection in the Norwegian reindeer PPV outbreaks was sheep (Klein and Tryland, 2005; Tryland et al., 2001).

In Finland, contagious pustular dermatitis caused by a PPV is an important disease of reindeer which has been observed since the early 1990s. The disease is characterised by erosions, papules, pustules and ulcers in the mouth of reindeer, but also wart-like lesions and necrotic/purulent erosions have been observed. The disease occurs typically in winter and according to a survey based on interviews of reindeer herding co-operatives and veterinarians, it is only in the most northern parts of the reindeer herding districts in the communes of Utsjoki, Inari, Enontekiö and Savukoski, that the disease has never been found (Hirvelä-Koski, 2001). The most severe outbreak occurred in winter 1992-1993, when approximately 400 reindeer died and about 2800 reindeer showed clinical signs of disease. Some sheep and human cases were also reported in the same period. At that time, the presence of parapoxvirus in reindeer and human samples was demonstrated by electron microscopic (EM) studies and the diagnosis was later confirmed by polymerase chain reaction (PCR) analyses of partial sequences of viral fusion peptide and the major envelope antigen p37K genes (Buttner et al., 1995). Although the diagnosis was based mainly on clinical symptoms, pathology and EM studies, ORFV was generally considered to be the main causative agent of the disease. Since the 1992-1993 outbreak, the disease has occured to a varying extent among semi-domesticated reindeer and is considered an economically significant disease to the reindeer husbandry.

The factors affecting morbidity and mortality of reindeer pustular dermatitis are thought to be manifold, but include supplementary feeding provided for reindeer during the hardest winter months, which had been increasing over the 1990s and is nowadays a common practice. According to the questionnaire survey, the clinical signs of the disease were observed in all areas where the proportion of reindeer kept in enclosures for feeding during wintertime was higher than 50% (Hirvelä-Koski, 2001). Artificial feeding with hay and silage can damage the mucous membrane

causing broken skin as a precondition for PPV infection, and herding reindeer closely together and shared feeding places facilitate virus transmission either directly between animals or indirectly via feeding troughs. Mouth infection makes eating difficult and can promote malnutrition which weakens the immune system of the animals. At least a proportion of the high mortality is likely to be attributable to secondary suppurative and necrotic bacterial infections often found during post-mortem examinations.

1.4 DIAGNOSIS OF PARAPOXVIRUS INFECTIONS

PPV infection can be detected on the basis of clinical signs but specific laboratory diagnosis is achieved by one or a combination of the following methods: 1) isolation and characterization of the virus in cell culture, 2) direct demonstration of virions, viral antigens, or viral nucleic acids and 3) detection and measurement of antibodies (Barthold et al., 2011). Laboratory tests used in PPV diagnostics are described in the sections 1.4.1 – 1.4.5.

1.4.1 VIRUS ISOLATION IN CELL CULTURE

Virus isolation in cell culture is regarded as the “gold standard“ method for detection of poxviruses. Although the method does not provide fast diagnosis of infectious agent it is important in detection of unknown viruses, and it is the only method for generating stock of live virus for further studies.

Various primary and continuous cell lines including primary lamb testis, kidney, turbinate and muscle (Delhon et al., 2004; Kottaridi et al., 2006a; McInnes et al., 2001; Rosenbusch and Reed, 1983), bovine fetal spleen and muscle (Hessami et al., 1979; Inoshima et al., 1999; Lard et al., 1991), primary bovine testis and lung (Kuroda et al., 1999; Mercer et al., 1994; Rosenbusch and Reed, 1983; Suzuki et al., 1993), Madin-Darby ovine kidney (MDOK) (Guo et al., 2003) and Madin-Darby bovine kidney (MDBK) (Klein and Tryland, 2005) cells have been reported to be suitable for cultivation of parapoxviruses. Cytopathic effect (CPE) is commonly seen as cell rounding, clumping and detachment, but it may take several blind passages to appear (Dal Pozzo et al., 2011; Kottaridi et al., 2006a; Kuroda et al., 1999). Generally, parapoxvirus cultivation in cell culture is regarded difficult because of many unsuccessful attempts to isolate virus, but the reasons for this are unknown (Nagington, 1968; Suzuki et al., 1993).

1.4.2 ELECTRON MICROSCOPY

Electron microscopic (EM) examination is a direct and rapid method for visualization of parapoxviruses in clinical specimens. PPVs appear as ovoid, 220-300 nm long and 140-170 nm wide, particles that are covered with a single thread-like tubule arranged in criss-cross fashion over the virion when viewed with negative staining EM (Naginton and Horne, 1962). The method is good for initial diagnosis of PPVs because the morphology of the virion is clearly distinguishable from those of the other poxvirus genera and all other viruses (Naginton, 1964). This allows differential diagnosis between ORFV and capripoxviruses, all of which cause disease with similar clinical manifestations in sheep and goats, as well as to distinguish between PCPV/BPSV and *Bovine herpesvirus 2*, which cause disease in cattle. EM is also useful in diagnosis of often slowly and irregularly growing PPVs, but it cannot be used to speciate viruses (Dal Pozzo et al., 2011; Naginton and Whittle, 1961; Suzuki et al., 1993). Disadvantages of the method, besides the inability to differentiate viruses at the species level, include low sensitivity (requires a minimum of 10^6 particles per ml) (Hazelton and Gelderblom, 2003), high cost and low availability of the EM facilities with experienced personnel.

EM examination is usually performed on scabs or biopsy specimens. After initial preparation of the samples virions are released from the cells e.g. by ultrasonic disruption, concentrated by centrifugation and examined by electron microscopy with phosphotungstate as a contrasting agent (Guo et al., 2004; Kuroda et al., 1999; Naginton, 1964).

1.4.3 SEROLOGICAL METHODS

Parapoxviruses have been shown to elicit detectable antibody responses in the host (Inoshima et al., 1999; Kuroda et al., 1999; McKeever and Reid, 1987; Yirrell et al., 1989; Yirrell et al., 1994). The viruses are immunologically closely related and exhibit serological cross-reactivity (Lard et al., 1991; Rosenbusch and Reed, 1983; Wittek et al., 1980), although a panel of monoclonal antibodies has been shown to be capable of discriminating between different parapoxvirus species (Housawi et al., 1998). Various serological methods have been used to measure parapoxvirus antibodies in different animal species, but most of the conventional serological methods are laborious and time consuming which makes them not well suited for primary diagnosis. However, serological methods are valuable in confirmatory testing and in epidemiological studies.

Parapoxvirus antibodies have commonly been detected with agar gel immunodiffusion tests (AGID), enzyme-linked immunosorbent assays (ELISA), and to a lesser extent with serum neutralisation tests. AGID is a simple method that detects parapoxvirus antibodies on the basis of a precipitation reaction between

a serum sample and the virus antigen. The test is performed in agar gel, where the serum sample is placed in a round well cut into the agar opposite a similar well containing virus antigen. The liquid in different wells diffuse towards each other, and if the sample contains antibodies against the test virus, a visible line of precipitation will form. AGID cannot discriminate between different parapoxvirus species (Papadopoulos et al., 1968) nor between ORFV and the capripoxviruses (Chand et al., 1994), and it is a less sensitive test than the ELISA (Housawi et al., 1992). The method has been used for parapoxvirus surveys and for confirmation of positive ELISA results (Housawi et al., 1992; Inoshima et al., 1999; Inoshima et al., 2001; Kuroda et al., 1999).

ELISA is a rapid method that enables screening of large number of samples at the same time. In an ELISA test, serum samples are incubated in 96-well microtitre plates coated with purified virus antigens with antibodies being detected with alkaline-phosphatase conjugated secondary antibodies (McKeever et al., 1987), peroxidase conjugated protein A (Suzuki et al., 1993) or protein AG (Inoshima et al., 1999). Although ELISA does not discriminate between different parapoxvirus species either, it has been applied successfully in the diagnosis of ORFV in humans (Yirrell et al., 1989), camels (Azwai et al., 1995) and parapoxvirus infections in California sea lions (Nollens et al., 2006a).

The serum neutralization test measures neutralizing antibodies in a serum sample against a known titre of the test virus in cell culture. If a fourfold rise in the antibody titer between acute and convalescent-phase serum is observed, the test is considered to confirm serological diagnosis. The method has been applied in detection of ORFV in domestic and wild ruminants in Alaska (Zarnke et al., 1983), but because immunity to ORFV infection is predominantly cell-mediated and virus-neutralizing antibodies are usually undetectable or develop only at low level (Haig and Mercer, 1998), the test is not reliable for primary diagnostic purposes.

1.4.4 HISTOPATHOLOGY AND IMMUNOFLUORESCENCE ASSAY

Parapoxvirus infection causes epidermal proliferation, which is seen as downgrowths penetrating the dermis. Other characterized features include epidermal hyperplasia, hyperkeratosis, vacuolation and ballooning of keratinocytes and accumulation of scale-crust. Furthermore, the presence of immune and inflammatory cells underneath and adjacent to virus-infected cells and marked capillary dilation and proliferation of the dermal lesions have been described (Griesemer and Cole, 1961; Groves et al., 1991; McKeever et al., 1988). These virus-induced morphological changes can be seen with the light microscope using haematoxylin and eosin staining of thin sections of the affected skin, and have been used in parapoxvirus diagnostics (Guo et al., 2004; Leonard et al., 2009; Nollens et al., 2006b; Vikoren et al., 2008).

Immunofluorescence assays (IFA) are based on detection of virus antigen with fluorophore-labelled primary antibody (direct method) or secondary antibody (indirect IFA): the fluorophore-labelled antibody fluoresces under UV illumination indicating the presence of virus antigen when viewed with a microscope. IFA is a useful and sensitive method because the diagnosis can be made on the basis of only few cells containing fluorescence of the right color and expected antigen distribution. However, because of this, correct diagnosis requires highly skilled personnel for reading the results. In detection and characterisation of parapoxviruses, both polyclonal convalescent sera from parapoxvirus-immunized animals and a panel of monoclonal antibodies against ORFV have been used in indirect IFA (Inoshima et al., 2001; Kanou et al., 2005; Lard et al., 1991; Rosenbusch and Reed, 1983). The results indicated significant antigenic overlap between ORFV, PCPV and BPSV (Lard et al., 1991; Rosenbusch and Reed, 1983) although six monoclonal antibodies against ORFV were found capable of differentiating ORFV from PCPV, BPSV and PVNZ confirming the ORFV diagnosis (Inoshima et al., 2001).

1.4.5 MOLECULAR METHODS

Molecular methods that have generally been used in parapoxvirus diagnostics include restriction fragment length polymorphism (RFLP), nucleic acid hybridization and nowdays especially conventional and real-time PCR. These nucleic acid based-methods, particularly PCR, offer fast, sensitive and specific identification of the target virus and are thus the preferred diagnostic methods used today.

RFLP is a method that exploits variations in genomes of even closely related virus species. In RFLP analysis, the DNA is digested with one or more restriction enzymes and the resulting restriction fragments are separated according to their size, by agarose gel electrophoresis, to generate distinct restriction fragment profiles. The RFLP analyses of parapoxvirus genomes revealed genetic heterogeneity that has enabled species (Gassmann et al., 1985; Wittek et al., 1980) and even strain differentiation (Rafii and Burger, 1985; Robinson et al., 1982). RFLP profiles generated by *Kpn*I, *Eco*RI, *Hin*dIII and *Bam*HI have been used to investigate parapoxviruses in various animal species including sheep, bovine, red deer, reindeer, musk ox, Japanese serow and Sichuan takin (Guo et al., 2004; Inoshima et al., 2001; Klein and Tryland, 2005; Rafii and Burger, 1985; Robinson and Mercer, 1995).

Nuclecic acid hybridization has been used together with RFLP analysis to confirm virus classification. Digested DNA samples are hybridized with labeled probes derived from the central and terminal regions of parapoxvirus genomes, and although the results indicate that a strong inter-species homology exists between regions within the central parts of the genomes, terminal regions hybridize only to the same virus species (Gassmann et al., 1985; Robinson and Mercer, 1995).

PCR is nowadays one of the most powerful and applied methods in virus diagnostics (Mackay et al., 2002). It is based on the ability of thermostable DNA polymerase to synthesize a new strand of DNA during repeated cycles of heat denaturation, annealing and extension. The target sequence is defined by specific oligonucleotide primers complementary to the target DNA which in turn allow the amplification of the desired region. The amount of target DNA is doubled in each cycle resulting in billions of copies of the original sequence being produced. The identity of the amplified product can be verified using DNA hybridization (Inoshima et al., 2000) or more commonly by direct sequencing (Chan et al., 2009; Guo et al., 2004; Hosamani et al., 2006; Inoshima et al., 2001; Kottaridi et al., 2006b; Lojkic et al., 2010). The benefit of sequencing is that it allows comparison of the amplified sequence with existing data and thus enables molecular epidemiological and evolutionary studies. Moreover, it can be used to exclude false-positive results due to possible laboratory contamination, a disadvantage associated with the sensitivity of the PCR. In parapoxvirus diagnostics, PCR methods detecting a number of target genes have been developed. These include the most widely used PCR for the major envelope protein B2L (Inoshima et al., 2000) and PCRs for the interferon resistant protein (VIR) (Kottaridi et al., 2006b), ATPase (A32L), dsRNA-binding protein (E3L) (Chan et al., 2009) and GM-CSF/ interleukin-2 inhibitory factor (GIF) genes (Hosamani et al., 2007).

In real-time PCR (RT-PCR), the accumulation of PCR product is monitored in each cycle during the PCR reaction by a thermocycler instrument. The amplified product can be detected using either non-specific DNA-binding fluorogenic molecules such as SYBR Green I, or by using specific fluorogenic probes such as TaqMan® oligoprobes. RT-PCR can be used for both qualitative and quantitative analysis, it is a fast and safe method due to short reaction times and because real-time detection eliminates the need of post-PCR prosessing; a reason that also decreases the risk of cross-contamination (Mackay et al., 2002). Furthermore, the use of specific fluorogenic probes provide an additional level of specifity, although this can be of disadvantage if it is used to detect various virus species or if the target sequence is not well known.

Two different RT-PCR methods for detecting parapoxviruses in clinical specimens have been developed (Gallina et al., 2006; Nitsche et al., 2006). Both methods target ORFV major envelope antigen B2L (Sullivan et al., 1994) and can be used either in the diagnosis and quantification of ORFV (Gallina et al., 2006) or in the diagnosis of several parapoxvirus species including ORFV, PCPV, BPSV and sealpoxvirus (Nitsche et al., 2006).

2 AIMS OF THE STUDY

The general aim of this study was to identify and characterize the causative agent of pustular stomatitis in reindeer in Finland and to establish specific and rapid methods for the detection of PPVs in clinical samples. The specific aims were:

1. To develop PCR methods for detection of PPVs and RanHV-1, which were initially considered as possible candidate viruses causing infectious stomatitis in reindeer. The aim was to study the genetic relationship between Finnish reindeer PPVs from the 1999-2000 pustular stomatitis outbreak, the PPV strains previously detected in Finland and the established species within the genus, as well as selected members of the subfamily *Chordopoxvirinae*.

2. To determine the full-length sequence of reindeer PPV (F00.120R) isolated from the outbreak of pustular stomatitis of reindeer in 1999-2000 together with that of a reference strain of PCPV (VR634). This was in order to understand the genetic relationship between the virus causing disease in Finnish reindeer and the other PPVs at the genome level. The corollary of this was to define the origin of reindeer PPV.

3. To determine whether the six gene deletion found in the F00.120R virus isolate was a result of genomic rearrangements due to culture of the virus, and determine whether these genes are present in other isolates of reindeer and bovine PCPVs isolated in Finland. This was with the aim of developing PCR methods to distinguish between ORFV and PCPV in clinical reindeer samples without the need to use DNA sequencing for species identification.

3 MATERIALS AND METHODS

Materials and methods described in original publications are referred to here with the Roman numerals I-III.

3.1 VIRUS STRAINS (I, II, III)

Parapoxvirus and herpesvirus reference strains used as positive controls in PCRs, phylogenetic analyses or genome comparisons are described in Table 1.

3.2 CLINICAL ISOLATES (I, III)

Clinical isolates from reindeer, sheep and cattle were collected during the years 1992-2010 (I, III). Reindeer samples were scabs and vesicle swabs from animals originating from different parts of Northern Finland showing signs of pustular stomatitis during the winters of 1992-1993, 1994, 1999-2000 and 2007. Sheep and cattle scab and swab samples were collected from animals showing clinical signs of PPV infection. Samples were homogenized in Hank's balanced salt solution (Gibco BRL) to obtain a 10% sample solution. The sample suspensions were clarified by centrifugation at 2000 rpm for 30 min at +4 ^{o}C. The supernatants were supplemented with 200 IU/ml penicillin (Oriola) and 400 μg/ml streptomycin (Sigma) and stored at -70 ^{o}C for further analyses. In addition, two paraffin wax embedded tissue blocks and two PPV DNA samples were included in the work (I). The paraffin embedded tissue samples were taken from clinically ill sheep in Northern Finland in 1997 and from a cow in South West Finland during a pox-disease outbreak in cattle in 1999. The DNA samples were isolated from a person who had had a close contact with sheep and had suffered typical orf lesions (isolate BO29) and from a lesion on a cow's teat (isolate BO35). Both isolates originate from Germany.

3.3 VIRUS ISOLATION IN CELL CULTURE (I, II)

PPV strains VR634, NZ2, Orf-11, D1701, F92.R and V660 used in the PCR analyses (I, III) were adapted and grown in sheep kidney or foetal bovine turbinate cells in 50% Minimum Essential Medium / 50% L-15 Leibovitz Medium (Gibco BRL) supplemented with 1% L-glutamine (Gibco BRL), 1% non-essential amino acids

Table 1. *Parapoxvirus and herpesvirus strains used in the thesis*

Virus	Strain	Isolation source and history	Country and year of isolation	Reference	Publication
PCPV	VR634	Lesion material from human after contact with cows having papular lesions in their udders. Passaged several times in cell culture.	USA, 1963	Friedman-Kien *et al.* 1963	I, II, III
PCPV	It1303	Mammary lesion material from naturally infected cow.	Italy, 2005	Hautaniemi *et al.*, 2011	III
ORFV	NZ2	Scab material from sheep with contagious ecthyma. The sheep had been inoculated with virus which had been purified from scab with two rounds of plaque purification in bovine testis cells prior to inoculation to sheep.	New Zealand, 1982	Robinson *et al.* 1982	I, II, III
ORFV	SA00	Scab material from a goat kid with severe, proliferative dermatitis. The virus was propagated in Madin-Darby ovine kidney cells.	Texas USA, 2000	Guo *et al.*, 2003; Delhon *et al.* 2004	II, III
ORFV	IA82	Nasal secretion material from sheep lamb with contagious ecthyma. Passaged in ovine fetal turbinate cells.	Iowa USA, 1982	Delhon *et al.* 2004	II, III
ORFV	Orf-11	Sheep scab material from a clinical contagious ecthyma case. Passaged 29 times in ovine kidney cells and subsequently maintained in foetal lamb muscle cells.	Scotland, 2001	McInnes *et al.* 2001	I
ORFV	D1701	Scab material from sheep with contagious ecthyma. Passaged several times in cell culture.	Germany, 1981	Mayr *et al.* 1981	I
ORFV	F92.R	Scab material from reindeer with pustular stomatitis during the outbreak in 1992-1993 in Finland. Passaged in bovine kidney cells.	Germany, 1995	Büttner *et al.* 1995	I
ORFV	India 67/04	Scab material from affected sheep during a severe contagious ecthyma outbreak.	India, 2004	Hosamani *et al.* 2006	III
ORFV	India 59/05	Scab material from affected goat during a severe contagious ecthyma outbreak.	India, 2005	Hosamani *et al.* 2006	III
ORFV	Korea	Scab material from goat with contagious ecthyma.	Korea, 2009	Oem *et al.* 2009	III
ORFV	Taiping	Lesion material from goat kid with contagious ecthyma.	Taiwan, 2007	Chan *et al.* 2009	III
BPSV	AR02	Bovine calf oral lesion material. Passaged in primary lamb kidney cells.	Arkansas USA, 2002	Delhon *et al.* 2004	II, III
BPSV	V660	Isolated from cattle. Passaged in bovine embryonic lung cells after plaque purification.	Germany, 1985	Gassman *et al.* 1985	I, III
PVNZ	DPV	Lesion material from velvet of farmed red deer, passaged in primary calf testis cells.	New Zealand, 1985	Robinson and Mercer, 1995	I
RanHV-1	Salla-82	Reindeer swab sample passaged in reindeer kidney cells and maintained in embryonic bovine trachea cells.	Finland, 1982	Ek-Kommonen *et al.* 1986	I

(Gibco BRL), 200 IU/ml penicillin (Oriola), 400 μg/ml streptomycin (Sigma) and 7% horse serum at +37°C until the CPE was found to effect more than 50% of the cell culture monolayer (3-4 days). Virus was harvested from cells by freezing and thawing three times and cellular debris was removed by low-speed centrifugation at 2000 rpm for 30 min at +4 °C. The supernatants were stored at -70°C.

In initial attempts to isolate reindeer PPV from PCR positive sample suspensions the following cell lines were used: foetal bovine lung, foetal bovine turbinate, foetal bovine kidney or sheep kidney cells cultured in the cell culture medium described above. Subsequently primary bovine oesophagus cells cultured in Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 1% L-glutamine, 1% Non essential amino acids, 200 IU/ml penicillin, 400 μg/ml streptomycin and 10% bovine serum were used. Cells that had been divided on the previous day were infected with 0.5 ml of the sample supernatant in 25-cm^2 cell culture flask (Nunc). Samples were allowed to adsorb for 1 h at +37 °C before adding the cell culture medium and the cells were checked daily for the presence of CPE. Cells were harvested when the CPE covered more than 50% of the cell culture monolayer or on the seventh day after infection by freezing and thawing three times, and cellular debris was removed by low-speed centrifugation at 2000 rpm for 30 min at 4 °C. The supernatants were stored at -70 °C. The passaging was repeated three times in an attempt to isolate the virus.

3.4 VIRUS CLONING WITH END-POINT DILUTION METHOD (II)

Reindeer PPV strain F00.120R was purified with three rounds of end-point dilution method in bovine oesophagus cells (Huovilainen et al., 1988) and passaged altogether nine times in bovine oesophagus cells before DNA purification (II). Briefly, $10^{-1} – 10^{-5}$ dilutions of F00.120R clinical sample suspension were seeded in microtitre plates (Nunc), one 96-well plate per dilution, and 20 000 bovine oesophagus cells were added per well. The plates were incubated at 37 °C and the wells showing CPE were identified by microscopy seven days post infection. Virus was harvested from wells showing CPE only in plates with one to three positive wells.

3.5 VIRUS DNA ISOLATION METHODS (I, II, III)

DNA was purified from clinical sample supernatants (I, III) and reference virus infected cell culture supernatants (I) with QIAamp® DNA Mini Kit (QIAGEN). The DNA from paraffin blocks was purified using the method described by Jackson et al. (1990) with slight modifications. Briefly, 100 mg of paraffin wax embedded

tissue was digested with 1 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K). After five days incubation with digestion buffer, DNA was extracted from 300 μl of the sample suspension with the standard phenol-chloroform method (Sambrook et al., 1989). Purified DNA was diluted 1/10, 1/100 and 1/1000 for PCR analyses (I).

Viral DNA of 9th passage (9p) cell culture grown reindeer PPV strain F00.120R was purified with the methods described by Esposito et al. (1981) and Guo et al. (2003) (II). In summary, cells from cell cultures infected with F00.120R were collected by scraping when about 80-90% cytopathic effect (CPE) was observed. Cells were washed in isotonic buffer (10 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA), resuspended in hypotonic buffer (10 mM Tris pH 8.0, 10 mM KCl, 5 mM EDTA) and placed on ice for 10 min. Then, 10% Triton X-100 (Sigma) and 0.25% 2-mercaptoethanol (Sigma) were added to the cell suspension and incubation continued on ice for another 10 min. The cell nuclei were removed by centrifugation at 3000 rpm at +4 °C for 10 min (Sorvall SS-34, DuPont). To purify the viral cores, supernatants were centrifuged at 13 000 rpm +4 °C for 60 min (SW 41Ti, Beckman Instruments) and the pellets were resuspended in 0.8 ml of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) with 0.1 mg/ml RNaseA (Sigma), 0.5 mg/ml proteinase K (Roche Applied Science), 1.5% 2-mercaptoethanol and 0.2 ml 20% (w/v) sodium N-lauroylsarcosinate (Sigma) and incubated +4 °C for 30 min. Incubation was continued at +37 °C for 16 h after adding 1 ml of TE buffer containing 1% SDS (Sigma). To inhibit non-specific binding of residual material, 0.4 ml of 5 M NaCl (Sigma) was added before the viral DNA was extracted with equal volume of phenol:chloroform, ethanol precipitated and resuspended in TE buffer.

VR634 DNA for genome sequencing (II) was purified with the method described by Ueda et al., (2003) by Norihito Ueda at Virus Research Unit of Department of Microbiology and Immunology, University of Otago, New Zealand.

3.6 PCR METHODS (I, III)

Various conventional, seminested and nested PCR methods were used to detect PPV (I, III) and RanHV-1 DNA (I) in clinical reindeer, sheep and cattle samples. Target genes and viruses are shown in Table 2. In addition, a primer pair was designed to amplify a 648 bp region spanning the deletion of the genes 116-121 in the F00.120R genome. Primer sequences and PCR assay conditions are described in detail in Publications I and III.

Table 2. *Target genes for PPV and RanHV-1 PCR methods*

Target gene	Gene number (PPV)	Amplicon size bp	Target virus	Reference	Publication
Virion core protein	080	458	ORFV, PCPV, PVNZ	Tikkanen *et al.* 2004	I
Virion core protein	080	745	ORFV, PCPV, BPSV	-””-	I
Viral fusion peptide	104	301	ORFV	-””-	I
EEV Envelope phospholipase	011	594, seminested reaction 235	ORFV, PCPV, BPSV, PVNZ	Inoshima *et al.*, 2000	I
Glycoprotein B	-	445, nested reaction 297	Ruminant alphaherpesviruses[1]	Ros & Belák, 1999	I
EEV Envelope phospholipase, complete CDS	011	1210	ORFV, PCPV	Hautaniemi *et al.* 2011	III
Unknown	116	523	ORFV	-””-	III
GM-CSF inhibitory factor gene (GIF)	117	530	ORFV	-””-	III
GIF, complete CDS	117	1070	ORFV	-””-	III
Unknown	118	237	ORFV	-””-	III
Unknown	119	414	ORFV, PCPV	-””-	III
Unknown	119	442	ORFV	-””-	III
Unknown	120	466	ORFV	-””-	III
NF-κB inhibitor	121	449	ORFV	-””-	III
Unknown	116	492	PCPV	-””-	III
GIF, complete CDS	117	1065	PCPV	-””-	III
Unknown	118	290	PCPV	-””-	III
Unknown	119	270	PCPV	-””-	III
Unknown	120	523	PCPV	-””-	III
NF-κB inhibitor	121	327	PCPV	-””-	III

[1] Bovine herpesvirus 1 and 5 (BHV-1 and 5), caprine herpesvirus 1 (CapHV-1), cervine herpesvirus 1 (CerHV-1) and rangiferine herpesvirus 1 (RanHV-1)

3.7 SEQUENCING AND PHYLOGENETIC ANALYSIS OF PCR PRODUCTS (I, III)

Positive amplicons from clinical samples and cell culture grown viruses were sequenced using the same primers as for PCRs. Sequencing was performed either at the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki (I), or at Evira (III). Sequencing reactions were performed using Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and assayed with the ABI 3100 Avant genetic analyzer (Applied Biosystems) (III). Sequence and phylogenetic analyses are described in detail in Publications I and III. Briefly, the predicted amino acid sequences were aligned with published poxvirus sequences (Table 3) using program ClustalX (Thompson et al., 1997) prior to performing phylogenetic analyses. The phylogenetic analyses were performed with the Phylip package version 3.6α1 (Felsenstein, 2003) (I) or with the MEGA 4.1 software (Tamura et al., 2007) (III). Phylogenetic trees were inferred with distance (I, III), parsimony and maximum likelihood (I) methods and the reliability of the trees were determined by 1000 data set bootstrap resampling. To take into account the rate heterogeneity in the data, we calculated the gamma distribution parameter alpha and tested the amount of gamma rate gategories to be used in the distance and maximum likelihood analyses using the program Tree-Puzzle version 5.1 (Schmidt et al., 2002) (I)

In addition, the amino acid alignments of the virion core and EEV envelope phospholipase sequences (I) were analyzed as concatenated data to gather further support for the results obtained from the separate sequence analyses. Concatenated data were analyzed with maximum likelihood method and the significance of the tree obtained was verified by bootstrap analysis of 100 replicates.

The percentage identities between paired nucleotide and deduced amino acid sequences were calculated with the Megalign program (DNASTAR).

Table 3. *Virus strains used in genome comparisons and phylogenetic analysis*

Virus	Strain	Accession number			References	Publication
		Complete genome[1]	ORF011	ORF117		
BPSV	Aomori		AB044797		Inoshima *et al.*, 2001	I
ORFV	Iwate		AB044795		Inoshima *et al.*, 2001	I
Sealpoxvirus (SPV)			AF414182		Becher *et al.*, 2002	I
Molluscum contagiosum virus subtype 1 (MOCV)		U60315			Senkevich *et al.*, 1996	I
Yaba-like disease virus (YLDV)	Davis	AJ293568			Lee *et al.*, 2001	I
Rabbit fibroma virus (SFV, "Shope fibroma virus")	Kasza	AF170722			Delange *et al.*, 1984	I
Lumpy skin disease virus (LSDV)	Neethling 2490	AF325528			Tulman *et al.*, 2001	I
Fowlpoxvirus (FWPV)	fowlpox challenge virus	AF198100			Afonso *et al.*, 2000	I
Monkeypox virus (MPXV)	Zaire-96-I-16	AF380138			Shchelkunov *et al.*, 2001	I
Swinepox virus (SWPV)	17077-99	AF410153			Afonso *et al.*, 2001	I
Variola virus (VARV)	Bangladesh 1975	L22579			Massung *et al.*, 1993	I
Vaccinia virus (VACV)	Copenhagen	M35027			Goebel *et al.*, 1990	I
Camelpox virus (CMLV)	M-96	AF438165			Afonso *et al.*, 2002	I
Cowpox virus (CPVX)	Brighton Red	AF482758			Pickup *et al.*, 1982	I
Sheeppox virus (SPPV)	A	AY077833			Tulman *et al.*, 2002	I
Goatpox virus (GTPV)	Pellor	AY077835			Tulman *et al.*, 2002	I
Ectromelia virus (ECTV)	Moscow	AF012825			Mossman *et al.*, 1995	I
PCPV	VR634		AB044792	EU999744	Inoshima *et al.*, 2001; Deane *et al.*, 2009	III
BPSV	AR02	AY386265			Delhon *et al.*, 2004	II, III
BPSV	V660			EU999745	Deane *et al.*, 2009	III
ORFV	NZ2	DQ184476	U06671		Sullivan *et al.*, 1994; Mercer *et al.*, 2006	II, III
ORFV	IA82	AY386263			Delhon *et al.*, 2004	II, III
ORFV	SA00	AY386264	AY278208		Guo *et al.*, 2003; Delhon *et al.*, 2004	II, III
ORFV	India 67/04		DQ263305		Hosamani *et al.*, 2006	III
ORFV	Taiping		EU327506		Chan *et al.*, 2009	III
ORFV	Korea		GQ328006		Oem *et al.*, 2009	III
ORFV	India 59/05		DQ263304		Hosamani *et al.*, 2006	III

[1] Virion core (ORF080) and EEV envelope phospholipase (ORF011) protein sequences (I, III) and GM-CSF inhibitory factor gene (ORF117) sequences (III) were edited from complete genome sequences for the purposes of the analyses

3.8 CLONING OF VIRAL GENOMIC DNA (II)

A cosmid library containing fragments of the F00.120R (9p) DNA was constructed using the vector SuperCos 1 (Stratagene) with protocols provided by the manufacturer. Viral DNA was partially restricted with *Sau* 3AI to obtain fragments ranging from 30 to 42 kb in length. These were dephosphorylated and ligated to the cosmid vector, according to the manufacturer's instructions and the subsequent ligation products were packaged using GigaPack II (Stratagene). The packaged cosmids were then used to transduce *E. coli* XL1-Blue MR. The cosmid library was amplified in accordance with the manufacturer's instructions. For purposes of preparing probes for screening the cosmid library, and later for sequencing the genome ends, the viral DNA was digested with *Hin*d III, *Kpn* I (Promega) and *Not* I (Roche) and the resulting fragments were cloned into the pBluescript SK- plasmid vector (Stratagene). The cosmids and plasmids selected for sequencing were purified with QIAprep Spin Miniprep Kit (QIAGEN) as recommended by the supplier prior to constructing the libraries.

3.9 DNA SEQUENCING STRATEGIES (II, III)

Details for genome sequencing strategies for F00.120R and VR634 are described in Publication II. The genomic sequence of F00.120R (9p) was determined by shotgun sequencing of plasmid sub-libraries of three cosmids covering the central region of the genome, and by sequencing transposon random insertion libraries of two plasmids derived from each terminus of the genome. Briefly, shotgun sublibraries were prepared using the TOPO Shotgun Subcloning Kit (Invitrogen) and the transposon insertion libraries were constructed using the EZ::TN<KAN-2> Insertion Kit (Epicentre). Both shotgun and transposon insertion clones were purified using the Wizard SV96 Plasmid DNA Purification System (Promega) either manually using the Vac-Man 96 Vacuum manifold (Promega) or with a Biorobot 8000 (QIAGEN) prior to sequencing. Shotgun library clones were sequenced using universal primers flanking the cloning site, and transposon insertion clones were sequenced using transposon specific forward and reverse primers provided with the kit.

The sequence between ORFs 115 and 122 in the wild type (wt) F00.120R genome was determined by sequencing two overlapping, cloned PCR fragments (III). The amplicons from three replicate PCR reactions were cloned into pSC-A plasmids and transfected into StrataClone SoloPack competent cells (StrataClone™ PCR Cloning Kit, Stratagene). The resulting plasmids were purified with QIAprep Spin Miniprep kit (QIAGEN), verified by restriction digestion and sequenced initially with the universal T3 and T7 primers and subsequently completed by primer walking. The sequence of the region corresponding to ORFs 116-121 in the It1303 genome

was determined by sequencing four overlapping PCR fragments with the primers used in the PCRs by Francesca Vaccari at University of Bologna. The details are described in Publication III.

All above mentioned sequencing reactions were performed using Big Dye v.3.1 chemistry (Applied Biosystems) and the reactions were assayed either on ABI3730xl or ABI3100 Avant genetic analyzers. For each cosmid, plasmid and PCR product, the individual sequences were edited and assembled into contigs using the programs SeqMan Pro and SeqBuilder of the Lasergene version 7.1 (DNASTAR). Gaps were closed by primer walking.

The genomic sequence of VR634 was determined with a 454/Roche GS FLX pyrosequencing system (Roche) by Norihito Ueda at Virus Research Unit of Department of Microbiology and Immunology, University of Otago, New Zealand. The details are described in Publication II.

3.10 ANNOTATION AND ANALYSIS OF VIRUS SEQUENCES (II, III)

Sequence analyses are described in Publications II and III. Briefly, open reading frames (ORFs) of F00.120R, VR634 and It1303 putatively encoding proteins were identified with the programs GATU (Tcherepanov et al., 2006) and SeqBuilder (DNASTAR) and verified by using BLASTP (Altschul et al., 1990) available through the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLASTP searches of predicted F00.120R proteins against a BLAST database created by formatdb from Vaccinia virus Copenhagen (VACV_Cop) predicted proteins were performed with Blastall (NCBI BLAST version 2.2.15). FASTA-formatted files of poxviruses and poxvirus proteins used in comparison were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) or the Viral Bioinformatics Resource Center (http://athena.bioc.uvic.ca). The percentage identities of the deduced amino acid sequences between F00.120R and PCPV strains VR634 and It1303, ORFV strains NZ2, IA82, SA00 and the BPSV strain AR02 were calculated with MegAlign (DNASTAR).

3.11 PHYLOGENETIC ANALYSES OF VIRUS GENOMES (II)

Phylogenetic analyses of the virus genome sequences are described in detail in Publication II. Briefly, individual amino acid sequences were aligned using ClustalW 1.83. (Thompson et al., 1994) and subsequently concatenated into three separate blocks of data comprising: 1) the genome ends comprising proteins 001 - 008 and proteins 112 -134; 2) the central core of the genome including proteins 009 –

111 and; 3) the entire genome, i.e. proteins 001 – 134. Phylogenetic analysis with maximum likelihood method was then performed separately on these blocks in order to see if the variable and conserved genome regions would generate different tree topologies. If a protein was missing from a particular virus it was coded as missing information in the analyses.

Phylogenetic relationships were calculated using the fast hill climbing method in the program RaxML-VI-HPC version 2.2.3 (Stamatakis et al., 2005). The validity of the results was determined by bootstrap analysis of 100 replicates with programs RaxML-VI-HPC and Consensus (PHYLIP package version 3.66 (Felsenstein, 2005)). The tree with bootstrap values was visualized in MEGA 4.1 software (Tamura et al., 2007).

3.12 ANALYSES OF PERCENTAGE G+C RESIDUES IN THE PPV GENOMES (II)

G+C contents of PPV and Molluscum contagiosum virus genomes were determined by moving average method with a 1000 bp window using the program R version 2.9.0 (R Development Core Team, 2008).

4 RESULTS AND DISCUSSION

4.1 DETECTION AND DIAGNOSIS OF REINDEER CONTAGIOUS PUSTULAR STOMATITIS (I, III)

Contagious pustular stomatitis is an important disease of reindeer which has been observed in Finland since the early 1990s. Prior to this study, the aetiological agent of the disease was not known, although based mainly on clinical symptoms and pathology, ORFV was thought to have been the major causative agent of the disease. In 1999, a project was started to establish the aetiology and epidemiology of the disease and to develop diagnostic methods for detection of PPVs. Local veterinarians were asked to take samples from sick animals and, because PPV isolation in cell culture is generally known to be difficult and time consuming, various PCR methods detecting virion core, fusion and EEV envelope phospholipase genes of PPVs were designed or set up according to the literature (Table 2; Publication I). In addition, since it had been shown that the rangiferine herpes virus 1 (RanHV-I) is prevalent in Finnish reindeer (Ek-Kommonen et al., 1982; Ek-Kommonen et al., 1986) and since the bovine herpesvirus-1-like virus had been isolated from clinically similar cases of disease in reindeer in Sweden (Rockborn et al., 1990), the samples were analysed with a PCR assay detecting ruminant alphaherpesviruses (Ros and Belak, 1999).

Altogether 81 clinical samples from 57 reindeer showing symptoms of pustular stomatitis during winter 1999-2000 were analysed with the PCR methods. As a result, ten reindeer (18%) were found to be positive in the PPV-specific virion core protein and EEV envelope phospholipase gene PCRs. The number of positive samples was lower than expected, but this might in part be due to the quality of the samples; the secondary bacterial infections often connected with the disease and the long distances travelled by samples can complicate the laboratory diagnosis. However, none of the samples were positive in ORFV-specific fusion protein PCR or in the PCR detecting RanHV-1, which suggested that at least a proportion of the pustular stomatitis observed in the winter 1999-2000 outbreak 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 the core protein gene-specific primers detected all known PPV species.

Later PCR methods to amplify the complete coding regions of the EEV envelope phospholipase gene and GM-CSF inhibitory factor (GIF) genes were designed for sequencing and phylogenetic analyses (Table 2; Publication III). The methods were used to identify the viruses causing contagious pustular dermatitis in reindeer in

2007 and the viruses isolated from the Finnish sheep and cattle pox-disease cases from the years 2005-2010. The EEV envelope phospholipase gene was chosen because it has been widely used for detection, diagnosis and identification of PPVs (Hosamani et al., 2006; Inoshima et al., 2001; Lojkic et al., 2010; Nagarajan et al., 2010; Thomas et al., 2003), and the GIF gene was selected due to its location in the region previously found to be missing in the sequenced reindeer PPV (strain F00.120R, 9p) genome. The EEV envelope phospholipase and GIF genes were amplified from six reindeer samples from the year 2007 and from three reindeer samples from the earlier outbreaks in the years 1992, 1994 and 2000. In addition, they were also amplified from two samples from sheep and cattle. The detailed results are described in Publication III.

4.2 CHARACTERIZATION OF REINDEER PARAPOXVIRUS AND PHYLOGENETIC ANALYSES OF FINNISH PPV ISOLATES (I, III)

To identify virus species the PPV PCR amplicons from reindeer, cattle and sheep were sequenced and the alignments of the conceptual virion core, EEV envelope phospholipase and GIF protein sequences were used to construct phylogenetic trees. All positive PPV isolates described in this thesis and the GenBank accession numbers for the amplified nucleotide sequences are presented in Table 4.

Table 4. *PPV-positive isolates and GenBank accession numbers for nucleotide sequences amplified by PCR*

Virus	Isolate	Host	Country and year of isolation	Virion core protein, ORF080; partial CDS		EEV Envelope phospholipase, ORF011; partial CDS	EEV Envelope phospholipase, ORF011; complete CDS	GM-CSF inhibitory factor gene (GIF), ORF117	Publication
				418 bp	703 bp	554 bp	1137 bp	798 bp	
PCPV	F00.120R	reindeer	Finland, 2000	AY455307	AY453686	AY453656			I
PCPV	F00.128R	reindeer	Finland, 2000	AY455306	AY453685	AY453657			I
PCPV	F00.91R	reindeer	Finland, 2000	AY455299	AY453679	AY453658			I
PCPV	F00.180R	reindeer	Finland, 2000	AY455305	AY453684				I
PCPV	F00.46R	reindeer	Finland, 2000	AY455303	AY453681				I
PCPV	F99.238R	reindeer	Finland, 1999	AY455300	AY453676				I
PCPV	F99.239R	reindeer	Finland, 1999	AY455301	AY453677				I
PCPV	F00.54R	reindeer	Finland, 2000	AY455302	AY453680				I
PCPV	F00.205R	reindeer	Finland, 2000	AY455304	AY453683				I
PCPV	F00.250R	reindeer	Finland, 2000	-	AY453682				I
PCPV	VR634	isolated from human	USA, 1963	AY455310	AY453688	AY453665			I
PCPV	F07.798R	reindeer	Finland, 2007				JF773692	JF773680	III
PCPV	F07.801R	reindeer	Finland, 2007				JF773693	JF773681	III
PCPV	F99.177C	cattle	Finland, 1999	AY455308	AY453678	AY453663			I
PCPV	F05.990C	cattle	Finland, 2005				JF773694	JF773682	III
PCPV	F10.3081C	cattle	Finland, 2010				JF773695	JF773683	III
PCPV	B035	cattle	Germany, 2000	AY455309	AY453687	AY453653			I
ORFV	NZ2	sheep	New Zealand, 1982	AY455296	AY453673	AY453667			I
ORFV	Orf-11	sheep	Scotland, 2001	AY455297	AY453674	AY453666			I
ORFV	D1701	sheep	Germany, 1981	AY455298	AY453675	AY453654			I
ORFV	F92.R	reindeer	Germany, 1995	AY455293	AY453671	AY453660			I
ORFV	F92.849R	reindeer	Finland, 1992	AY455291	AY453669	AY453659	JF773697	JF773685	I, III
ORFV	F94.848R	reindeer	Finland, 1994	AY455294	AY453670	AY453661	JF773696	JF773684	I, III
ORFV	F07.808R	reindeer	Finland, 2007				JF773698	JF773686	III
ORFV	F07.810R	reindeer	Finland, 2007				JF773699	JF773687	III
ORFV	F07.816R	reindeer	Finland, 2007				JF773700	JF773688	III
ORFV	F07.821R	reindeer	Finland, 2007				JF773701	JF773689	III
ORFV	F97.391S	sheep	Finland, 1997	AY455295	AY453672	AY453662			I
ORFV	F07.3748S	sheep	Finland, 2007				JF773702	JF773690	III
ORFV	F09.1160S	sheep	Finland, 2009				JF773703	JF773691	III
ORFV	B029	isolated from human	Germany, 1996	AY455292	AY453668	AY453652			I
BPSV	V660	cattle	Germany, 1985		AY453689	AY453664			I
PVNZ	DPV	red deer	New Zealand, 1985	AY455311		AY453655			I

Phylogenetic trees were inferred with distance (I, III), parsimony and maximum likelihood (I) methods and the reliability of the trees were determined by bootstrapping 1000 resamplings. The trees as well as the pairwise comparisons of both DNA and aa sequences revealed that there are two PPV species circulating in Finnish reindeer. The samples from the winter 1999-2000 outbreak were most closely related to PCPV and the samples from the earlier years (1992 and 1994) grouped together and showed the highest nucleotide and aa identity to ORFV strains (Fig 2 in Publication I). The results were the same regardless of the method used to infer phylogeny: PPVs always segregated into three to four phylogenetic lineages in accordance with the established PPV genera and separate from the other chordopoxviruses (ChPVs), depending on the virus species included in the analyses.

To confirm the tree topology obtained from the analyses of partial gene sequences, the amino acid alignments of the partial virion core and EEV envelope phospholipase gene were concatenated and subjected to maximum likelihood analysis with 100 data set bootstrap replicates (I). Results from the initial analyses with Tree Puzzle indicated that missing data of some virus strains (coded with question marks) impaired the analysis in a way that the likelihood to find fully resolved tree topologies decreased, and thus the actual analysis was performed including as little coded data as possible. The tree of concatenated data (Fig. 2) showed the same grouping as seen in single gene analyses: reindeer PPV strains from the years 1992-1994 grouped together with ORFV and the reindeer PPV strains from the winter 2000 grouped together with PCPV, both with high bootstrap support. The analysis also verified the same main groupings of ChPV seen in the single gene analyses and agrees with the previous results of combined phylogenetic analysis of 17 conserved proteins from all ChPV genera except the PPVs (Gubser et al., 2004).

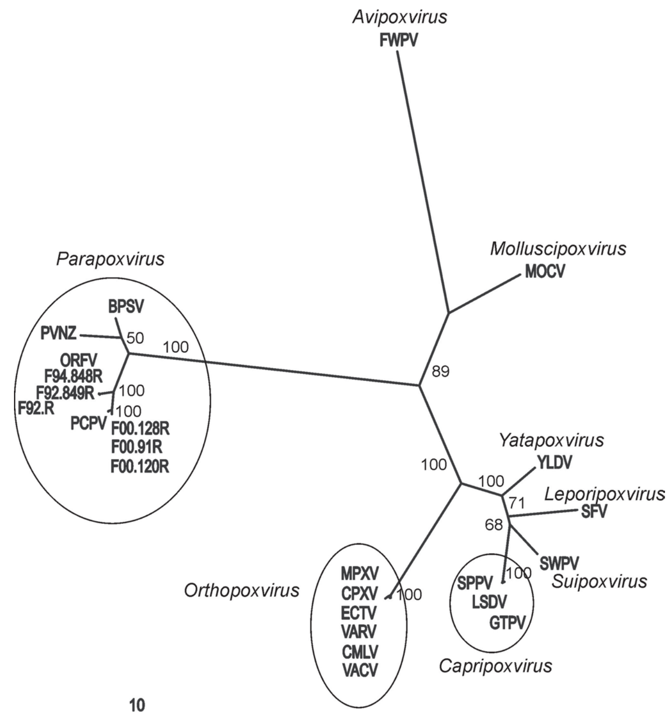

Fig. 2. *Phylogenetic tree constructed from concatenated data of core and envelope protein gene regions of PPVs isolated from Finnish reindeer, reference PPV strains and selected strains of ChPVs. Numbers on the tree represent percentage of bootstrap support calculated for 100 replicates and the scale bar beneath the tree indicates ten amino acid substitutions per site. Reprinted from Tikkanen et al. 2004, with permission.*

The trees inferred from the complete coding region of the EEV envelope and GIF proteins (Fig 1a and b in Publication III) showed the presence of both ORFV and PCPV in the 2007 reindeer pustular stomatitis outbreak: four of the six positive samples were identified as ORFV and two of them were identified as PCPV. Furthermore, the analyses indicated that the viruses causing the disease in reindeer over the years 1992-2007 are either identical or very closely related to the PCPVs and ORFVs infecting Finnish cattle and sheep, respectively. The variation in the nucleotide and predicted aa sequences of EEV envelope and GIF genes between Finnish ORFV strains was only 1.1% at the most, and similarly, the variation of these sequences between the Finnish PCPV strains did not exceed 1.4%. These results indicate potential transmission of virus strains between reindeer and sheep as well as between reindeer and cattle. Cattle husbandry is in general practice in northern Finland and it is not uncommon for reindeer and sheep to share the same pastures. However, in the light of these results, it is advisable that any direct contacts between reindeer, sheep and cattle should be avoided.

4.3 GENOME AND CLASSIFICATION OF REINDEER PARAPOXVIRUS (II, III)

4.3.1 GENOMIC FEATURES AND PREDICTED CODING REGIONS OF THE F00.120R AND VR634 GENOMES

To determine whether or not the virus isolated from the 1999-2000 reindeer pustular stomatitis outbreak was indeed PCPV rather than a separate, but closely related species of PPV, the full genome sequence of this virus (F00.120R) was determined together with the reference PCPV strain VR634. The detailed findings from the two virus genomes are described in Publication II. The main results include: 1) The sequenced F00.120R and VR634 genomes are 135 and 145 kbp in length, and contain 131 and 134 putative genes, respectively; 2) The genome size and organization of both viruses are similar to that of other PPVs, and both contain 88 predicted genes that are conserved across all sequenced poxviruses; 3) The G+C content of F00.120R and VR634 genomes are 64% and 65%, respectively; 4) F00.120R lacks six and VR634 lacks three genes normally found near the right terminus of other sequenced PPVs; 5) F00.120R has four genes at the left genome terminus and both viruses have one gene near the central region of the genome that appear to be fragmented paralogues of other genes within the genome; 6) VR634 contains nine genes that appear at both genome ends, which is most likely a result of genetic rearrangements. The genome sequences of F00.120R and VR634 have been deposited in GenBank with the accession numbers GQ329669 and GQ329670, respectively.

Both F00.120R and VR634 genomes were found to contain inverted terminal repeats (ITRs), which are identical terminal sequences but inverted with respect to each other. ITRs are a common feature to all poxviruses, but can vary in size (Fraser et al., 1990; Massung et al., 1995; Mercer et al., 1987). The size of the F00.120R ITR (2800 bp) compares well with the ITR sizes of BPSV (1161 bp) and ORFV (3092-3936 bp) (Delhon et al., 2004; Mercer et al., 2006) but VR634 ITR was found to be unexpectedly large at 14909 bp. Like ORFV and BPSV, F00.120R has one gene which appears twice due to its location in the ITR but VR634 contains nine genes in the ITR. However, this seems to be due to duplication and translocation of sequence from the right terminus of the genome to the left terminus of the genome, a common process which is often accompanied by a deletion of a sequence at the left terminus of the genome when a poxvirus is passaged in cell culture. This kind of genome rearrangement has been reported previously for ORFV (Cottone et al., 1998; Fleming et al., 1995; McInnes et al., 2001) and has most likely happened also to VR634, which has been passaged several times in cell culture.

The 131 putative F00.120R and 134 putative VR634 genes were predicted on the basis of their size and localization within the genome, the presence of promoter-like sequences or early gene transcription termination signal (TTTTTNT) and by their similarity with previously described poxvirus proteins. The likelihood of the ORFs corresponding to genuine poxvirus genes was assessed by comparing predicted amino acid sequences with the GenBank database using BLASTP. The results of gene order, spacing and the presence of conserved core region genes in F00.120R and VR634 genomes (Figure 2 and Table 2 in Publication II) were consistent with those of ORFV and BPSV genomes (Delhon et al., 2004; Mercer et al., 2006). These findings together with the PPV like genome G+C profile (Fig. 1. in Publication II) support the classification of F00.120R and VR634 as PPVs.

Despite the similarities between F00.120R, VR634, ORFV and BPSV genomes, some differences were found in relation to gene content. Both F00.120R and VR634 were found to contain genes that, deduced from the sequence similarity, appear to be remnants of genes found elsewhere in the genome. F00.120R has four such genes located in the left end of the genome (ORFs 001.6, 001.9, 004.6 and 004.8), and one near the central region of the genome (ORF 073.5), which is present also in the VR634. Interestingly, the results comparing the left end of F00.120R with that of BPSV indicate evidence of potential gene translocation and inter-species recombination, a phenomenon known to occur between other poxvirus genomes (Gershon et al., 1989; Moss, 2001). This region, containing ORFs 001.3, 001.6, and 001.9, shows the greatest sequence similarities to the region between ORFs 128 and 123 of BPSV. These include substantial predicted aa sequence similarities between 001.3 and BPSV 127, between 001.9 and BPSV 124 and non-coding nucleotide similarities between the region around 001.6 and BPSV 126/125. Moreover, the upstream promotor region of ORF 001.9 and its first six codons are similar to the corresponding sequences of ORF 132.5 of F00.120R / VR634 and ORF 002 of ORFV. Together these observations indicate the possibility that the region containing BPSV genes 124 – 127 may have recombined into the PCPV genome around the site of PCPV gene 002 sometime during the evolution of these viruses. They may also indicate that, prior to recombination, PCPV 002 had been duplicated and translocated to the right genome terminus to create 132.5. Because the F00.120R genes 001.6, 001.9, 004.6 and 004.8 are found in the region corresponding to that in the VR634 genome which is likely to have been lost during *in vitro* culture of the virus it cannot be verified if these genes are present in all PCPV isolates.

The other distinct genomic feature of the F00.120R and VR634 compared to ORFV and BPSV was that both appear to have suffered a deletion of genes towards the right terminus of the genome. These include F00.120R genes 116 to 121, together with the 5' end of gene 122, and VR634 genes 118-120 together with the 5' end of gene 121 (Figure 2 and Table 2 in Publication II). The function of these PPV specific genes are unknown except for gene 117, which is a known virulence gene targeted

against host immune defense (Deane et al., 2000; Haig and Mercer, 1998; McInnes et al., 2005), and for 121, which has been reported to inhibit the NF-kB pathway (Diel et al., 2011b).

Before sequencing the F00.120R genome, the virus had been cloned with three rounds of end-point dilution method (Huovilainen et al., 1988) and passaged altogether nine times in bovine oesophagus cells. Most of the previously described genome rearrangement events in multiple-passaged ORFV have included sequence duplication in the right end of the genome and subsequent translocation of the duplicated region to the left end of the genome without a loss of the original genes from the right genome end (Cottone et al., 1998; Fleming et al., 1995; McInnes et al., 2001). Because sequencing and size determination of uncloned F00.120R ITR did not show any evidence of the missing genes, and because nine cell culture passages was considered to be relatively low, the deletion was initially thought to be a special feature of the reindeer PCPV virus that could be used to differentiate viruses causing pustular stomatitis in reindeer. However, initial PCR analyses of wild-type and early passages of F00.120R described in the following section implied that the deletion of the six genes may have arisen as a result of passaging the virus in cell culture.

4.3.2 DISCOVERY OF GENES LOST DURING *IN VITRO* CULTURE OF REINDEER PARAPOXVIRUS

The original aim was to develop PCR methods that could be used to differentiate ORFV and PCPV in the diagnosis of reindeer pustular stomatitis taking advantage of the deletion found in the F00.120R 9p genome. We designed sets of primers to amplify separately genes 116, 117, 118, 119, 120 and 121 that were found to be missing in the F00.120R 9p genome but are present in ORFV genomes (Delhon et al., 2004; Mercer et al., 2006). The primer sequences were based on the published genomes of ORFVs NZ2 (Mercer et al., 2006) and IA82 (Delhon et al., 2004), and DNAs from NZ2 (Robinson et al., 1982) and F00.120R 9p were used as a template in all PCRs. In addition, a primer pair was designed to amplify a 648 bp fragment spanning the deleted region of the F00.120R genome. As a result, in contrast to what had been expected, PCR analysis of the F00.120R 9p produced an amplicon with the primer pair designed to detect ORFV gene 119. This suggested that the 9p virus was not clonal and contained either an ORFV or possibly a remnant from the initial isolate of the virus in which gene 119 had not been deleted. The latter possibility was supported by the presence of an ORF119 amplicon obtained from the wild type (wt) F00.120R DNA, and 100% sequence identity between the highly variable genes 109 (homologue to VACV A33R) and 132 (vVEGF) of the wt and 9p virus. These results together with the lack of amplicons obtained with other

ORFV specific primer pairs suggested that the presence of ORFV was unlikely and also raised the possibility that the deletion of genes 116-121 (estimated to be approximately 5.5 kb in length based on the ORFV genome) may have occurred as a result of culturing the virus *in vitro*.

Evidence in support of this was provided by analyzing F00.120R DNA by PCR for a 648 bp fragment spanning the deletion of the F00.120R (9p) genome. This fragment was found only in the 9p DNA and not in the wt DNA, indicating that a deletion had occurred at some point during the culturing of F00.120R. Subsequent analysis of DNA from passages 4-8 of the cell culture grown F00.120R virus revealed that the 648 bp fragment could be detected only from passages 7 and 8 but not from passages 4-6, suggesting that the deletion had occurred before the 7th cell culture passage of the virus.

Although genomic rearrangements are known to occur after serial passages of poxviruses in cell culture (Cottone et al., 1998; Fleming et al., 1995; McInnes et al., 2001), to our knowledge, the only other genomic deletions comparable to the deletion thought to have occurred during passaging the F00.120R virus is the loss of two sequences of about 2 – 3.7 kbp from the right and left ends of the ORFV B015 genome (Rziha et al., 2000). These deletions were observed after three cell culture passages, and similar to the deletion observed in the F00.120R genome, restriction analyses of wild type and cell culture passaged B015 indicated that the deleted sequences had not been translocated elsewhere in the genome.

The significance of the six gene deletion in F00.120R 9p and the deletion of three genes in the VR634 genome is unknown. In cell culture nonvital genes are commonly lost, including genes used to evade innate immunity such as those related to complement activation, interferon signaling, natural killer cells and regulatory T cells. Based on the knowledge that genomic rearrangements have often been found to result in virus attenuation *in vivo* (Fleming et al., 1995; McInnes et al., 2001; Rziha et al., 2000), together with their location in the variable terminal region, it is possible that all of the deleted F00.120R and VR634 genes are involved in virulence, host range or pathogenesis. Given that both F00.120R 9p and serially *in vitro* passaged VR634 have lost genes from the same region it seems possible that the deletion of these genes may give the virus a growth advantage *in vitro*.

4.3.3 CONFIRMATION OF THE PRESENCE OF ORFS 115-121 IN REINDEER PPV

The sequence between F00.120R ORFs 115-122 was determined by sequencing two cloned overlapping PCR fragments from wt virus isolated from original clinical sample obtained from lesions. At the same time, this region was sequenced from an Italian PCPV field isolate designated It1303. Comparison of the sequence obtained from the F00.120R wt DNA with the F00.120R 9p genome sequence revealed that

there was a deletion of 5431 bp starting at nucleotide 117052 in the F00.120R 9p sequence (Fig. 3). The corresponding sequence from It1303 wt virus was 5484 bp in length. Both F00.120R and It1303 sequences were found to contain orthologues to PPV ORFs 116, 117, 118, 119, 120 and 121 (Delhon et al., 2004; Mercer et al., 2006). Furthermore, the F00.120R ORF122 previously determined as 792 bp could now be confirmed to be 969 bp in length, the same as its ORFV and BPSV orthologs. Overall, the size, order and spacing of these genes in both F00.120R and It1303 are very similar to that found in ORFV, and to a lesser extent with BPSV, which lacks ORF118 (Delhon et al., 2004). The 5431 bp F00.120R and 5484 bp It303 sequences have been deposited in GenBank with the accession numbers JF792399 and JF800906, respectively.

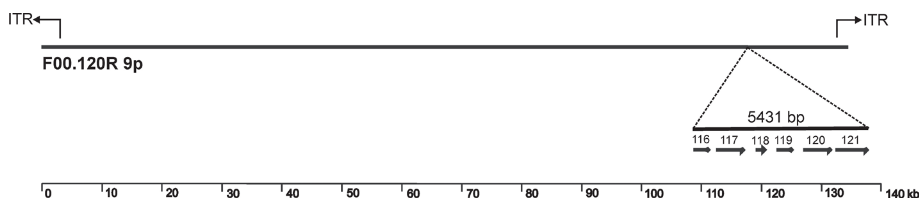

Fig. 3. *Schematic representation of the deletion that occurred during culturing of F00.120R. The thick line represents the F00.120R 9p genome and dashed lines mark the location of the additional 5431 bp region containing ORFs 116-121 detected in wild type F00.120R.*

To further confirm that the absence of genes 116-121 in the F00.120R 9p genome do not represent a virus variant commonly found in the wild, the presence of genes 116 and 118-121 was studied in the reindeer isolates of the 2007 outbreak and in the sheep and cattle isolates from the years 2005-2010. All isolates had been identified either as PCPV or ORFV with PCR and by sequencing genes 011 and 117. PCR methods for genes 116 and 118-121 were designed to target specifically either PCPV or ORFV. None of the isolates examined showed any evidence of deletions in the region covering genes 116-121. Two reindeer and two cattle isolates gave positive results in the PCPV-specific PCRs amplifying genes 116 and 118-121, and were negative in the corresponding ORFV-specific PCRs. Conversely, all four suspected reindeer ORFV isolates and the two sheep isolates gave negative results in the PCPV-specific PCRs, but all produced the appropriate amplicon in the ORFV-specific PCRs.

These results confirmed the presence of genes 116-121 in the wild type viruses and re-affirmed that the deletion is not a common phenomenon in wild-type PCPV.

4.3.4 COMPARISON OF THE PREDICTED PROTEIN SEQUENCES OF PPV GENOMES

Whole genome phylogenetic analyses and comparisons of the predicted protein sequences of F00.120R and VR634 with three strains of ORFV and one strain of BPSV provided evidence for the close relationship between F00.120R and VR634 viruses as well as the relationship between these viruses and other PPVs. Alignments of individual amino acid sequences were concatenated into three blocks of data comprising either variable terminal regions (ORFs 001-008 and 112-134), the conserved core protein region (ORFs 009-111) or the entire genome (ORFs 001-134). The trees inferred separately from variable and conserved regions gave similar tree topologies and high bootstrap support for the tree inferred from the entire genome (Fig. 3 in Publication II) placing F00.120R and VR634 together. A similar result was seen from the comparisons of individual proteins which showed the highest amino acid identity (~95%) between F00.120R and VR634 genomes (Fig. 4; Table 2 in Publication II and Table 3 in Publication III). These results indicate that F00.120R and VR634 should be classified as the same species of virus, namely PCPV, and confirms the classification of PCPV as a distinct species of the *Parapoxvirus* genus.

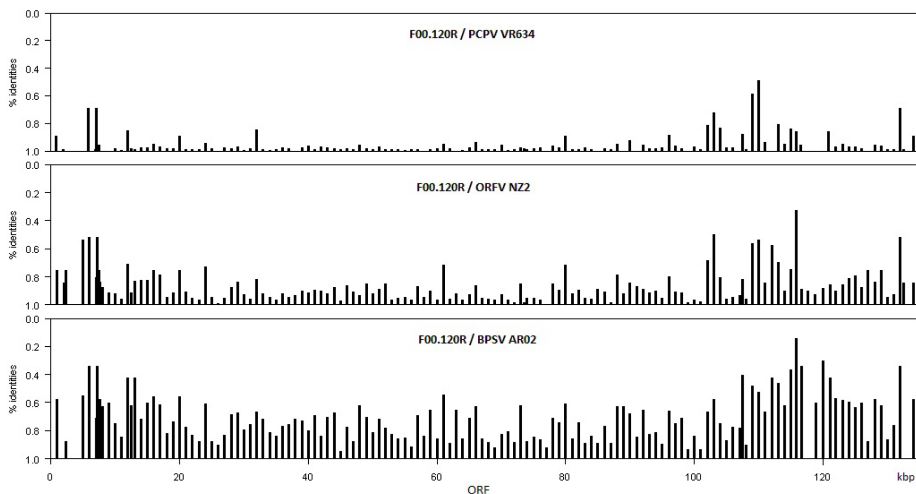

Fig. 4. *Analysis of variation between the predicted protein sequences of F00.120R and the corresponding proteins of VR634 (top panel), ORFV strain NZ2 (middle panel) and BPSV strain AR02 (bottom panel). Within the panels each bar depicts one of the 137 predicted proteins of F00.120R genome arranged in the order in which they occur in the genome. Shown is the percent amino acid sequence identity between the indicated pair of proteins. Where there is no bar present, there is no counterpart in the virus in question.*

The analyses also revealed PCPV's relatively closer genetic relationship to ORFV, in comparison to BPSV. This is despite the fact that BPSV and PCPV share the same bovine host whereas the natural hosts of ORFV are sheep and goats. The average amino acid identity between the predicted proteins encoded by ORFV and F00.120R/VR634 was approximately 87% in contrast to 72 % identity between F00.120R/VR634 and BPSV (Fig. 4; Table 2 in Publication II and Table 3 in Publication III). This confirms the proposition from previous studies of partial gene sequences that PCPV and ORFV are genetically more closely related to each other than either of them is to BPSV (Hosamani et al., 2006; Inoshima et al., 2001; Thomas et al., 2003; Tikkanen et al., 2004).

Most of the PPV-specific genes previously described in the ORFV and BPSV genomes (Delhon et al., 2004; Mercer et al., 2006) were found in both F00.120R and VR634 supporting the classification of PPVs as a separate genus in the family *Chordopoxvirinae*. The majority of these genes (ORFs 001/134, 005, 012, 012.5, 013, 024, 073, 107.5, 113, 115, 116, 121, 124, 125, 006/132) are located in the genome ends and have unknown function (Table 2 and 3 in Publication II and III, respectively). The substantial inter-isolate sequence variation seen in terminal regions of three strains of ORFV (Mercer et al., 2006) was also evident between F00.120R and PCPV (Fig 4; Table 2 and 3 in Publication II and III, respectively) again supporting the conclusion that ORFV and PCPV may share similar biological properties. Five of the most variable ORFs between F00.120R and VR634 (103, 109, 110, 112, and 132) showed only 49.1 – 72.4% amino acid identity. In addition, the aa identity between It1303 ORF116 and that of F00.120R and VR634 ORF116 was found to be very low (37.2% and 38.0%, respectively) whereas the aa identity between F00.120R and VR634 ORF116 was 84.8%. This protein is among the five most variable proteins of ORFV (Mercer et al., 2006); for example, the aa identity between the ORF116 proteins from NZ2 and IA82 is 92% but between NZ2 and SA00 it is only 50% and therefore it may not be surprising that a similar degree of variability is found in ORF116 proteins from different PCPV isolates.

Although terminal genes are generally variable and specific to PPVs, two of the most variable genes located in the right terminus, ORFs 109 and 110, are orthologues of envelope glycoproteins present in all mammalian poxviruses (A33R and A34R in VACV_Cop, respectively). VACV A33R and A34R have been shown to be important to virus egress, dissemination and extracellular virus infectivity (Duncan and Smith, 1992; McIntosh and Smith, 1996; Roper et al., 1996; Tan et al., 2009) and furthermore, A33R has been shown to be one target of neutralizing antibodies that protected mice during experimental VACV infection (Fogg et al., 2004). It has been suggested that the inter-isolate variation of these proteins could be important in host-range selection of the PPVs (Delhon et al., 2004) and that they may also be responsible for the ability of different virus isolates to re-infect its host (Mercer et al., 2006). Our analysis did not support the association in host-range selection

since ORF109 and ORF110 proteins of F00.120R are more similar to those from SA00 (Delhon et al., 2004), the ORFV isolate from a goat (70.6% ORF109, 89.2% ORF110) than they are to those from VR634 (58.4% ORF109, 49.1% ORF110). In contrast, those sequences from VR634 show highest amino acid identity to sheep ORFV strains NZ2 and IA82 (Table 3 in Publication II).

4.3.5 F00.120R VIRULENCE AND IMMUNOMODULATORY GENES

Comparative studies between different poxvirus species have shown that genes responsible for genome replication, transcription and virus assembly are located in the central part of the genome whereas genes involved in virulence and/or host range are commonly found at either end of the genome (Delhon et al., 2004; Mercer et al., 2006; Upton et al., 2003). The F00.120R genome was not predicted to encode any additional virulence factors to those previously described in ORFV and BPSV, with the possible exception of the seemingly fragmented genes at the left terminus of the genome (ORFs 001.6, 001.9, 004.6 and 004.8). In contrast, F00.120R lacks the 149 amino acid long protein ORF133 and the two additional ANK/F-box proteins (encoded by ORFs 003 and 004) found in the BPSV genome termini (Delhon et al., 2004). The importance of this is not known, but almost all chordopoxviruses have several genes encoding either ANK repeat or ANK/F-box proteins implying they are of significance to poxviruses (Mercer et al., 2005). It has been suggested that poxviruses use their ANK/F-box proteins to remove unwanted host cell proteins in order to favour viral replication (Mercer et al., 2005; Sonnberg et al., 2008; Zhang et al., 2009). In addition, it has been suggested that ANK/F-box proteins influence virus host-range and tissue tropism (Ink et al., 1995; Johnston et al., 2005; Shchelkunov et al., 1993). The sequence comparisons of ANK/F-box proteins between different PPV species does not clearly support a role as host-range determinants since those from VR634 are more similar to the corresponding proteins from viruses isolated from sheep (ORFVs NZ2 and IA82) and reindeer (F00.120R) than those from a virus isolated from cattle (BPSV AR02). Specifically, the F00.120R and VR634 ANK/F-box proteins share on average 96 % identity whereas there is approximately 80 % identity with the corresponding proteins from ORFV and only approximately 60% identity with those from BPSV.

In addition to ANK/F-box genes, F00.120R has orthologues of other viral virulence, immunomodulatory and apoptosis-inhibitory genes found in ORFV, BPSV and PCPV VR634 sequenced it this study. These genes include IFN-resistance and chemokine-binding proteins, vVEGF, vIL-10, GM-CSF/IL-2 inhibitory factor protein, Bcl-2-like inhibitor of apoptosis and the NF-κB inhibitor.

The interferon (IFN) resistance protein ORF020 is an orthologue of VACV_Cop E3L, a double stranded RNA (dsRNA)-binding protein kinase inhibitor, which is

essential for the broad host-range of VACV *in vitro* and affects virulence *in vivo* (Brandt and Jacobs, 2001; Vijaysri et al., 2003; Vijaysri et al., 2008). The E3L protein has two domains, both of which are required for viral pathogenesis in a mouse model (Brandt and Jacobs, 2001). The low sequence identity between the N-terminal domains of the ORFV and BPSV proteins has raised a thought that this domain might have a role in virus host range and pathogenesis in their respective hosts (Delhon et al., 2004). Although our analysis also revealed that the N-terminal domain is more variable between different PPV strains than the C-terminal domain, the results did not clearly suggest host-range properties for this protein as there were no greater similarity between BPSV and PCPV 020 proteins than there was between them and the corresponding ORFV proteins.

F00.120R vVEGF is located in the right terminus of the genome similar to where it is found in ORFV. PPV vVEGFs are orthologues of mammalian VEGF gene family, and they have been shown to be responsible for the highly vascularized and proliferative nature of ORFV lesions (Savory et al., 2000; Wise et al., 2003). PPV and mammalian VEGFs share a VEGF homology domain (VHD), which contains eight cysteine residues essential to VEGF structure and receptor binding (Stacker and Achen, 1999; Wise et al., 2003). F00.120R VEGF was found to contain all eight of these cysteine residues as well as the glycine/serine-rich insertion in the loop 3 region of the VHD described in other PPVs, together with the potential O-linked glycosylation sites at the C-terminal part of the protein (Delhon et al., 2004; Inder et al., 2007; Ueda et al., 2003; Ueda et al., 2007; Wise et al., 2003). These findings would imply, together with the early transcription control elements found in the vicinity of the VEGF gene, that F00.120R possesses a biologically active VEGF gene. However, the loop 3 glycine/serine insertion in the F00.120R VHD contains eight amino acids and the potential O-linked glycosylation site contains two amino acids that are not found in ORFV (Lyttle et al., 1994; Wise et al., 2003), PCPV (Ueda et al., 2003) or BPSV (Delhon et al., 2004) vVEGFs. The importance of these insertions is yet to be determined. It is however known, that the vVEGF gene is highly variable among different PPV species and even between different isolates of the same species; for example VEGFs of sheep ORFV strains NZ2 and NZ7 are only 41% identical to each other (Lyttle et al., 1994). Despite this low sequence identity, both NZ2 and NZ7 VEGFs are capable of inducing highly vascularized and proliferative lesions in infected animals (Wise et al., 2007). The F00.120R and VR634 VEGF sequences share 69% amino acid identity which is similar to the previous analyses of vVEGF variation (Inder et al., 2007; Mercer et al., 2002).

The vIL-10 gene is found at the left end of the F00.120R genome (ORF 001.3) in contrast to the ORFV and BPSV genomes where it is located near the right end of the genome (ORF 127). VR634 was not found to have a vIL-10 gene, but this is thought to have been lost during *in vitro* culture of the virus. Indeed Klein and Tryland (2005) have reported the partial sequence of PCPV IL-10 isolated from

cattle in Norway. The F00.120R IL-10 gene has an insertion of 12 amino acids (residues 116-127) in comparison to other vIL-10s and those from cattle, sheep, goats, red deer (*Cervus elaphus*) and caprine, the functional significance of which is not known. Phylogenetic analyses of vIL-10 orthologs from ORFV, BPSV, Epstein-Barr virus, *Equid herpesvirus 2* and their vertebrate hosts have suggested that these viruses have acquired the IL-10 gene from their hosts independently at different times during evolution (Delhon et al., 2004; Fleming et al., 2007; Hughes, 2002; Rziha et al., 2003). The C-terminal two thirds of PPV and cellular IL-10s are highly conserved, but the genes have certain amino acid differences that are helpful in predicting the genetic origin of the sequence. The F00.120R IL-10 sequence has six amino acids that are found in bovine IL-10, but not in ovine IL-10. Although the IL-10 sequence from reindeer has not yet been published, only two of these six amino acids are also found in the IL-10 from red deer, another species of deer belonging to the same family *Cervidae* as reindeer, and thus supports the view that F00.120R is a cattle virus that has been transmitted to reindeer. Further supporting evidence for this might come from the fact that F00.120R and BPSV strains AR02, B177 and V660 share 14 amino acids in the highly variable N-terminal domain of the vIL-10 that are not found in ORFV, ovine, bovine, red deer or caprine IL-10 proteins (Fig. 4 in Publication II). This suggests that a recombination event may have occurred between the ancestor of F00.120R and BPSV in which, as discussed earlier, a region of the BPSV genome that includes the vIL-10 may have recombined into the PCPV genome.

In addition, poxviruses encode a variety of immunomodulatory proteins that interfere with the cytokine networks of infected hosts. F00.120R was found to encode homologues of two ORFV cytokine binding proteins: GM-CFS/IL-2 inhibitory factor (GIF), which binds to and inhibits ovine cytokines granulocyte-macrophage colony-stimulating factor and interleukin-2, and a chemokine-binding protein (CBP) encoded by ORF112. The ORFV CPB shares some amino acid and functional identity with poxvirus type II CC-chemokine-binding (CBP-II) proteins of the *Orthopox* and *Leporipox* genera (Lalani et al., 1998; Seet et al., 2003a) and with VACV_Cop A41L chemokine binding virulence factor (Clark et al., 2006). ORFV GIF has also some amino acid identity with VACV A41L, but the amino acid identity is higher between the PPV GIFs and the PPV CBPs (Delhon et al., 2004). In agreement with the previous results, we found that the predicted F00.120R GIF and CBP proteins share 22% amino acid identity and that F00.120R GIF was 11% and 17% identical to VACV CBP-11 and A41L proteins, respectively. It has been suggested that the genes encoding CBP and GIF proteins are derived from a common poxvirus ancestral gene, which has been evolved towards different binding specificities during evolution (Seet et al., 2003b).

So far, homologues of ORFV GIF have only been found from PCPV (Deane et al., 2009) and BPSV (Delhon et al., 2004). The predicted F00.120R GIF showed

94.0% amino acid identity with VR634 and was found to contain the six cysteine residues, four asparagine-linked glycosylation sites and the WDPWV motif critical to biologically active VR634 GIF (Deane et al., 2009). This indicates a close genetic relationship between the genes and suggests that the function of the gene may also be conserved. The predicted CBP protein was found to be considerably more variable between F00.120R and VR634 (68% identity) than it is found between different ORFV isolates (85% identity). It has been suggested that the absence of immunomodulatory gene conservation between members of the poxvirus family may reflect differences in host and tissue tropism and may therefore help to explain the phenotypic differences of diverse poxviral diseases (Seet et al., 2003a; Seet et al., 2003b). Future analyses of the F00.120R and VR634 proteins are needed to determine whether they possess functional differences which may have evolved due to the adaptation of F00.120R to reindeer.

5 CONCLUDING REMARKS

In Finland, contagious pustular stomatitis is an important disease of reindeer which has been observed for many years. Prior to this study, the aetiological agent of the disease was not known. ORFV was generally considered to be the main causative agent of the disease, although the diagnosis was based mainly on clinical symptoms and pathology. This thesis describes the development of PCR methods for detection of PPVs in clinical samples as well as identification and characterization of the causative agent of reindeer pustular stomatitis. The PCR methods targeting the conserved core protein in particular was found to be an acceptable target for diagnostic PCR since the combination of the core protein primers detected all known PPV species. In addition, methods developed to differentiate ORFV and PCPV in clinical reindeer samples were proven to be applicable without the need to use sequencing for species identification.

Both ORFV- and PCPV-like PPVs were identified from reindeer showing symptoms of pustular stomatitis. The phylogenetic analysis in this study confirmed that PPVs are a distinct group of poxviruses and form the fifth main group within the subfamily *Chordopoxvirinae*. Furthermore, the phylogenetic analyses of Finnish reindeer, bovine and sheep isolates indicated that the viruses causing the disease in reindeer are very closely related to the PCPVs and ORFVs infecting Finnish cattle and sheep, respectively. This indicates that these viruses are circulating among Finnish reindeer, cattle and sheep which suggest that direct or indirect contacts between reindeer, sheep and cattle should be avoided if possible.

This study also presents a complete genomic sequence of a PCPV-like PPV isolated from reindeer (F00.120R) together with a genomic sequence of an established strain of PCPV (VR634). The genomes of F00.120R and VR634 viruses were found to consist of a central core region of conserved genes, flanked by more variable terminal regions as seen in other poxvirus genomes. The protein-coding contents and G+C profile of the genomes, as well as gene order and predicted protein homologies indicated that F00.120R is an isolate of PCPV and that PCPV is correctly classified as a member of the genus *Parapoxvirus*. These results expand the host range of PCPV to reindeer. In addition, the analysis revealed that PCPV is more closely related to the ovine PPV, ORFV, than to its fellow bovine PPV, BPSV. This close relatedness between PCPV and ORFV may explain why both these viruses but not BPSV have been observed to cause infection in reindeer. Differences in immunomodulatory and virulence genes of PCPV and ORFV may be linked to the different disease phenotype seen in the PCPV outbreak in the winter 1999-2000 (mostly mild inflammatory spots and ulcers in the mouth of reindeer) compared to wart-like lesions seen in earlier outbreaks caused by ORFV-like viruses.

The F00.120R genome was found to have undergone genomic rearrangements. The results showed that a 5431 bp sequence containing genes 116-121 was likely to have been deleted from the genome between the 6th and 7th passages in cell culture. These findings conclude that the genome of reindeer PCPV is 140 kbp in length and has 137 genes. Detailed information of the molecular biology and genetic variability of the reindeer PPV is essential for the development of diagnostic methods and vaccines as well as to understand the transmission of PPVs between different animal species, which will provide insights into control measures that are needed to reduce morbidity during disease outbreaks.

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