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Peste-des-petits-ruminants virus– in the field and in the host

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Cover: Goats gathered for sample collection, Ngorongoro Conservation Area, Tanzania (photo: Emeli Torsson)

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Abstract

Sheep and goats are one of the most important sources of food and income for many people around the world. They are especially important among vulnerable groups in Africa and Asia who may depend solely on them for their livelihood. The disease peste des petits ruminants (PPR) mainly affects domestic sheep and goats, and is caused by the highly contagious PPR virus (PPRV). PPRV is currently the goal of a control and eradication program launched by the Food and Agriculture Organ of the United Nations (FAO) and the World Organization for Animal Health (OIE). This thesis has explored aspects of PPR with the hope of helping the eradication effort. Among other things, the thesis has looked at prevalence and risk factors for PPRV, development of better diagnostic methods, and studied the virus-host interactions.

The prevalence and risk factors for infection among sheep and goats in Tanzania was studied during two years. PPRV circulated with a prevalence of 49.3% in 2014 and 10.0% in 2015. The main risk factors for the animals were being female and increasing age. Interaction with wildlife was also evaluated as a risk factor, but did not lead to increased infection. The transport of serum samples was improved by validating the use of filter papers in a commercial cELISA. By adjusting the cut-off for a positive result, filter papers were a viable option for transport with unreliable cold-chains.

Long transports are also a problem in molecular diagnosis, as the sensitive nucleic acid may degrade. A protocol was developed for a field-adapted full genome sequencing of PPRV. A portable miniPCR and a minION sequencing device, allow analysis at the disease outbreak or in a minimally equipped laboratory. A genetic marker of 255 nucleotides is commonly used for molecular epidemiology, but use of the full genome allows more precise tracing of the infection and viral evolution.

A major symptom of PPR is a severe immunosuppression, mainly produced by the PPRV C and V proteins. The effects of these proteins were studied on the type I and II interferon (IFN) signaling pathways. The V protein was a strong inhibitor of both pathways, whereas the C protein inhibited the type I pathway, but stimulated the type II.

Keywords: peste-des-petits-ruminants virus, sheep, goat, epidemiology, serology, sample transport, field diagnostics, molecular epidemiology, interferon modulation

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Peste-des-petits-ruminants virus—i fält och i värddjuret

Sammanfattning

Får och getter är en av de viktigaste källorna till mat och inkomst för många människor runt om i världen. De är särskilt viktiga bland utsatta grupper i Afrika och Asien som kan vara beroende av dem för försörjning. Sjukdomen peste des petits ruminants (PPR) drabbar främst får och getter och orsakas av det mycket smittsamma PPR-viruset (PPRV). PPRV är för närvarande fokus för ett kontroll- och utrotningsprogram som lanserats av FN:s livsmedels- och jordbruksorgan (FAO) och Världsorganisationen för djurhälsa (OIE). Denna avhandling har undersökt aspekter av PPR med hopp om att hjälpa till med utrotningen. Avhandlingen har bland annat tittat på prevalens och riskfaktorer för PPRV, utveckling av bättre diagnostiska metoder och studerat virus-värdinteraktioner.

Förekomst och riskfaktorer för infektion bland får och getter i Tanzania studerades under två år. PPRV cirkulerade med en prevalens på 49,3% 2014 och 10,0% 2015. De viktigaste riskfaktorerna för djuren var att vara hondjur och ökande ålder. Interaktion med vilda djur utvärderades också som en riskfaktor, men ledde inte till en ökad risk. Transporten av serumprover förbättrades genom validering av användningen av filterpapper i en kommersiell cELISA. Genom att justera gränsvärdet för ett positivt resultat var filterpapper ett användningsbart alternativ för transport med opålitliga kylkedjor.

Långa transporter är också ett problem vid molekylärdiagnostik, eftersom den känsliga nukleinsyran kan brytas ner. Ett protokoll utvecklades för fältanpassad fullgenomsekvensering av PPRV. En bärbar miniPCR och en minION-sekvenator möjliggör analys vid sjukdomsutbrottet eller i ett minimalt utrustat laboratorium. En genetisk markör på 255 nukleotider används ofta för molekylärepidemiologi, men användning av det fullständiga genomet möjliggör en mer exakt spårning av smittan och den virala evolutionen.

Ett huvudsymptom på PPR är en kraftig immunhämning, främst producerat av PPRVs C och V proteiner. Dessa proteiners effekt på typ I och II-interferon-signaleringen studerades. V proteinet var en stark hämmare av båda, medan C proteinet inhiberade typ I interferon, men stimulerade typ II.

Nyckelord: peste-des-petits-ruminants virus, får, getter, epidemiologi, serologi, provtransport, fältdiagnostik, molekylärepidemiologi, interferonmodulering

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Dedication

To the goats

Mischief managed
J.K. Rowling

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Torsson, E**.*, Berg, M., Misinzo, G., Herbe, I., Kgotlele, T., Päärni, M., Roos, N., Blomström, A.L., Ståhl, K. & Wensman, J.J. (2017). Seroprevalence and risk factors for Peste des petits ruminants and selected differential diagnosis in sheep and goats in Tanzania. *Infection Ecology and Epidemiology,* 7 (1)**,** 1368336.

II **Torsson, E**.*, Abubakar, M., Alvåsen, K., Manzoor, S., Roos, N., Kgotlele, T., Zahur, A. B., Misinzo, G., Berg, M. & Wensman, J.J. (2019). Filter paper is a simple and cost-effective transport medium for serological diagnosis of Peste des petits ruminants. *Small Ruminant Research,* 170**,** pp. 154-159.

III **Torsson, E.,** Kgotlele, T., Misinzo, G., Wensman, J.J., Berg, M., Karlsson Lindsjö, O. (2019) Field-adapted full genome sequencing of peste-des-petits-ruminants virus using Nanopore sequencing. Manuscript.

IV **Torsson, E**., Wensman, J.J., Blomström, A.L., Berg, M. (2019) Peste-des-petits-ruminants virus C protein modulate the type I and type II interferon signaling pathways. Manuscript.

* Corresponding author

The contribution of Emeli Torsson to the papers included in this thesis was as follows:

I Planning of the study together with co-authors. Performed part of the sample collection and questionnaire study. Performed part of the lab work. Performed analysis of results. Wrote the manuscript with input from co-authors and handled correspondence with the journal.

II Planning of the study together with co-authors. Performed part of the sample collection and part of the lab work. Performed analysis of results. Wrote the manuscript with input from co-authors and handled correspondence with the journal.

III Designed study together with OKL. Performed main part of the lab work with help from OKL. Performed analysis of results together with OKL. Wrote the manuscript with input from co-authors.

IV Planning of study together with co-authors. Performed all practical lab work. Performed analysis of results together with MB. Wrote the manuscript with input from co-authors.

Related publications

I Abubakar, M., Manzoor, S., Wensman, J.J., **Torsson, E.**, Qurban, A. & Munir, M. (2016). Molecular and epidemiological features of Peste des petits ruminants outbreak during endemic situation. *British Journal of Virology,* 3(4), p. 123-129.

II Fisher, K., Chenais, E., **Torsson, E.** & Wensman, J.J.. (2016). Where is the participation in participatory epidemiology? How engagement with social science could lead to improved understanding and control of peste des petits ruminants. *British Journal of Virology*, 3(4), pp. 105-114.

III Kgotlele, T., Chota, A., Chubwa, C.C., Nyasebwa, O., Lyimo, B., **Torsson, E.**, Karimuribo, E., Kasanga, C.J., Wensman, J.J. & Misinzo, G. (2018). Detection of peste des petits ruminants and concurrent secondary diseases in sheep and goats in Ngorongoro district, Tanzania. *Comparative Clinical Pathology,* 28(3), pp. 755-759.

IV Kgotlele, T., **Torsson, E.**, Kasanga, C., Wensman, J.J. & Misinzo, G. (2016). Seroprevalence of peste-des-petits-ruminants virus from samples collected in different regions of Tanzania in 2013 and 2015. *Journal of Veterinary Science & Technology,* 7, p. 394.

V **Torsson, E.***, Kgotlele, T.*, Berg, M., Mtui-Malamsha, N., Swai, E.S., Wensman, J.J. & Misinzo, G. (2016). History and current status of peste des petits ruminants virus in Tanzania. *Infection ecology & epidemiology,* 6(1), p. 32701. * = contributed equally

VI Wensman, J.J., Lindahl, J., Wachtmeister, N., **Torsson, E.**, Gwakisa, P., Kasanga, C. & Misinzo, G. (2015). A study of Rift Valley fever virus in Morogoro and Arusha regions of Tanzania–serology and farmers' perceptions. *Infection ecology & epidemiology,* 5(1), p. 30025.

Abbreviations

ANOVA	Analysis of variance
BDV	Border disease virus
bp	Base pair
BT	Bluetongue
BTV	Bluetongue virus
BVD	Bovine viral diarrhea
BVDV	Bovine viral diarrhea virus
CCPP	Contagious caprine pleuropneumonia
CDV	Canine distemper virus
cELISA	Competitive enzyme-linked immunosorbent assay
CP	Chromatography paper
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
FAO	The Food and Agriculture Organization of the United Nations
FeMV	Feline morbillivirus
FMD	Foot-and-mouth-disease
FMDV	Foot-and-mouth-disease virus
GAS	Interferon-γ-activated sequence
HTS	High-throughput sequencing
IFN	Interferon
IRF	Interferon regulatory factor
ISG	Interferon stimulated genes
ISRE	Interferon-stimulated response element
MAVS	Mitochondrial antiviral-signaling protein
Mccp	*Mycoplasma capricolum* subspecies *capripneumoniae*
MDA-5	Melanoma differentiation-associated protein 5
MeV	Measles virus
mRNA	Messenger RNA

NFS	Nobuto filter strip
NK cell	Natural killer cell
nts	Nucleotides
OIE	World Organization for Animal Health
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PPR	Peste des petits ruminants
PPRV	Peste-des-petits-ruminants virus
PPV	Positive predictive value
PRC	Precision recall curve
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLU	Relative light units
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPV	Rinderpest virus
RT-PCR	Reverse transcription polymerase chain reaction
SLAM	Signaling lymphocytic activation molecule
STAT1/2	Signal transducer and activator of transcription 1/2
ZIKV	Zika virus

1 Introduction

1.1 History of peste-des-petits-ruminants virus

The first description of the disease Peste des petits ruminants (PPR) is from 1942 in Côte d'Ivoire, Western Africa (Gargadennec & Lalanne, 1942). The disease was described to be symptomatic in goats, while cattle in contact with the goats were unaffected. At this time, PPR was believed to be caused by a strain of rinderpest virus (RPV) that had lost its ability to infect cattle and was only able to spread in small ruminants (Hamdy & Dardiri, 1976). However, in 1979 it became clear that PPR was caused by a unique virus, peste-des-petits-ruminants virus (PPRV), and PPRV was classified as the fourth member of the genus *Morbillivirus,* together with measles virus (MeV), canine distemper virus (CDV), and RPV (Gibbs *et al.*, 1979). Analysis of full genome sequences from 14 isolates of PPRV suggests that the virus has been around much longer than previously thought. The most recent common ancestor of the current four lineages is estimated to be from approximately 1904 (Muniraju *et al.*, 2014).

The highly similar clinical signs of RPV and PPRV infection in small ruminants make it difficult to distinguish the two viruses from each other (Roeder & Obi, 1999). Therefore, before the eradication of RPV, many outbreaks of PPRV are suspected to have gone unnoticed as they were misdiagnosed as rinderpest. In addition, the RPV vaccine provides cross-coverage against PPRV. So following the eradication and discontinuing of RPV vaccinations, and the development of more precise diagnostics, the distribution and awareness of PPR have increased (Banyard *et al.*, 2010).

1.2 Biology of peste-des-petits-ruminants virus

1.2.1 Taxonomy

Peste-des-petits-ruminants virus belongs to the family *Paramyxoviridae,* subfamily *Orthoparamyxovirinae* and genus *Morbillivirus.* The genus *Morbillivirus*, alongside PPRV, includes measles virus, canine distemper virus, dolphin morbillivirus, phocine morbillivirus, the now eradicated rinderpest virus, and the newest member, the feline morbillivirus (FeMV) (Woo *et al.*, 2012) (Figure 1). In 2018, an update of the entire order *Mononegavirales* was done, in which PPRV was made the only virus in the species *Small ruminant morbillivirus* (Maes *et al.*, 2019). Thus, some articles refer to PPRV as Small ruminant morbillivirus or SRMV. However, in this thesis I will use PPRV and PPR as these are the most known and used terms for the virus and for the disease it causes.

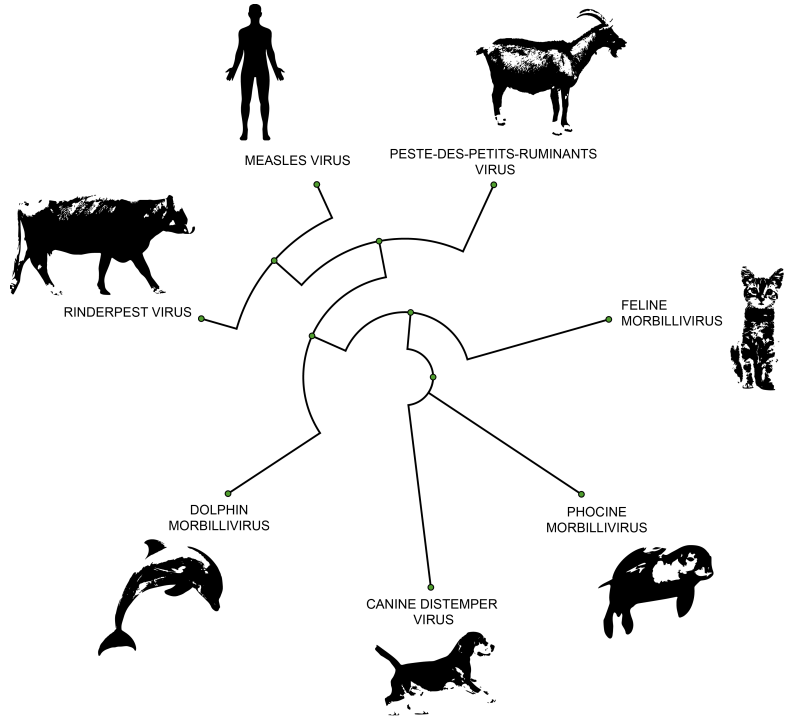

Figure 1. Phylogenetic tree depicting all current members in the genus *Morbillivirus.* Illustration by Emeli Torsson.

1.2.2 Genome and virus structure

Peste-des-petits-ruminants virus is a single-stranded negative-sense RNA virus. The genome length is 15,948 nucleotides (nts), the second longest of the morbilliviruses after FeMV (16,050 nts) (Woo *et al.*, 2012). The genome encodes for six structural proteins, that are contiguous and non-overlapping, with non-coding intergenic regions of varying length between them (Figure 2). The two non-structural proteins, V and C, are encoded for within the phosphoprotein (P) gene (Mahapatra *et al.*, 2003). The V protein is produced via post-transcriptional editing of the mRNA, for which an additional G is added at the editing site, position 751. This leads to the V and P proteins sharing the same N-terminus, but differing in the C-terminus (Mahapatra *et al.*, 2003). The C protein is produced via initiation of translation from a second initiation codon, due to leaky scanning.

The genomic RNA in the virion is surrounded by several copies of the nucleoprotein (N), together with copies of the polymerase and the phosphoprotein (Figure 2). The proteins and the viral genome form a nucleocapsid with helical symmetry within a cell-derived envelope. Covering the inside of the envelope are copies of the matrix protein, that via association with the nucleoprotein facilitate the budding of the virus through the membrane of the host cell. The virion has two surface glycoproteins. These are the hemagglutinin (H) and the fusion (F) proteins, responsible for the attachment (H) and fusion (F) of the virus particle with a new host cell (Munir *et al.*, 2013a).

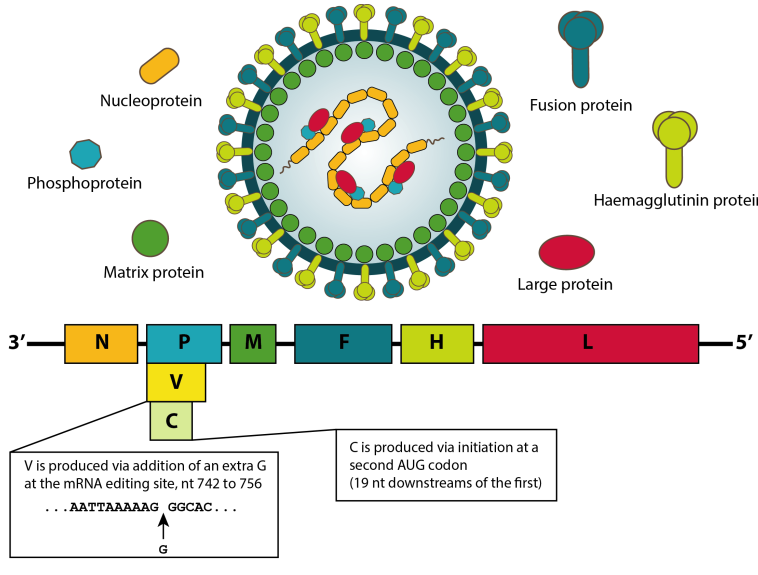

Figure 2. PPRV virion and genome structure (not drawn to exact scale), including the editing site for the V protein. Illustration by Emeli Torsson.

Two different cellular receptors have been described for PPRV, the signaling-lymphocytic activation molecule (SLAM/CD150) on lymphocytic cells, and the nectin-4 receptor on epithelial cells (Prajapati *et al.*, 2019). The use of two receptors on different cell types enables PPRV to be both lymphotropic and epitheliotropic, thereby causing a wide variety of clinical signs including a severe immunosuppression. The virus-host protein-protein interaction occurs through H. The interaction with the SLAM receptor is more species-specific than the interaction with nectin-4, thereby controlling the host range of PPRV (Prajapati *et al.*, 2019).

1.3 Virus-host interactions

When a virus infects a host, the interactions between virus and host are immediate on many levels. For example, the host immune system is activated and attempts to hinder replication of the virus and further spread within the host. The virus equally attempts to avoid and modulate the host immune system so it can use the infected cells for its own replication. The first host defense against a viral infection is a group of signaling proteins called interferons (IFNs).

1.3.1 Interferon response

Interferons are secreted cytokines that control the host antiviral response by activating or downregulating specific genes (Lee & Ashkar, 2018). There are three classes of IFNs (type I, II and III IFN) differentiated by their amino acid sequences and their binding receptors (Randall & Goodbourn, 2008). In mammals, type I IFN can further be divided into eight separate subtypes, of which IFNα and IFNβ are the two most important for the viral immune response (Randall & Goodbourn, 2008). Type II IFN comprises of a single member, IFNγ, produced predominantly by activated T lymphocytes and natural killer (NK) cells during an infection (Lee & Ashkar, 2018). Type III IFN consists of three subtypes. They mainly use the same pathways as type I IFN, but they use a different receptor that has a limited tissue distribution (Uzé & Monneron, 2007). For this reason, the focus of this thesis will be on types I and II IFN.

Type I interferon

The type I IFN signaling pathway starts with the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Subgroups of PRRs recognize different pathogens, and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) recognize foreign RNA (Iwasaki,

2012). The helicases RIG-I and melanoma differentiation-associated protein 5 (MDA-5) are present in the cytoplasm and recognize pathogen-specific RNA structures. RIG-I recognizes uncapped single-stranded RNA with a 5' phosphate and MDA-5 recognizes double-stranded RNA (Figure 3) (Kato *et al.*, 2006). Both proteins contain two caspase-recruitment domains, which recruit the mitochondrial antiviral signaling protein (MAVS), a protein bound to the mitochondrial membrane (Seth *et al.*, 2005). MAVS then activates the kinases TBK1 and IKK-α/β, which in turn phosphorylates the transcription factors interferon-regulatory factor-3 (IRF-3), IRF-7 and NF-κB (Schmitz *et al.*, 2014; Kato *et al.*, 2006). These factors are then nuclearized and activate the IFNβ promotor, leading to transcription of type I IFN mRNA and release of IFNβ from the cell.

Another pathway to activate type I IFN transcription is via the toll-like receptors (TLRs) (Figure 3). These are transmembrane proteins located in the cell membrane and within lysosomes and endosomes (Dhanasekaran *et al.*, 2014; Randall & Goodbourn, 2008). Humans, cattle, goats, and sheep have ten different TLRs (1 to 10) (Raja *et al.*, 2011), where RNA viruses are detected by TLR3, TLR7 and TLR8. When a ligand binds to a TLR, a domain in their cytoplasmic tail (Toll/interleukin-1 receptor domain) binds an adaptor protein.

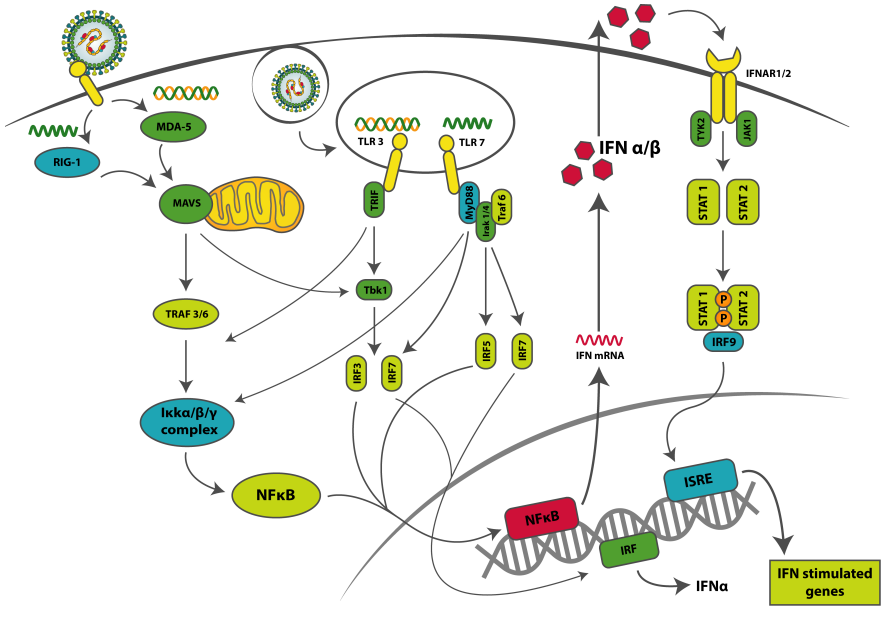

Figure 3. The type I IFN induction and signaling pathway for RNA viruses. Illustration by Emeli Torsson.

TLR3 binds to TRIF and TLR7 and 8 binds to myeloid differentiation primary response protein 88 (Myd88). This binding starts the signaling pathway activating the IFN-β promotor (Figure 3) (Randall & Goodbourn, 2008). Upon release from the cell, IFNβ, binds to a heterodimeric receptor, composed of the IFNα/β-receptor 1 and 2 (IFNAR1/2). The receptor is bound both in a feed-back loop to the IFN producing cell and to neighboring cells. The cytoplasmic tail of IFNAR1/2 associates with tyrosine kinase 2 (Tyk2) and tyrosine kinase JAK1, which together phosphorylate STAT1 and STAT2 to form a stable heterodimer (Randall & Goodbourn, 2008). The STAT1/2 complex then associates with IRF-9 to nuclearize and bind to the interferon-stimulated response element (ISRE) located in the promotor region of IFN-responsive genes leading to transcription of IFN stimulated genes (ISGs) (Randall & Goodbourn, 2008). The production of type I IFNs is the first defense of the host to a viral infection. It is essential for activating the innate immune response and for inducting an antiviral state. The effects of type I IFNs include, but are not limited to, the upregulation of up to 300 ISGs. For example; the induction of protein kinase R (PKR), which inhibits cellular translation functions essential for the virus; the activation of RNA degradation via OAS and RNase; T-cell activation; the enhancement of MHC II expression; and the recruitment of inflammatory monocytes (Lee & Ashkar, 2018). Another effect of type I IFN induction is the activation of NK cells, which, in turn, can produce IFNγ (type II IFN) (Abboud *et al.*, 2016).

Type II interferon

Type II IFN is produced by NK cells and cytotoxic T-cells. The activation of these cells is carried out either by cytokines (type I IFN, interleukin (IL)-12, IL-15, and IL-18) or by direct stimulation via activating receptors (Brandstadter & Yang, 2011). Once the NK cell is activated, it can secrete IFNγ, enhance the antiviral state and facilitate the activation of the adaptive immune response. Secreted IFNγ binds to the double heterodimer interferon-gamma receptor 1 and 2 (IFNGR1/2), which then associates with the intracellular JAK1 and JAK2 (Figure 4). This leads to phosphorylation and homodimerization of STAT1. The STAT1 complex is nuclearized and binds the IFNγ-activated sequence (GAS), a promotor for ISGs (Platanias, 2005). The IFNγ enhances the antiviral state and facilitates the activation of the adaptive immune response by a variety of means, including activating T-cells, promoting dendritic cell maturation, increasing MHC I and II expression, and inducing nitric oxide production by macrophages (Lee & Ashkar, 2018).

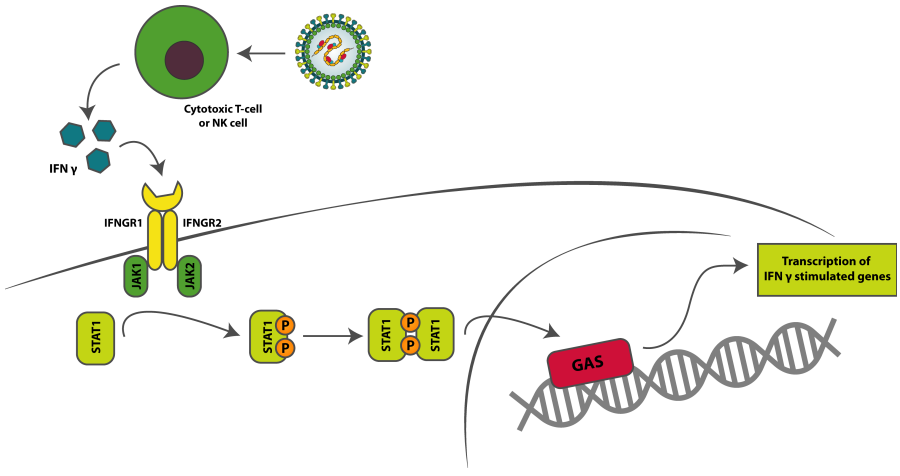

Figure 4. The type II IFN signaling pathway, stimulated by IFNγ secreted from cytotoxic T cells or natural killer (NK) cells upon viral infection. Illustration by Emeli Torsson.

1.3.2 Viral modulation of the interferon response

Every virus has developed one or more strategies to evade the interferon response of the host. There are five main ways by which they do this: i) globally interfering with gene expression and/or protein synthesis; ii) minimizing the IFN production by limiting production of viral PAMPs; iii) inhibiting IFN signaling; iv) blocking the action of IFN-induced antiviral enzymes; and, v) having a replication strategy insensitive to the actions of IFNs (Randall & Goodbourn, 2008). Exactly which of these strategies is used varies among viruses and most viruses use two or more of them. PPRV replicates within the cytoplasm of the host cell following fusion of the cell-derived virus envelope and the cell membrane (Goodbourn & Randall, 2009). As the viral replication takes place in the cytoplasm, there is a risk of the cell recognizing the free 5' triphosphate on the viral RNA via the RIG-I helicase (see section 1.3.1.). To avoid this, PPRV, as all other paramyxoviruses, encapsulates its genomic and antigenomic RNAs within the nucleoprotein during replication. Nevertheless, the RIG-I is still activated during infection (Goodbourn & Randall, 2009; Plumet *et al.*, 2007; Kato *et al.*, 2006). The limited amount of free RNA produced during replication of paramyxoviruses should also regulate the activation of MDA-5. However, this pathway is also activated to some degree during infection (Goodbourn & Randall, 2009). A common property of paramyxoviruses is the production of non-structural proteins via RNA editing (see section 1.2.2.). These proteins are rarely essential for the virus replication, even if the virus often is attenuated and the growth slowed down by their deletion (Bernardo *et al.*, 2017). The functions of these proteins are more often to control the interferon response of the host

(Goodbourn & Randall, 2009). Among the morbilliviruses, the non-structural proteins are named the V and C proteins, and are the main viral proteins responsible for the modulation of this interferon response (Bernardo *et al.*, 2017).

1.3.3 Non-structural proteins V and C

The non-structural V and C proteins of morbilliviruses play an important part in the modulation of the host immune response to infection (Chinnakannan *et al.*, 2013; Shaffer *et al.*, 2003). Studies suggest that the cysteine-rich C-terminus of the V protein interacts with the cytoplasmic helicase MDA-5 and prevents its activation of downstream signaling pathways (Bernardo *et al.*, 2017; Childs *et al.*, 2009; Kato *et al.*, 2006). This inhibition of MDA-5 is not complete, so additional mechanisms of immune modulation by the virus are needed. The V protein of some paramyxoviruses are believed to bind to TBK-1 and IKK-ε, as a decoy substrates, to prevent phosphorylation and activation of IRF-3 (Lu *et al.*, 2008). The V protein of morbilliviruses blocks the phosphorylation of STAT1/2 and thereby inhibits its nuclear translocation, and the following activation of the ISRE and transcription of ISGs (Bernardo *et al.*, 2017; Chinnakannan *et al.*, 2013).

The V protein is considered as the main immunomodulatory protein of PPRV, however, the C protein is also important in some way for the modulation. One strategy viruses can use to circumvent the immune response is to hide the fact that they are there at all, by regulating the expression of PAMPs. This has been suggested to be the primary function of the C protein (Goodbourn & Randall, 2009; Fontana *et al.*, 2008; Shaffer *et al.*, 2003). However, it has been reported that the C protein of MeV might be able to interfere with type I IFN transcription in the nucleus (Sparrer *et al.*, 2012).

1.4 Clinical signs

Peste des petits ruminants is mainly a disease of domestic sheep and goats, but wild small ruminants, camels, and pigs can also develop clinical signs (Schulz *et al.*, 2018; Abubakar *et al.*, 2011; Khalafalla *et al.*, 2010). In general, goats develop more severe clinical signs than sheep, but this can, for unknown reasons, vary in outbreaks (Roeder & Obi, 1999). Cattle can be infected and develop antibodies to PPRV, but they do not show any signs of disease and do not transfer the virus further (Parida *et al.*, 2015).

The clinical signs of a PPRV infection are related to the cell types infected by the virus, i.e. the epithelial cells and lymphocytic cells. A after an incubation

period of 2-6 days, the first sign is high fever, at least 40-41°C (Roeder & Obi, 1999). The animal appears lethargic and depressed, and soon develops a clear nasal and ocular discharge that later turns purulent due to secondary bacterial infections (Balamurugan *et al.*, 2014). As the discharge dries in the nose and nasal cavity, it can cause obstructed breathing (Roeder & Obi, 1999). A couple of days after the fever has set in, painful lesions appear in the mucous membranes and the animal may stop eating due to the pain. The lesions start as multiple pin-prick lesions, but grow and finally join together leaving the mucous membrane covered with foul smelling, painful necrotic tissue (Roeder & Obi, 1999). The next clinical sign to appear is diarrhea. In mild cases this is only a soft and blood-stained feces, but in severe cases is watery, foul-smelling, blood-streaked, and containing pieces of dead intestinal lining (Roeder & Obi, 1999). Infected animals additionally develop a severe pneumonia sometimes with forced and abdominal breathing. Because the virus replicates in lymphoid organs, the result is severe lymphopenia and immunosuppression, which may lead to secondary infections (Tatsuo & Yanagi, 2002). Due to the diarrhea and the unwillingness to eat, animals become severely dehydrated. In combination with secondary infection, this is the cause of death within seven to ten days after onset of clinical signs (Munir *et al.*, 2013b). Animals that do not succumb to the infection recover after a long period of convalescence.

Clinical signs can be less severe or the disease can have an even faster progression depending on the animal infected. Age, breed, general condition, previous immunity, and other co-infections, can affect the outcome. In an immunologically naïve population, the morbidity and case fatality rate can be as high as 80-100%, whereas in an endemic setting it can be as low as 10% (Pope *et al.*, 2013). Animals that recover from the disease develop a life-long immunity. To date, no carrier state or subclinical reservoirs have been identified (Parida *et al.*, 2015).

1.4.1 Differential diagnosis

Most cases of PPR are diagnosed in the field. Diagnosis is then based only on clinical signs, which makes it easy to confuse with other diseases, especially those present in the same geographic area (Roeder & Obi, 1999). Before it was eradicated, rinderpest was the most likely differential diagnosis because the clinical manifestation of these two diseases are more or less the same in small ruminants (Balamurugan *et al.*, 2014).

The lesions in the mucous membranes can lead to an incorrect diagnosis of foot-and-mouth disease (FMD). However, these do not grow to occlude the oral

mucous membranes nor have a foul smell. Furthermore, pneumonia and diarrhoea are additionally not present in FMD (Roeder & Obi, 1999).

Another viral disease that gives rise to oral lesions is bluetongue (BT) (Maclachlan, 2011). However, apart from fever, other clinical signs of BT (e.g. edema, bluish discoloration of oral cavity, and lameness) are not shared with PPR, and the indigenous breeds of Africa and Asia do not in general show severe clinical signs of BT (Munir *et al.*, 2013b; Roeder & Obi, 1999). A co-infection of bluetongue virus (BTV) with PPRV in small ruminants can exacerbate clinical signs and complicate the clinical outcome (Mondal *et al.*, 2009).

The difficulty in breathing as well as the fever and pneumonia seen in PPR can—especially in goats—be confused with contagious caprine pleuropneumonia (CCPP), caused by the bacteria *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) (Roeder & Obi, 1999). However, CCPP does not cause any oral lesions or diarrhea. It is mainly a disease seen in goats, with sheep remaining unaffected (Munir *et al.*, 2013b).

Other diseases worth mentioning as differential diagnosis or co-infections are: contagious ecthyma or orf, which leads to scabbed lips, pneumonic pasteurellosis, a purely respiratory disease in sheep and goats, and diarrhea due to helminths or coccidiosis (Munir *et al.*, 2013b; Roeder & Obi, 1999).

1.5 Diagnostic methods

An initial tentative diagnosis of PPR can be done based on the clinical signs and the epidemiological setting of the outbreak (Roeder & Obi, 1999). This is, however, not optimal due to the numerous differential diagnoses easily mistaken for PPR. Laboratory testing is needed for a definitive diagnosis.

1.5.1 Serological methods

The detection of antibodies is a good method for surveillance of vaccination efficiency and seroprevalence to ascertain PPR disease spread and status. Because the antibodies produced following a natural infection or following a vaccination provides a life-long immunity, serological methods are seldom a good option for diagnosis. The most sensitive and specific method is the virus neutralization test (VNT) (OIE, 2019b), but it is very time-consuming, not available in many countries, and unsuitable for routine diagnostic analysis. Commercial competitive enzyme-linked immunosorbent assays (cELISAs) are available and are based on either a recombinant nucleoprotein or the viral attachment H protein (OIE, 2019b; Libeau *et al.*, 1995). In this thesis, we have used the cELISA developed by Libeau et al., which is based on the use of a

recombinant nucleoprotein expressed by a modified baculovirus. This cELISA has a sensitivity of 94.5% and specificity of 99.4% compared to the VNT (Libeau *et al.*, 1995).

It is not yet possible to differentiate between vaccinated and naturally infected animals using any serological methods, as the current vaccine uses an attenuated live virus (Santhamani *et al.*, 2016).

1.5.2 Molecular methods

A specific diagnosis of PPRV requires molecular methods for detection of virus. The most commonly used, and most sensitive method, is the reverse transcription polymerase chain reaction (RT-PCR) (OIE, 2019b). Several different protocols have been developed for this purpose, based on either the nucleoprotein gene or the fusion protein gene (Couacy-Hymann *et al.*, 2002). The protocol used in this thesis is a one-step quantitative RT-PCR that uses a primer pair and a probe for detection of a 96 nts segment of the nucleoprotein gene (Kwiatek *et al.*, 2010).

Before an RT-PCR can be performed, the viral RNA needs to be purified from the sample. For PPRV, viral RNA can be purified from whole blood, buffy coat, ocular, oral or nasal swabs, or from different tissues (liver, spleen, lung, or lymph nodes) (OIE, 2019b). The viremia, i.e. the period during which viral RNA can be detected in blood following an infection, is short, about 6-8 days after first clinical signs are seen. RNA can also be detected in ocular and nasal discharges for about 8-10 days (Truong *et al.*, 2014). This leaves a short time window for sample collection. In addition, PPRV is a single-stranded RNA virus, and therefore highly sensitive to the environment and to high temperatures (Parida *et al.*, 2015). Because of this sensibility, the nucleic acid can degrade during transport with a broken cold chain, leading to false negative results. A certain level of expertise and laboratory equipment is also needed to perform a RT-PCR analysis. To solve the problem with sample transport, several protocols for loop-mediated isothermal amplification (LAMP) have been developed. These can be performed in a single tube using a simple heat block without advanced laboratory equipment (Mahapatra *et al.*, 2019; Dadas *et al.*, 2012; Li *et al.*, 2010). The Pirbright Institute, UK, has also developed a lateral flow device assay that is commercially available and can easily be used for diagnosis of PPR in the field (Baron *et al.*, 2014).

1.5.3 Molecular epidemiology

Previously, one of the major problems was to differentiate outbreaks of PPR from outbreaks of rinderpest. For this reason an RT-PCR protocol was developed based on the well conserved fusion protein (F) gene (Forsyth & Barrett, 1995) to type the isolates phylogenetically. However, later the nucleoprotein (N) gene, the most abundant transcript produced due to the transcriptional gradient across the genome (3'-N-P/V/C-M-F-H-L-5'), was suggested as the most appropriate gene for phylogenetic analysis (Kwiatek *et al.*, 2007; Couacy-Hymann *et al.*, 2002). The partial segment of the N gene (255 nts) used for phylogenetic analysis is located at the 3' end and has been shown to be a more powerful sequence for clustering isolates based on their geographic distribution (Figure 5) when compared to analysis using the F gene (Kwiatek *et al.*, 2007).

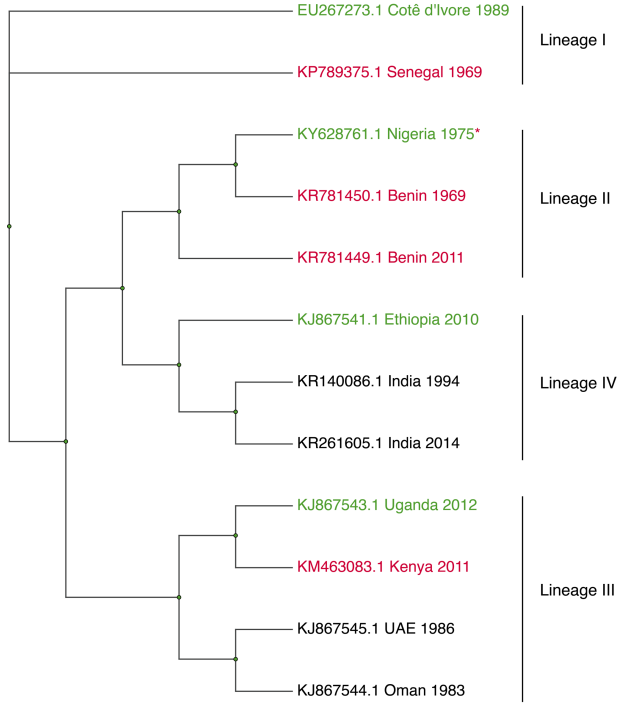

Figure 5. Phylogenetic tree using selected full genomes of PPRV, available at NCBI GenBank. The tree was produced using the Bayesian method in the UGENE software (Okonechnikov *et al.*, 2012). Isolates in green text are included in Paper IV and isolates in red are included in Paper III. The Nigeria 75 isolate, labelled with a *, was used in both studies. Illustration by Emeli Torsson.

With the advances in the field of high-throughput sequencing (HTS), with shorter runtimes and reduced costs, full genome sequencing has become a possible tool for molecular epidemiology (Deurenberg *et al.*, 2017). The use of full genomes, compared to partial sequences, are able to trace microevolution within the viral genome and detect a more specific source of an outbreak (Tang *et al.*, 2017).

1.6 Distribution and epidemiology

As stated in section 1.1., PPR was first described in Côte d'Ivoire in 1942 (Gargadennec & Lalanne, 1942). For the subsequent 30 years, outbreaks were confined to western Africa, but between 1970-80, PPR was also described in Sudan in north-eastern Africa and in India. Between 1990-2010 PPRV spread exponentially until it reached its current distribution (Figure 6) (Banyard *et al.*, 2010). Most likely, this does not represent the actual spread of the virus, but rather an increased awareness and improved diagnostic methods. During the eradication of RPV, differentiation of the two diseases became more important, leading to a clearer picture of the spread of PPRV (Taylor, 2016). PPRV is still expanding its distribution, threatening the countries in southern Africa. In 2018, PPR was reported in the European Union, following an outbreak among sheep in Bulgaria, close to the Turkish border (OIE, 2019a).

PPRV can be divided into four separate lineages on the basis of a partial nucleotide sequence of either the N or F genes (Libeau *et al.*, 2014). Lineage I is mainly found in Western Africa, lineage II is found in Western and Central Africa, and lineage III is found in Eastern Africa. Lineage IV is the currently most prevalent lineage with a distribution including Asia, the Arabic Peninsula, and a majority of the African countries (Figure 6) (OIE, 2019a; Parida *et al.*, 2015). For a long time, lineage IV was considered to be the Asian lineage of PPRV; however, during the 1990s it was introduced to the African continent and spread quickly. This could possibly be due to the establishment of a strain that has the ability to outcompetes the other lineages (Kwiatek *et al.*, 2011).

PPR is mainly a disease of domestic sheep and goats, but can affect camels (Saeed *et al.*, 2015; Khalafalla *et al.*, 2010), pigs (in an experimental setting) (Schulz *et al.*, 2018), and wild small ruminants of different species (Aziz-Ul-Rahman *et al.*, 2019; Mahapatra *et al.*, 2015; Abubakar *et al.*, 2011; Hamdy & Dardiri, 1976). Large ruminants (e.g. cattle, African buffalos and yak) can be infected and seroconvert, but do not show any clinical signs. They do not transfer the virus further, so they are dead-end hosts (Li *et al.*, 2018; Lembo *et al.*, 2013; Anderson & McKay, 1994). Currently there is no way to differentiate between antibodies due to a vaccination or a natural infection in small ruminants and

large ruminants do not receive the PPRV vaccine. Therefore large ruminants, kept in close proximity to small ruminants can be used as sentinel animals (Khan *et al.*, 2008). A sentinel animal can then be tested for seroconversion to reveal possible circulation of the virus within the ruminant population.

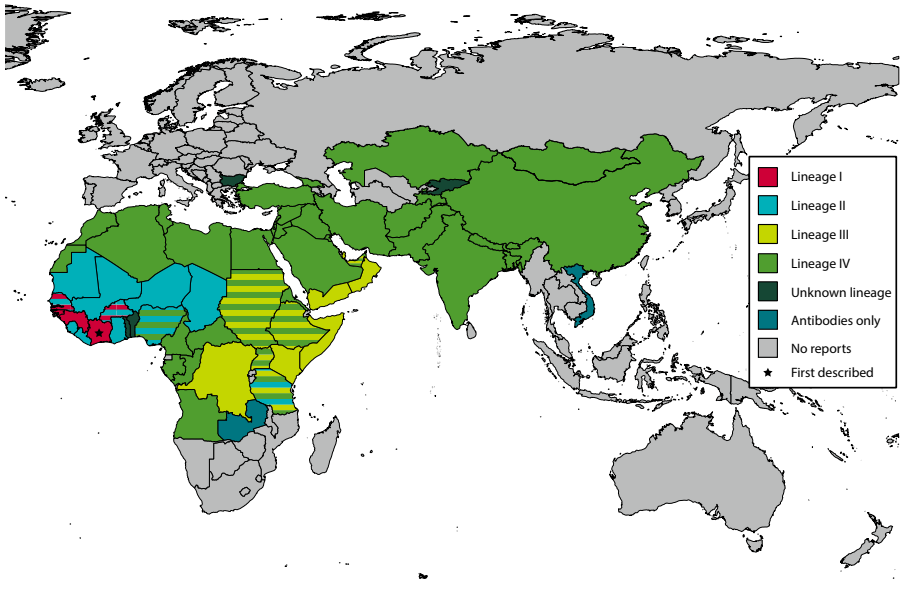

Figure 6. PPRV distribution of the four currently known lineages. Illustration by Emeli Torsson.

Infected animals transmit the virus mainly by close or direct contact with susceptible animals through excretions (ocular and nasal discharges, saliva, urine or feces) or through exhaled aerosol at short distances (Parida *et al.*, 2015). Transmission via fomites is almost negligible, because the virus is sensitive to dehydration, high and low pH, heat, and UV light and therefore does not survive long in the environment (Baron *et al.*, 2011). The importance of wild ruminants in the epidemiology of PPR is still not clear (Aziz-Ul-Rahman. *et al.*, 2016; Munir, 2014). In an area where PPRV is endemic, wild ruminants do not seem to contribute to the disease spread in a significant way. However, as we move closer to PPRV eradication, with more control of the disease in domestic animals, the importance of wildlife may increase. When most domestic animals are vaccinated, the unvaccinated wildlife may be a susceptible reservoir population. Herds in the nomadic cultures share pastures with wildlife and often have contact in a way that allows for transmission of virus. Reports of clinical signs in African wildlife infected by PPRV is limited to one (Asil *et al.*, 2019), but clinical signs have been seen in captive gazelles (Kinne *et al.*, 2010). In Asia, reports of PPR in wildlife are more common. In 2017, an outbreak of PPR

decimated the Mongolian Saiga population (Aguilar *et al.*, 2018); between 2014 and 2016, over 1000 wild sheep and goats died due to PPRV infection in Iran (Marashi *et al.*, 2017); and in China, several different wild species have been tested positive for PPRV (Li *et al.*, 2017). Phylogenetic analysis of these isolates place them all in lineage IV, but apart from that, no other significant difference between the isolates from wildlife and those from domestic animals have been identified (Aziz-Ul-Rahman *et al.*, 2019).

1.6.1 Tanzania

Tanzania is a country on the eastern coast of Africa with around 56 million people (WorldBank, 2019). The rural population consists of 37 million people, and three out of five rural households earn some part of their income (on average 22% of the total income) from livestock (WorldBank, 2019; Covarrubias *et al.*, 2012). Tanzania's total sheep population is around 6 million and goat population is around 15 million, with 99% of the animals held by smallholders (United Republic of Tanzania, 2012). The use of Tanzania as a study area for PPR, and performing the studies for Paper I and II, in this country has several benefits. PPRV is endemic in the country and attempts to control the disease with vaccination campaigns have begun. Tanzania is also the home of many wild ruminants in a variety of national parks and conservations areas, so there is opportunity for both direct and indirect contact between these wild ruminants and domestic livestock.

PPR in Tanzania

The first description of PPR in Tanzania was reported from the northern Ngorongoro district in 2008, following reports of increased death rates in local sheep and goats (Kivaria *et al.*, 2013; Swai *et al.*, 2009). It is suspected that the disease had spread from northern countries, such as Uganda and Kenya, by animals walking across the borders. Both Uganda and Kenya had reports of PPRV in 2007 (Dundon *et al.*, 2015a; Luka *et al.*, 2012). After this initial report in Tanzania, archived serum samples from Ngorongoro district collected between 1998 and 2004 were analyzed for presence of PPRV antibodies (Karimuribo *et al.*, 2011). The results show that PPR was present in Tanzania as early as 2004 (Karimuribo *et al.*, 2011).

Following the introduction of PPRV in northern Tanzania, the disease spread to the southern parts of the country and in 2011 it was confirmed to be present on the border to Mozambique (Muse *et al.*, 2012). Today, PPRV is considered endemic in the domestic sheep and goat population of Tanzania, and limited to one or more zones in the wild population (OIE, 2019a; Kgotlele *et al.*, 2016;

Torsson *et al.*, 2016). Serum samples from 46 wild ruminants (buffalo, Grant's gazelle, Thomson's gazelle, wildebeest, and impala) were collected in the Ngorongoro Conservation Area in 2014 and showed a prevalence of 63% for PPRV antibodies (Mahapatra *et al.*, 2015). One Grant's gazelle tested positive with RT-PCR, though no clinical signs were reported. This sample collection was done during a concurrent outbreak among sheep and goats in the area, suggesting a spillover of virus from domestic animals to wild animals (Mahapatra *et al.*, 2015).

1.7 Implications of PPR

There are currently around 1.2 billion sheep and 1 billion goats in the world (FAOSTAT). Of these 2.2 billion animals, 1.7 billions in around 70 countries are at risk for infection with PPRV, and 30 million animals are affected every year (FAO, 2013). Small ruminants are mainly kept by vulnerable populations that depend on them for income and livelihood, and for whom an outbreak of PPR can be detrimental (FAO, 2013). Small ruminants can provide meat, milk, wool, and skins to their owners, and are important assets for resource-constrained people. They serve as a sort of bank account and payment method when quick cash is needed (e.g., when paying for school fees, medical visits, etc.) (FAO, 2013). Small ruminants are easier to keep and cheaper to buy than large ruminants, making their health and continued production important for poverty elimination (FAO, 2013). PPR is estimated to cause economic losses between USD 1.45 to 2.1 billion due to reduced production, death of animals, and cost for caring for sick animals, including vaccinations (FAO & OIE, 2015). A calculation of the economic impact of PPRV eradication resulted in a suggested net benefit of USD 74.2 billion (Jones *et al.*, 2016), in addition to the benefits to the millions of animals that would suffer this severe and painful disease. The importance of small ruminants as a form of saving and payment method are not included in this calculation, so the actual benefit of PPRV eradication is most probably even higher.

1.8 Control and eradication

In March 2015, the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) launched a joint program to control and eradicate PPR (FAO & OIE, 2015). The close relative of PPRV, RPV, was declared eradicated in 2011 and the many lessons learned from that eradication campaign are seen as highly useful in this next eradication effort (Roeder *et al.*, 2013). The PPRV program aims to reach its goal by 2030 by

following four separate stages (Figure 7). Countries are assigned to these stages depending on their current PPR status (FAO & OIE, 2015). In stage 1, the epidemiological situation of PPRV is to be ascertained, serological methods are to be established, and the ground work for prevention and control to be started. In stage 2, the laboratory capacity is to be strengthened by molecular methods to characterize field isolates, surveillance and response to outbreaks improved, and vaccination campaigns started. In stage 3, laboratory capacity is to be strengthened further by introducing quality assurance systems, improving surveillance with an emergency response mechanism, and increasing vaccination and/or policies to achieve eradication. In the final stage, stage 4, laboratory capacities are to be maintained and differential diagnostics are to be enhanced. Surveillance is to consist of proving the absence of PPRV, vaccinations are suspended and outbreaks are controlled by stamping out, biosecurity measures, and risk analysis of possible reintroduction (FAO & OIE, 2015).

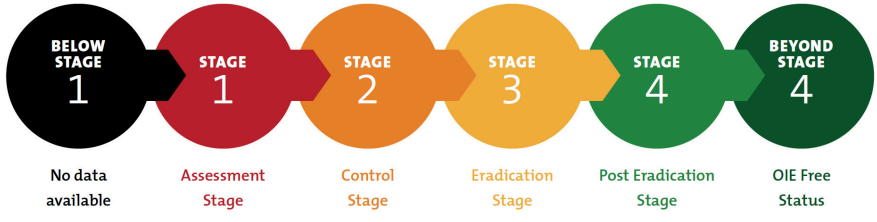

Figure 7. The four stages of the FAO and OIE control and eradication program. Illustration: *Global strategy for the control and eradication of PPR*, FAO and OIE, 2015.

1.8.1 Vaccine

The eradication of PPRV is highly dependent on vaccination campaigns, as was the RPV eradication program (FAO & OIE, 2015). The PPRV vaccine consists of a wild-type virus, attenuated via serial passages in cell cultures (Eloiflin *et al.*, 2019). There are currently several vaccines available on the market, based on different wild-type isolates (such as Nigeria75/1 and Sungri/96), but they are all believed to protect against all four lineages of PPRV (Kumar *et al.*, 2017; FAO & OIE, 2015). Following survival of a natural PPRV infection, the surviving animal mounts a life-long immunity (Parida *et al.*, 2015). Immunity developed after vaccination with a single dose lasts from three years to life-long (Kumar *et al.*, 2017; Parida *et al.*, 2015). The goal within the eradication program is to reach post-vaccination immunity in 80% of the epidemiological units (flock, area, or farm) in order to control and contain the disease (Kumar *et al.*, 2017; FAO & OIE, 2015). To reach this value of 80% post-vaccination immunity it is estimated

that the vaccination coverage need to be almost 100% in animals over three months of age (FAO & OIE, 2015).

PPRV is mostly prevalent in hot and humid climates and is, in addition, often found in areas lacking developed infrastructure. The current vaccine is not thermostable and requires an unbroken cold chain during transport (Kumar *et al.*, 2017). Development of more thermostable vaccines and also of DIVA vaccines (Differentiating Infected from Vaccinated Animals) are seen as highly important for the eradication of PPRV and for the differentiation of vaccinated and naturally infected animals during the post-eradication stage (Kumar *et al.*, 2017; Mariner *et al.*, 2017; FAO & OIE, 2015).

Figure 8. The author performing sample collection on filter papers in Tanzania in 2015.

2 Aims of the thesis

The overall objective of this thesis was to gain a better understanding of the peste-des-petits-ruminants virus, and in this way contribute to its eradication. This was done by studying the virus both in a field setting (to understand the epidemiology and to improve the diagnostic methods), and in the cell (to understand how the virus interacts with the host). Studying how both the wild-type virus and vaccine isolates, interacts with and modulates the host's immune response could improve understanding of the gained adaptive immunity and effectivity of vaccinations.

The specific aims were to:

- Assess the current seroprevalence in domestic small ruminants in Tanzania, and study the role of domestic and wild species in peste-des-petits-ruminants virus epidemiology (Paper I)

- Provide methods and protocols to improve diagnostics and molecular epidemiology in resource limited areas (Paper II and III)

- Study the immunomodulation of type I and II interferon by the non-structural C and V proteins of peste-des-petits-ruminants (Paper IV)

3 Comments on materials and methods

Detailed descriptions on material and methods are presented in Papers I-IV. This section provides an overview of the methods and materials used in the four papers, with comments and considerations on why these specific methods were chosen.

3.1 Study design and sample collection (Papers I and II)

The studies in Papers I and II were conducted in Tanzania, and for study II, also in Pakistan. In Paper I, the aim was to study animals in the wildlife–livestock interface in Tanzania. Tanzania is divided into 26 administrative regions, that are divided into districts, and further subdivided into wards. Four districts in two different regions were purposively selected because the districts contain national parks or game reserves and had had previous reports of PPR outbreaks (Kgotlele *et al.*, 2014; Kivaria *et al.*, 2013). The Ngorongoro district in the Arusha region (which contains the Ngorongoro Conservation Area), and the Mvomero, Ulanga, and Kilombero districts in the Morogoro region, (which contain the Mikumi National Park and the Selous Game Reserve) were selected (Figure 9A). Wildlife move freely across the park or reserve boundaries, but are present in much higher concentration within them. The residents within the conservation areas or reserves are different groups of the indigenous population of Tanzania. In the northern Arusha region, the indigenous groups are mainly semi-nomadic pastoralists, and in the south-central Morogoro region they are semi-nomadic pastoralists or agro-pastoralists (IFAD, 2012). These groups herd their animals between different pastures over varying distances, where the semi-nomadic groups cover a greater area. During herding, there is an almost constant intermingling with wildlife.

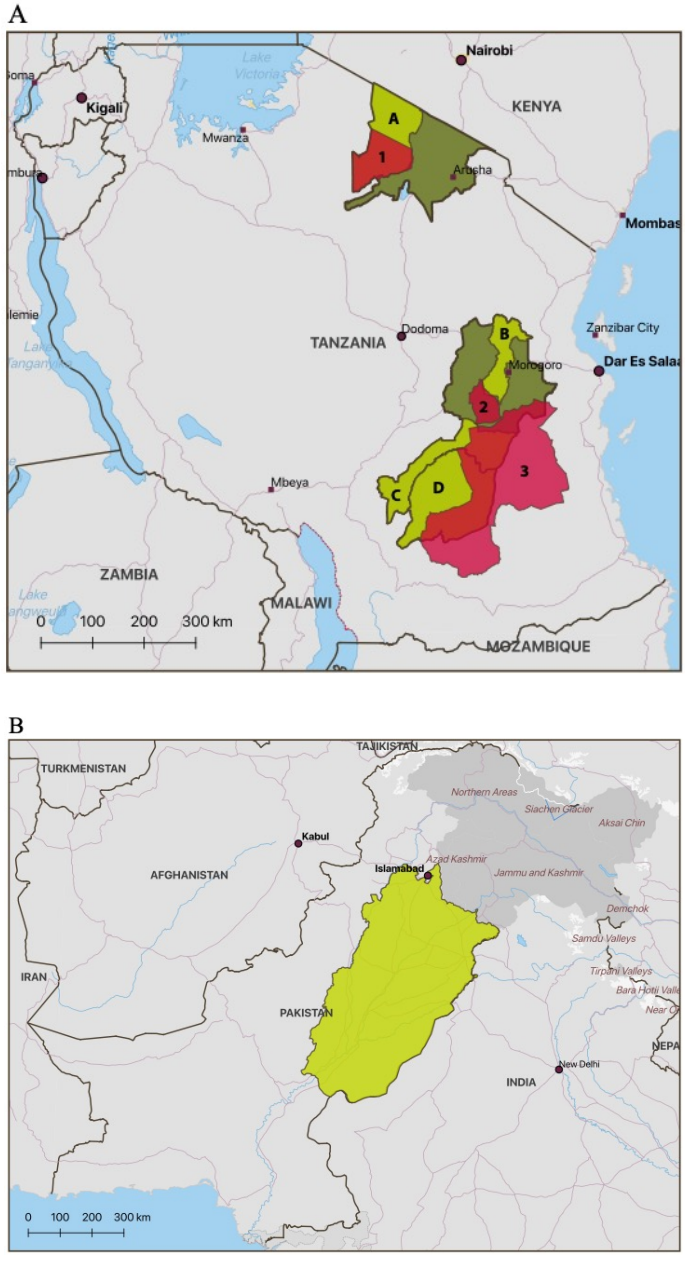

Figure 9. Study areas in Tanzania (A) and Pakistan (B). A) Dark green areas indicate the northern Arusha region and the central-southern Morogoro region. In green are districts where samples were collected: A = Ngorongoro, B = Mvomero, C = Kilombero, D = Ulanga. Red areas indicate parks or reserves: 1 = Ngorongoro Conservation Area, 2 = Mikumi National Park, 3 = Selous Game Reserve. B) Green area indicates sample area, Punjab province. Figure 9A is previously published in Torsson *et al*. (2017), with slightly modifications.

In Paper I, blood samples from animals belonging to indigenous groups and traditional farmers (who keep animals tethered or in enclosures close to the home and/or within a village) were sampled to study the risk factors for contracting PPR or one of its differential diagnoses. In addition to blood samples, nasal swabs from each animal were collected and analyzed for presence of PPRV. The samples were collected in Tanzania during September-October 2014 and June-July 2015 (Figure 10). The blood samples from 2015 were additionally stored on filter papers and used in Paper II, which also includes blood samples collected in Pakistan. Pakistan is divided into seven administrative units, and the samples were collected in the central Punjab province as part of a separate study on PPR performed by our collaborators in Pakistan (Figure 9B).

Figure 10. Herd of sheep and goats in the Ngorongoro Conservation Area during sample collection in 2014. Photo: Emeli Torsson.

3.2 Serology (Paper I and II)

All serological analyses were done using commercially available enzyme-linked immunosorbent assays (ELISAs): *ID screen PPR competition ELISA* (ID. Vet, France), *IDEXX CCPP Ab test* (IDEXX, The Netherlands), *ID Screen FMD NSP competition* (ID. Vet, France), *Bluetongue Virus (BTV) Antibody Test Kit* (IDEXX, The Netherlands), and *BVDV p80 Ab Test Kit* (IDEXX, The Netherlands). All kits were used and interpreted according to the manufacturers' instructions as described in Paper I.

For Paper II, the serum samples were analyzed in the same way as in Paper I. For whole blood stored on filter papers, the saturated and dried filter papers were cut into 10-15 mm^2 pieces, added to 150 µl dilution buffer and incubated

for 1 hour in room temperature. After that, 50 µl of the solution was used in the PPR cELISA analysis that was otherwise performed according to the manufacturer's instructions.

3.2.1 Filter papers

Two kinds of filter papers were used in Paper II, the Nobuto Filter Strips (NFS) (Advantec, US) and a chromatography paper (CP) (Sartorius AG, Germany). The NFS was developed for the purpose of sample transport and was expected to perform well; it was used both in Tanzania and Pakistan. The reason to include the CP in the study in Tanzania was its much lower price, approximately 30 times less than the NFS, which makes it interesting for use in budget constrained areas.

3.3 Screening for PPRV in Tanzania

During the sample collection in Tanzania in 2014 and 2015, nasal swabs were collected in addition to blood samples from the animals. Nucleic acid (viral RNA) was extracted from 120 µl swab fluid using the GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions, with the addition of a Proteinase K step. The samples were then analyzed at the Sokoine University of Agriculture, Tanzania, using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) according to (Kwiatek *et al.*, 2010).

3.4 Full genome sequencing (Paper III)

3.4.1 Samples

To develop the protocol described in Paper III, a selection of samples were used. First the protocol was tested and optimized using PPRV (Nigeria 75/1) grown in cell cultures, kindly provided by Dr. Siamak Zohari (National Veterinary Institute, Uppsala, Sweden). When the protocol was finalized, it was tested on PPRV isolates from all four currently known lineages, cultured on CV-1 cells (African green monkey kidney cells) to evaluate the method for use in outbreaks of all lineages of PPRV: Lineage I: Senegal 1969, lineage II: Benin 1969 and Benin 2011, lineage III: Kenya 2011, and lineage IV: Turkey 2012 (samples kindly provided by Dr. William G. Dundon, International Atomic Energy Agency, Vienna, Austria). As a final step, the protocol was tested on field

samples from Tanzania, collected by Tebogo Kgotlele and Professor Gerald Misinzo (Sokoine University of Agriculture, Morogoro, Tanzania). This was to assess the method on field samples that was not transported or stored under optimal conditions, thus reflecting the use in an outbreak situation in remote areas with poor infrastructure.

3.4.2 Primer design and polymerase chain reaction (PCR)

Two sets of multiplex full genome primers were designed using the Primal Scheme (http://primal.zibraproject.org). The two sets of primers amplified differently sized amplicons: 800 bp and 600 bp. This was done to evaluate the best primer set-up for optimal coverage of the genome. The RNA was then converted to cDNA and amplified according to (Quick *et al.*, 2017), a protocol developed for full genome sequencing of Zika virus (ZIKV).

3.4.3 Full genome sequencing and bioinformatic analysis

Following first strand synthesis and PCR amplification, the samples were purified using a magnetic bead purification kit and the sequencing library was prepared using an altered protocol for the SQK-LSK109 Ligation Sequencing Kit (Oxford Nanopore, UK) (Hu & Schwessinger, 2018; Quick *et al.*, 2017). The alterations in the protocol included reducing volumes to minimize costs and substituting some reagents for more cost-efficient versions, while still providing the same quality and desired results.

The full wet lab protocol, from cDNA synthesis to loading of sample to minION sequencer is available from DOI: dx.doi.org/10.17504/protocols.io.pnxdmfn.

The raw reads were base-called and demultiplexed. Reads were then aligned to a reference genome, and a consensus genome was extracted using the UGENE software (Okonechnikov *et al.*, 2012). Full bioinformatic methodology is available at: github.com/Ackia/Field_Seq.

The consensus sequences were then used to perform phylogenetic analysis using two approaches. The commonly used, partial 255 nts sequence from the nucleoprotein gene, was extracted from the consensus sequences. These sequences were aligned and a tree constructed using Mrbayes (Bayesian model). The whole genome consensus sequences were equally aligned and a phylogenetic tree constructed in the same way. The two trees were then visualized and compared using a tanglegram. The whole genome sequence from four isolates previously published and available on the NCBI GenBank, was

compared to the sequences produced using this protocol in a separate tanglegram.

3.5 Cell cultures (Paper IV)

In Paper IV, HEK293T (human embryonic kidney cells) and A549 (human alveolar epithelial carcinoma cells) cells, purchased from the American Type Cell Culture Collection (ATCC), were used to study the modulation of the interferon response by PPRV C and V proteins. These cells were selected for their reliable growth and use in several similar studies. In addition to HEK293T and A549 cells, the original aim was to perform the experiments in cells deriving from the natural hosts of PPRV: sheep and/or goats. For this purpose, we kindly received four cell lines from the Friedrich-Loeffler-Institut (FLI), Germany. Two cell lines were derived from sheep—SFN-R (kidney) and SFT-R (kidney)— and two cell lines were derived from goat: ZN-R (kidney) and ZZ-R (fetal tongue).

3.6 Luciferase reporter assay system (Paper IV)

3.6.1 Expression and reporter plasmids

Eight plasmids expressing the C or V protein from four different isolates of PPRV were designed and ordered from GenScript Biotech Corp (Piscataway, USA) (Table 1). The four isolates are available on the NCBI GenBank as full genomes. They were selected because all four originated from the African continent and had different characteristics. For example, one was a highly virulent field strain and another was a vaccine strain often used in research. In addition to the PPRV expression plasmids, three plasmids containing the C protein of other morbilliviruses were designed and produced: canine distemper virus (KF914669), feline morbillivirus (KR014147), and measles virus (DQ227319). All proteins were expressed with a FLAG-tag for expression monitoring.

Table 1. *The PPRV isolates from which the C and V genes used in Paper IV originated.*

Lineage	Country	Year	Accession no.	Comment
I	Côte d'Ivoire	1989	EU267273	Highly virulent field isolate
II	Nigeria	1975	KY628761	Vaccine strain
III	Uganda	2012	KJ867543	Field isolate
IV	Ethiopia	2010	KJ867541	Field isolate

To study both the type I and type II interferon (IFN) pathways, reporter plasmids were selected for both signaling pathways (Table 2). To study where in the more complex type I IFN signaling pathway the inhibition occurs, two reporter plasmids at different steps in the pathway were included (Figure 3).

Table 2. *Reporter plasmids used in Paper IV.*

Reporter plasmid	Transcription factor	Signaling pathway	Stimulus	Source
p-125Luc	IFNß promotor	IFN type I	dsRNA	Prof. Takashi Fujita[a]
pISRE-Luc	ISRE	IFN type I	dsRNA	Agilent
GL4[luc2P/GAS-RE/Hygro]	GAS	IFN type II	IFNγ	Promega
pGL4.75[hRluc/CMV][b]	N/A	N/A	N/A	Promega

[a] Graduate School of Biostudies, Kyoto University
[b] Internal control plasmid containing renilla luciferase

3.6.2 Transfections

All transfections were performed in 24-well plates, 24 hours after seeding and at a confluency of around 70%. Transfections were done using TransIT-LT1 (Mirus Bio, Madison, USA) according to the manufacturer's instructions. The concentration of reporter plasmid and internal control plasmid were based on preliminary experiments validating the performance and concentration ratio in the selected cell lines. During this validation, the concentration of stimulus (dsRNA or IFNγ) was also optimized. In the final protocol, cells were transfected with a combination of either three or four plasmids: a) 2 ng/ml of pGL4.75[hRluc/CMV] as an internal transfection control, b) luciferase reporter plasmid at a concentration of 200 ng/ml for the pISRE-Luc and p-125luc, or 500 ng/ml for the pGL4[luc2P/GAS-RE/Hygro], and c) 200 ng/ml expression plasmid (C, V, or a combination of the two). Equal concentrations of both expression plasmids were used when C and V were co-transfected. Twenty-four hours after transfection, cells were treated with either 1 µg/ml poly I:C, or 1000 IU/ml human IFNγ, depending on which signaling pathway was studied. As a control in each experiment, cells were transfected with an empty backbone pcDNA3.1(+) and treated as described above. All transfections in HEK293T cells were done in technical and biological triplicates, and transfections in A549 cells were done in technical triplicates and biological duplicates.

The protocol was also tested on the cell lines originating from sheep and goat. All four cell lines were found to be extremely difficult to transfect with this protocol. Other transfection reagents (e.g. Lipofectamine 2000 and 3000, and TransIT X2 and 2020) or reverse transfection did not sufficiently improve the transfection efficiency. Electroporation was also evaluated; however, the sheep

and goat cell lines were not adequately fast-growing, and not a high enough proportion of cells survived the electroporation to consider this method viable.

3.6.3 Luciferase assay

Luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and Tecan Infinite M1000 plate reader. The reagents (Luciferase Assay Reagent II and Stop&Glo Reagent) in the Dual-Luciferase Reporter Assay System were diluted 10x in Milli-Q water; otherwise the assay was performed according to the manufacturer's instructions. This dilution of the reagents does not affect the results of the analysis, but only dilutes the luciferase value. The luciferase activities are normalized by dividing the luciferase value from the reporter plasmid by the luciferase value from the internal control, so this ratio is not affected by the dilution of the reagents. The mean value of the replicates was used for calculations and expressed as relative light units (RLUs); and presented as a fold change compared to a control plasmid. Activation of the reporter plasmids was calculated by comparing stimulated cells with non-stimulated ones, and the effect of the viral proteins was compared to that with the control cells.

3.7 Western blotting (Paper IV)

Western blotting was performed to analyze the expression levels of the PPRV C and V proteins. Transfections for this purpose were performed in parallel to transfections for luciferase assays using a concentration of 200 ng/ml, and cells were lysed 48h after transfection. Western blotting was also performed to study the phosphorylation of the STAT1 protein. Due to the need to add a protease inhibitor during cell lysis, these transfections were done separately from the luciferase assays. Expression plasmids for PPRV C or V protein were transfected, as previously described, and stimulated with 1000 IU/ml IFNγ 24h after transfection, then lysed 2h, 6h, or 24h after stimulation. The lysates were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked (using a blocking buffer containing milk powder) at room temperature for at least one hour. The membranes were then incubated overnight with continuous agitation with primary antibodies raised in rabbits targeting the FLAG-Tag (1 µg/ml) for expression levels of viral proteins, unphosphorylated STAT1 (0.5 µg/ml), or phosphorylated STAT1 (1:3000, anti-phospho-STAT1-PTYR701). After washing with washing buffer (TBS, Tween 0.1%), membranes were incubated with secondary, peroxidase-conjugated, anti-rabbit IgG

antibodies (1:10 000) for one hour with continuous agitation. After developing and visualizing the membrane, it was stripped from antibodies, washed thoroughly, and blocked again for at least three hours before re-probing with a primary antibody produced in rabbit against human β–actin for loading control (1:1000, 4°C, overnight).

3.8 Statistical analyses (Papers I, II and IV)

The statistical analyses in Papers I-II and IV were performed using the statistical software *R* (R Core Team, 2015). All analyses were done in collaboration with the Statistics@SLU support team.

In Paper I, possible risk factors for a positive result in the serological analysis were evaluated both at individual and herd levels. On the individual level, the factors included were: sex, species (sheep or goat), age group, and for PPR and CCPP, vaccination (as there is no possibility to differentiate between naturally infected and vaccinated animals). These factors were first analyzed for all five pathogens separately with an univariable analysis (Chi-squared), and factors with p-value < 0.2 were used in the generalized linear mixed-effect model. This was done to minimize the number of factors used in the multivariate analysis and to avoid possibly confounding factors. The factors included in the analysis on herd level varied between the samples collected in 2014 and 2015, due to an updated and improved questionnaire for 2015. For the samples collected in 2014, the factors *district* and *interaction with wildlife* were included, and for samples collected in 2015, the factors *interaction with other domestic herds* and *introduction of new animals (in the last 12 months)* were added.

In Paper II, the performance of serum samples, whole blood stored on Nobuto Filter Strips (NFS) and whole blood stored on chromatography paper (CP) in the *ID screen PPR competition ELISA* was compared. The results from the serum samples were used as the reference or gold standard. The manufacturer's instructions specify that samples with a competition percentage < 50% are considered positive, those > 60% are considered negative, and samples that fall between 50-60% are a doubtful result and is suggested to be re-analyzed. In the statistical analysis, these doubtful results were considered as negative.

The agreement between serum samples and NFS or CP samples was analyzed with several statistical methods. Bland-Altman plots were used to visualize the differences. The dots in the Bland-Altman plot represent the difference between the measurements (serum vs. the filter paper of each individual sample) on the y-axis and the average of the measurements on the x-axis (Bland and Altman, 1999). In other words, the difference of the measurements is plotted against the mean of the measurements. The colored line in the plot represents the mean

difference for all samples. If the two tests were to give the same results, all the dots would be centered around 0 on the y-axis (marked by the black horizontal line) and the colored line would overlap with the black line. Receiver operating characteristic curves (ROC-curve) and Precision-Recall curves (PRC) further compared the different methods of sample transport and storage, and the ROC-curve was additionally used to determine adjusted cut-offs for the NFS and CP samples. Cohen's Kappa for agreement between serum samples and NFS/CP samples was calculated on both unadjusted and adjusted cut-offs for NFS/CP to compare the cut-offs.

Paper IV looked at the ability of PPRV C and V proteins from four different isolates, to modulate the host's interferon response was studied. Possible differences in inhibitory abilities between lineages, comparing the four C or V proteins separately, were calculated by a one-way analysis of variance (ANOVA). Confidence intervals (95%) were calculated using the standard error of the mean using the biological replicates.

4 Results and discussion

4.1 Seroprevalence of PPR and its differential diagnoses in Tanzania (Papers I and II)

For paper I, 476 animals were sampled in 2014 and 481 animals were sampled in 2015, giving a total of 957 animals. Samples were collected in four different districts, with the Ulanga district included both years (no herds were visited both years). Samples were tested for antibodies to PPRV and selected differential diagnoses to PPR: i) contagious caprine pleuropneumonia (CCPP) caused by the bacteria *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp); ii) foot-and-mouth disease virus (FMDV); iii) bluetongue virus (BTV); and iv) bovine viral diarrhea virus (BVDV). Antibodies to all pathogens were found in both 2014 and 2015 (Table 3). As the two sample collections were not performed in the same area, comparisons between the two should be done cautiously. For PPRV, the observed decrease from 49.3% to 10.0% is most likely because we sampled animals of all ages in 2014, but in 2015 we sampled animals 3-12 months of age, to better access the current circulation of PPRV.

Table 3. *Calculated true prevalence based on positive proportion and the sensitivity and specificity of the analysis method. Confidence intervals at 95% are presented in parentheses.*

Disease	2014 (%)	2015 (%)
PPR	49.3 (44.5;54.0)	10.0 (7.1;12.8)
CCPP	14.6 (11.0;19.0)*	18.8 (14.9;23.5)*
FMD	39.0 (33.8;44.3)	14.6 (10.9;17.2)
BT	98.9 (90.1;100)	74.5 (68.4;80.6)
BVD	3.9 (0;8.0)	1.7 (0.1;3.4

* = It was not possible to calculate the true prevalence because there was no information on the sensitivity of the ELISA used. Showed are the positive proportion.

In 2013, a vaccination campaign was carried out in the Mvomero and Ulanga districts, and even though we excluded animals from the analysis if their owners had reported vaccinations against PPRV, the positive proportion was not reduced (see Table 1, Paper I). A natural PPRV infection or vaccination gives rise to a life-long immunity, so by including older animals in 2014, a higher prevalence was expected.

The highest seroprevalence, for both years, was for BTV. BTV is spread not by direct or close contact as the other included pathogens, but by *Culicoides* biting midges (Maclachlan, 2011). This effective way of spread, in combination with the mild or even unapparent disease in small ruminants, could be a reason for the high seroprevalence.

4.2 Risk factors for PPR and its differential diagnoses in Tanzania (Paper I)

The serological results, from each year, were used to calculate possible risk factors for animals to be seropositive to PPRV and the selected differential diagnoses. The risk factors were calculated first with an univariable analysis on individual level, and then with a multivariate analysis on individual and herd level. In the multivariate analysis on individual level, female animals were found to be more likely to be seropositive than the male animals (Tables 4 and 5).

Table 4. *Summary of multivariate analysis for risk factors in samples collected in Tanzania in 2014. Included values are p < 0.05. BVD was not included in the multivariate analysis due to a low positive percentage. Confidence intervals at 95% are presented in parentheses Full tables in Paper I, Table 3-6.*

	Goat	Female	Age 1-2 y	Age > 2 y	Interaction with wildlife
PPR	#	2.78 (1.5; 5.4)	**	**	*
CCPP	81.9 (17.4; 726)	4.5 (1.2; 19.0)	*	5.2 (1.5; 21.1)	*
FMD	*	3.8 (1.6; 9.5)	*	8.7 (2.8; 30.5)	*
BT	#	7.5 (1.3; 63.9)	*	183 (15.2; 23216)	*

= not included in multivariate analysis due to p >0.2 in univariable analysis

* = p > 0.05

** = interaction with vaccination was found in multivariate analysis

Table 5. *Summary of multivariate analysis for risk factors in samples collected in Tanzania in 2015. Included values are p < 0.05. BVD was not included in the multivariate analysis due to a low positive percentage. Confidence intervals at 95% are presented in parentheses. Full tables in Paper I, Table 3-6.*

	Goat	Female	Age 1-2 y	Age > 2 y	Interaction with wildlife	Interaction with domestic animals
PPR	#	6.2 (2.0; 28.2)	3.5 (1.1; 12.4)	17.6 (3.8; 113)	*	*
CCPP	9.2 (1.7; 84.0)	#	#	#	0.008 (0; 0.2)	0.05 (0; 0.5)
FMD	4.2 (1.5; 13.0)	4.7 (1.9; 13.1)	*	9.1 (3.1; 30.8)	*	20.7 (3.1; 262)
BT	2.32 (1.1; 4.9)	*	3.0 (1.5; 6.3)	18.4 (6.6; 61.4)	*	3.9 (1.6; 10.5)

= not included in multivariate analysis due to p >0.2 in univariable analysis
* = p > 0.05

A similar odds ratio for female animals was also found for FMD. This risk factor has been previously described for PPR (Aziz-Ul-Rahman. *et al.*, 2016; Kihu *et al.*, 2015; Kivaria *et al.*, 2013), however, other studies have found the opposite, with male animals at higher risk for seropositivity (Mbyuzi *et al.*, 2014; Mahajan *et al.*, 2012; Sarker & Islam, 2011). Precisely why this is so is not yet known. Female animals are in general kept longer due to their reproductive value, and are therefore older, which was found to be another risk factor. However, we did not find an interaction between the variables sex and age in our multivariate analysis, so this cannot be the full explanation. It might be that females are selected for vaccination to a greater extent if only some of the herd is vaccinated and that owners are unable to recollect exactly which animals were vaccinated and which ones were not.

As previously mentioned, increasing age was a second risk factor in our dataset. We divided the animals into three age groups: < 1 year, 1-2 years, and > 2 years. Increasing age was found to be a risk factor in the multivariate analysis for PPR (2015), CCPP (2014), FMD (2014 and 2015), and BT (2014 and 2015) (Tables 4 and 5). All these pathogens are endemic in Tanzania and most animals likely encounter them at a young age. For FMD, age is a well-known risk factor for seropositivity; a higher age gives a longer time to encounter the virus, possibly several times, and the increased case fatality rate in young individuals leaves only the older ones to be sampled (Megersa *et al.*, 2009; Rufael *et al.*, 2008).

CCPP is a disease with clinical signs seen predominantly in goats. Sheep can show clinical signs, but very rarely (OIE, 2014). Not surprisingly, goats in our

data analysis were at a higher risk for a seropositive CCPP result (Table 4 and 5). In 2015, goats were also at higher risk for being seropositive for FMD and BT (Table 5). In 2015, 73% of the samples we collected and analyzed were from goats, which might have biased these results.

Multivariate analysis was additionally performed on herd level. The districts where the samples were collected, as well as interactions with either wildlife or other domestic herds were included as fixed effects. In 2014, animals in the district Mvomero were at higher risk for a positive result for PPR. In this area, vaccinations had been performed the previous year, and even though we removed animals with reported vaccinations, this was only based on the information from owners, which can be incorrect for many reasons. Interactions with wildlife, as reported by the owners, were not in any of the pathogens identified as a risk factor (Table 4 and 5).

The epidemiological role of wild ruminants is still unclear for PPR. For a long time, clinical signs of PPR have mainly been reported in wild ruminants in Asia (Aziz-Ul-Rahman *et al.*, 2019). Recently, PPR was reported in a Dorcas gazelle in Sudan, which is the first report of clinical signs in wild ruminants in sub-Saharan Africa (Asil *et al.*, 2019). Interestingly, as pointed out by Rahman *et al*, most PPRV samples collected from wildlife belongs to lineage IV, which also is true for the gazelle in Sudan (Asil *et al.*, 2019). As lineage IV of PPRV now is spreading to more of the African continent, it is possible that reports of PPR in wild ruminants will increase. A comparative study of the currently available PPRV genome sequences isolated from wild ruminants (37 isolates) does not reveal any common mutations in the genomes explaining the change of host. The PPRV isolates grouped together with isolates from domestic animals collected in the same geographic region, suggesting a rather wide host range for the virus (Aziz-Ul-Rahman *et al.*, 2019). It is possible that the increased spread of lineage IV is due to a broader host range, driven by an as yet unknown mechanism. To elucidate this possible mechanism, more PPRV genome sequences from wild hosts of PPRV are needed.

For CCPP and FMD, the interaction with other domestic animals was statistically significant in the multivariate analysis. For CCPP, the interaction with other domestic herds, as well as interaction with wildlife, was associated with an odds ratio < 1 (Table 5). The cELISA used to detect antibodies to Mccp is very specific for this mycoplasma bacteria (Peyraud *et al.*, 2014). It is possible for mycoplasma subspecies to induce cross-protection to other subspecies and in that way give a natural protection for one another (Kanyi Kibe & Smith, 1984; Smith & Oliphant, 1981). If, by interaction with other species, the small ruminants in this study were exposed to other mycoplasma subspecies, this could induce a protection against Mccp.

For FMD, the interaction with other domestic herds, but not wildlife, was a risk factor in our data. Of the wild ruminants in Africa, only the African buffalo is an important reservoir for FMDV (Thomson *et al.*, 2003). Small ruminants are highly susceptible to infection, but not efficient at maintaining an infection within the population (Kitching & Hughes, 2002). FMDV is endemic within the cattle population of Tanzania (Picado *et al.*, 2011) and small ruminant herds often interact with cattle, giving an opportunity for virus transmission.

4.3 Filter paper as a transport medium for serological diagnosis (Paper II)

During the collection of serum samples in 2014 (Paper I), the maintenance of a cold chain for the collected serum samples was sometimes a difficult task. For example, on days when we did not return to a laboratory facility, we were forced to store the samples as best we could, in hotel refrigerators/freezers or similar. For this reason, we wanted to evaluate an alternative transport medium. Therefore, during the sample collection in 2015, we sampled animals in triplicate: serum samples in tubes without additives using a vacutainer system; whole blood on Nobuto filter strips (NFS); and whole blood on chromatography paper (CP). Serum samples were transported as cool as possible, while filter papers were left to air dry away from direct sunlight and then transported at ambient temperature. We also collaborated with colleagues in Pakistan, and were able to include samples from a region with a different epidemiological setting.

The use of NFS or CP as a transport medium for serological diagnosis, requires validation and optimization of the protocol (Hopkins *et al.*, 1998). We chose to validate the use of filter papers in the cELISA, developed by one of the OIE PPR reference laboratories, CIRAD (Montpellier, France) (Libeau *et al.*, 1995) and now commercially available from ID. vet (Grabels, France).

In Tanzania, samples from 196 animals were transported by the three methods above. In Pakistan, samples from 60 animals were transported as serum or on NFS. The results from the filter papers were compared to the results from the serum samples, which was the reference material. In Tanzania, 10.7% (21 animals) were seropositive for PPRV when analysis was run on serum samples. Pakistan, 80% (48 animals) were seropositive when analysis was run on serum samples. Initial analysis of samples stored on filter papers, diagnosed 5.6% of the samples in Tanzania (NFS or CP) and 66.7% of the samples in Pakistan (NFS) as positive using the manufacturer's cut-off of a positive sample having a competition percentage < 50% (Table 6 and 7).

Table 6. *Results from the serological assay (cELISA) from samples collected in Tanzania. Shown are results from analysis run on serum, Nobuto filter strips (NFS), and chromatography paper (CP). For NFS and CP, results both from manufacturer's cut-off at < 50% and adjusted cut-offs are included. Cohen's kappa describes the agreement between results from serum and the filter papers (with 95% confidence intervals included in parentheses).*

Medium	Serum	NFS	NFS	CP	CP
Cut-off	< 50%	< 50%	< 84.6%	< 50%	< 84.3%
Positive	21	11	25	11	20
Negative	175	185	171	185	176
Total	196	196	196	196	196
Cohen's kappa		0.66 (0.47-0.85)	0.85 (0.74-0.97)	0.66 (0.47-0.85)	0.92 (0.83-1)

Table 7. *Results from the serological assay (cELISA) from samples collected in Pakistan. Shown are results from analysis run on serum and Nobuto filter strips (NFS). For NFS, results both from manufacturer's cut-off at < 50% and adjusted cut-off are included. Cohen's kappa describes the agreement between results from serum and the filter papers (with 95% confidence intervals included in parentheses).*

Medium	Serum	NFS	NFS
Cut-off		< 50%	< 69.0%
Positive	48	40	47
Negative	12	20	13
Total	60	60	60
Cohen's kappa		0.67 (0.46-0.87)	0.95 (0.85-1)

The agreement between analyses performed on serum samples and those on either NFS or CP were compared using several different statistical methods. Using the manufacturer's cut-off for both methods, the initial agreement was calculated using Cohen's kappa. Before adjusting the cut-off, the result of the Cohen's kappa was 0.66-0.67, which is considered as a moderate to substantial agreement (McHugh, 2012). A receiver operating characteristics (ROC) curve and a Precision-Recall curve (PRC) were used to analyze the agreement and find an adjusted cut-off for samples transported on filter paper (Figures 2 and 3, Paper II). For both methods, the results from serum samples were used as the reference results. The ROC curve describes the agreement between the two methods, where an area under the curve (AUC) of 1 indicates a perfect match. For the NFS in Tanzania, the AUC was 0.988, for CP in Tanzania 0.983, and for the NFS in Pakistan 0.996, i.e., an almost perfect fit.

However, for unevenly distributed results, such as ours (with a higher proportion of negative results in Tanzania and a higher proportion of positive

results in Pakistan), an ROC curve can be misleading and indicate a better fit than what is actually the case. For this reason, a PRC was used. The PRC plots the precision, equal to the positive predictive value (PPV), for all values of recall, equal to the sensitivity of the test. The PPV is a value that describes the probability of an animal with a positive result being a true positive. This is calculated by the true prevalence divided by the positive results from the test, with a perfect PPV being 1.

The PPV is, however, influenced by a number of variables, such as the true prevalence and the sensitivity of the test. The sensitivity describes the proportion of true positive results that are correctly identified as positive (in this case having antibodies to PPRV). Once again, a perfect test would have an AUC of 1 in a PRC. For the NFS in Tanzania, the PRC gave an AUC of 0.938, for the CP, 0.925 and for the NFS in Pakistan the 0.999. These indicate an excellent performance of the filter papers (Saito & Rehmsmeier, 2015). Adjusted cut-offs for the filter papers were calculated from the ROC curve. Cohen's kappa was re-calculated for the results using these new cut-offs, and was improved to 0.85 for NFS in Tanzania, 0.92 for CP in Tanzania, and 0.95 for NFS in Pakistan, which is considered a near perfect agreement (McHugh, 2012) (Tables 6 and 7). The agreement of the samples on filter papers and the serum samples was additionally visualized using Bland-Altman plots (Figure 11) (Bland & Altman, 1999). The Bland-Altman plots show a systemic difference between the methods equal over the test result interval, with a majority of samples' results falling between two standard deviations from the mean bias. This indicates that the difference in the test results between the two methods is equal over the full test result interval.

The use of filter papers to transport samples for either serological or molecular diagnosis has been suggested previously, for example: PCR detection of viruses (Michaud *et al.*, 2007), serological and molecular diagnosis of Chikungunya virus (Matheus *et al.*, 2015), serological and molecular diagnosis of African Swine fever virus (Randriamparany *et al.*, 2016), and serological diagnosis of dengue virus (Tran *et al.*, 2006; Ruangturakit *et al.*, 1994). For PPRV, filter paper has been proposed as a tool for long-term storage before detection and genotyping using a PCR method (Michaud *et al.*, 2007).

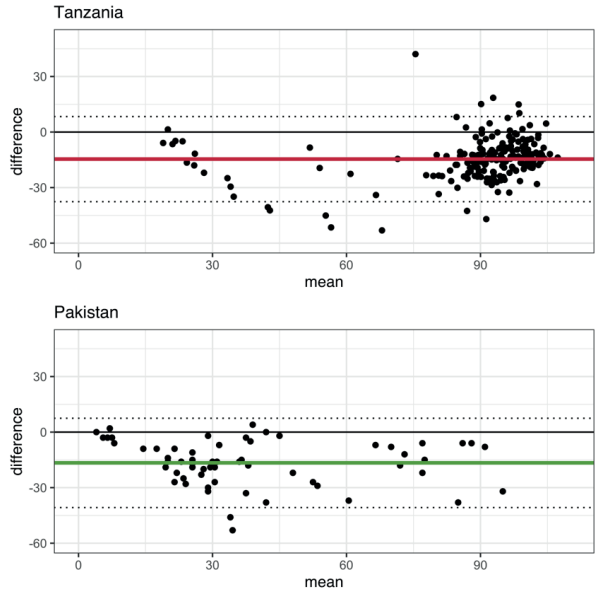

Figure 11. Bland-Altman plots for serum samples and whole blood transported on Nobuto filter strips (NFS) in Tanzania (top) and Pakistan (bottom). A Bland-Altman plot visualizes the differences between two measurements on the y-axis and the average of these samples on the x-axis. If the two tests were to give the exact same results, all the dots would be centered around 0 on the y-axis (marked by the black horizontal line) and the colored line would overlap with the black line. The colored line represents the mean difference (Tanzania: -14.65, Pakistan: -16.63), the dotted lines represents the highest and lowest limit of agreement (2 *sd* from the mean bias). This figure is slightly modified from the one published in Torsson *et al.* (2019).

Here we optimized the use of filter paper in serological diagnosis for PPRV. This was done in samples both from Tanzania and from Pakistan to study two different epidemiological settings. In Tanzania, the samples gave a positive proportion of 10%, whereas in Pakistan, the positive proportion was 80%. This highlights the need to adjust the cut-off depending on where and why the analysis is being performed. In an area with a high prevalence, the sensitivity of the test needs to be higher, to avoid false negatives. Our protocol achieves this by lowering the cut-off value for a positive result, as seen in the samples from Pakistan (< 69% vs. < 84%). During the eradication of PPRV, the aim of the analysis is to detect all possibly infected animals and as early as possible. To do this, a higher proportion of false positives results needs to be accepted, and a higher cut-off value is suggested. When the prevalence of PPRV decreases during the control and eradication program, so will the PPV of the test, as the PPV is influenced by the true prevalence of the disease. This illustrates, that for tests with a continuous result scale such as the cELISA, the cut-off value for a

positive result is not universal for all epidemiological settings. Rather, it needs to be adjusted for different settings and conditions (Habibzadeh *et al.*, 2016).

4.4 qRT-PCR screening

During the collection of serum samples in Tanzania in 2014 and 2015, we also collected nasal swabs for PPRV nucleic acid detection. In this way, we aimed to discover and isolate a collection of PPRV field isolates to be used in further studies. We were, however, unable to find any animals positive for PPRV using qRT-PCR. The analysis was judged appropriate for the screening of nasal swab samples and to be performing well. We believe the reason for our lack of positive samples was the study design for sample collection. As described in section 1.5.2., the window for sample collection to detect nucleic acid is relatively short following debut of clinical signs (Truong *et al.*, 2014). Tanzania does not have a system in place for immediate notification of outbreaks of infectious diseases among animals, leading to difficulty in locating and reaching outbreaks of PPR in time for sample collection.

4.5 Field-adapted full genome sequencing (Paper III)

Keeping a cold chain during sample transport for serological diagnosis is important and can be difficult. The cold chain during transport of samples is even more important for viral nucleic acid detection, because viral RNA is very heat sensitive and can degrades quickly when not stored properly. Precise molecular epidemiology to trace the source of an outbreak is highly desirable for the control and eradication program. Long, time-consuming and uncertain transports to well-equipped laboratories are counterproductive for this purpose. If the sample and outbreak can be analyzed as close and as quickly as possible, the risk of further spread is reduced. For this reason, we developed a protocol using the miniPCR (Amplyus) and the Oxford Nanopore minION sequencer.

4.5.1 Protocol performance

The first trial of the protocol was done using a good quality, cell-culture grown, Nigeria 75/1 virus isolate. This sample was tested using three versions of the primers: the 800-bp primer set, the 600-bp primer set, and the 800-bp primer set in a combination to produce 2400-bp amplicons. We found that the 800-bp amplicons gave the best and most even average coverage over the full genome, while the 600-bp failed mainly in producing good coverage at the ends of the genome. For this reason we continued working with the 800-bp primer set for

all samples. The Nigeria 75/1 sample was run in duplicate to evaluate the reproducibility. The duplicates produced 709,440 and 636,171 reads that mapped against PPRV, with an average coverage of 4,454 and 4,749 reads. This was considered as an equal performance of the duplicates, which were henceforth presented as a mean of the two. These reads gave a genome coverage of 98.4% (above 50x coverage).

The two field isolates from Tanzania performed equally well in the protocol, producing 771,053 and 1,197,778 reads mapping against PPRV respectively and genome coverage of 91.1% and 93.5% (above 50x coverage) (Paper III, Table 2). The five samples cultured on CV-1 cells did not produce as distinct bands on the gel electrophoresis done to verify the PCR amplification (Paper III, Figure 1). Sequencing of these samples produced a genome coverage (above 50x) between 49.6% and 85.0%. Instead, most reads produced in these samples instead mapped against the human genome, which was suspected to have originated from the cell line used to culture the viruses. The viral RNA in the samples was believed to be degraded and in low concentration. This is not a perfect result; however, it reveals that the method performs well, even with degraded and low concentrations of RNA, which may be the case in the field.

Good quality samples produced a 98-99% genome coverage with an average coverage of around 4500 reads, while lower quality samples produced, on average, 69% genome coverage with an average coverage around 800 reads (range 416-2312). An attempt to define standards for sequencing of viral genomes suggests five standards, based on the percentage of genome recovered and the coverage (Ladner *et al.*, 2014). For molecular epidemiology, the recommended standard is the "Complete coding", which means 90-99% of the genome is sequenced with no gaps in the genome, all open reading frames (ORFs) are complete, and the coverage is 100x (Ladner *et al.*, 2014). The method suggested here more than sufficiently meets these requirements when using good quality samples.

The protocol does not require an expert laboratory- or sequencing technician, but a basic understanding of avoiding contamination and handling with laboratory equipment is however needed. We estimate that, assuming previous basic pipetting skills, this protocol can easily be performed following one full run-through auscultation. The protocol takes, from RNA purification to produced sequences, around 22 hours.

This protocol is aimed at a field environment or a less well-equipped laboratory and at production of full genome sequences in a cost sensitive way. A full list of reagents and costs, based on prices indicated on manufacturers' homepages in September 2019, is included in Paper III, Table 3. A full genome is possible to produce for between USD 80 and USD 100 with this protocol.

4.5.2 Sequence analysis

The full genome consensus sequences were used to perform a phylogenetic analysis. Four of the isolates used in the study have previously been sequenced using Sanger sequencing and are published (Adombi *et al.*, 2017; Dundon *et al.*, 2015a; Dundon *et al.*, 2015b). These sequences were used to evaluate the performance of the suggested method, and this was done using a tanglegram (Paper III Figure 2). The isolates grouped in the same way; however, the branches divided slightly differently, with an addition of one node and removal of another node. The compared set of sequences were produced using different methods and would therefore be expected to differ slightly. The suggested protocol did not produce an equally high coverage over the ends of the genome, leading to a decreased quality of the consensus sequences in these parts; this can somewhat affect the phylogenetic analysis (Shrivastava *et al.*, 2018).

The full genomes produced using this protocol were also compared to the commonly used genetic marker, a partial sequence of the nucleoprotein gene extracted from the full genome consensus sequence. This comparison was done to highlight the added benefit of using the full genome compared to the genetic marker. The full genome produces a phylogenetic tree with higher resolution, but does not change the placement of the isolates within the tree, indicating that the nucleoprotein is a good genetic marker (Paper III, Figure 3). The higher resolution produced by the full genomes will improve the possibility to pinpoint the source of infection and also improve the ability to follow the virus evolution. The isolates included in Paper III, Figure 3 are all separated over both geography, time, and genotypes; however, during an on-going outbreak the details of the nodes and branches could give important information about the transmission. If time of sample collection is included in the sample information, a transmission tree reconstruction could further help in tracing the source (Wohl *et al.*, 2016).

4.6 Immunomodulatory abilities of PPRV C and V proteins (Paper IV)

The production of more full-genome sequences, and from a wider variety of hosts, makes it possible to study virus evolution and its adaptation to the host. One characteristic shared by all morbilliviruses is the severe immunosuppression they induce during an infection. Even vaccination against a morbillivirus with an attenuated vaccine isolate leads to immunosuppression. In morbilliviruses, the most important proteins modulating the host immune response are the non-structural C and V proteins. Knowledge of how these viral proteins modulate the immune response could improve the current vaccine, possibly by eliminating the

immunosuppression. The first and most important defense the host has against a virus infection is the interferon response.

4.6.1 Type I interferon

In Paper IV, the C and V proteins of PPRV and how they modulate the host's interferon (IFN) response were studied, both separately and combined. The modulation of the type I IFN response was assessed by measuring the gene activity at two different points in the IFN pathway. Early in the pathway, gene activity was represented by the IFNβ promotor region, and by the interferon-stimulated response element (ISRE) in the later part of it (Figure 3). Included in the study was also possible differences between four isolates, with different characteristics. These were the highly virulent ICV89 isolate and the vaccine strain Nigeria75/1, and two additional isolates to include all the four currently know lineages (I-IV) of PPRV. The experiments were done using expression plasmids carrying the C or V protein gene of the selected isolates.

The C and the V proteins of PPRV are both inhibitors of the type I IFN signaling pathway (Figure 12A and B). The inhibition measured at the very end of the signaling pathway, via the ISRE activity, indicates the full effect that the viral proteins have on the type I IFN signaling pathway. The C proteins of PPRV inhibited the pathway to 32.6-72.0% of the activity compared to the control (100%), and the V protein inhibited it to 27.2-32.9% (Figure 12B). A combination of both proteins did not increase inhibition result in a statistically significant way, nor was there a difference for the two proteins be found when transfected separately.

To study if this inhibition of the type I IFN signaling pathway took place mainly in the early or the later stage (following secretion of type I IFN), an additional reporter plasmid was used to measure the activity at the IFNβ promotor. The C and V proteins were both strong inhibitors, 18.4-55.4% and 10.6-33.5% respectively (Figure 12A). This indicates that most of the PPRV type I IFN modulation is done in the early stages of the pathway. A comparison of the four different C and V proteins to each other showed no statistically significant difference in inhibition. Co-transfection of both proteins did not increase this inhibition in a statistically significant way either.

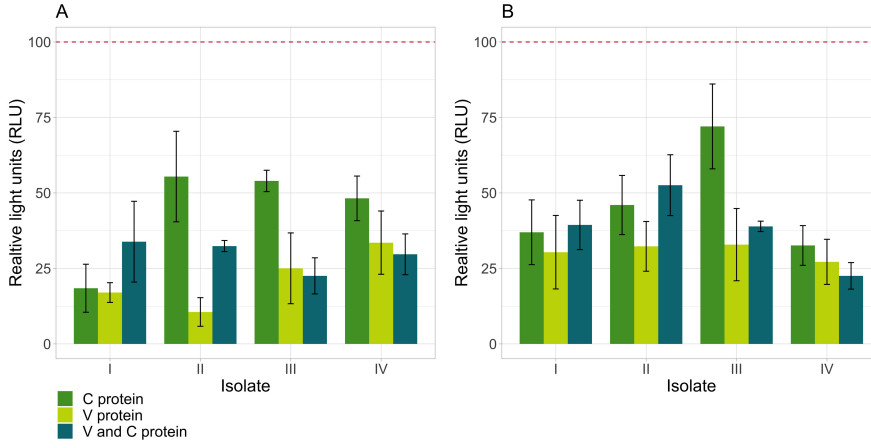

Figure 12. Modulation of the type I IFN signaling pathway by the PPRV V and C proteins, separately and combined, measured as the activity of: A) the IFNβ promotor and B) the interferon-stimulated response element (ISRE). HEK293T cells were transfected with expression plasmids for PPRV V protein, C protein, or both, together with the p125–Luc or ISRE-Luc reporter plasmid to measure the activity of these promotors with viral proteins present. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. No statistically significant difference between lineages was found. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

The modulation of type I IFN by other morbilliviruses, such as CDV, RPV, and MeV, report similar results, with the V protein being a strong inhibitor (Svitek *et al.*, 2014; Chinnakannan *et al.*, 2013; Nakatsu *et al.*, 2008). Fontana *et al.* (2008) studied, as we did, the possible differences between attenuated and wild-type isolates of MeV, but did not find any correlation between the interferon modulatory ability and the source of the virus isolate. This indicates that the attenuation lies elsewhere in the viral genome. For the MeV V protein, the need of a tyrosine residue at amino acid 110 has been linked to the ability to strongly inhibit type I IFN (Fontana *et al.*, 2008; Ohno *et al.*, 2004). Changing this tyrosine residue to a cysteine impairs the ability to interfere with type I IFN (Fontana *et al.*, 2008). Of the four isolates included in Paper IV, isolates from lineages I-III have a tyrosine at position 110, while the isolate from lineage IV (Ethiopia 2010) has a cysteine residue. However, in contrast to MeV, this change in position 110 of PPRV did not lead to a clear change in type I IFN modulation.

For the MeV V protein, another important residue for interferon inhibitory abilities is the cysteine at position 272 (Ohno *et al.*, 2004), and all PPRV isolates used in Paper IV have a cysteine at this position. The 272 cysteine is in the C-terminus of the V protein. Both the N- and the C-termini of the MeV V protein are able to interfere with type I IFN signaling, though the interference is strongest with both termini present, suggesting the two termini participate in

different ways (Caignard *et al.*, 2007). The N-terminus is suggested to bind to Jak1 and STAT1, and the C-terminus to interact with MDA-5 (Caignard *et al.*, 2007).

The type I IFN inhibitory effect of the MeV C protein is described differently in different studies, from no effect on the pathway (Nakatsu *et al.*, 2008) to a strongly inhibitory one (Sparrer *et al.*, 2012; Shaffer *et al.*, 2003). The RPV C protein has additionally been shown to inhibit type I IFN (Boxer *et al.*, 2009; Nanda & Baron, 2006). The exact mechanism for how the C protein of RPV interferes with the type I IFN signaling is not clear; however, a virus with a non-functional C protein is less virulent and its growth is stunted in cell cultures (Bernardo *et al.*, 2017). The MeV C protein has been suggested to act one step downstream of the activation of IRF3, and, thus, possibly interferes with the IFN response within the nucleus (Sparrer *et al.*, 2012). This conclusion is also proposed for the RPV C protein (Boxer *et al.*, 2009). The two steps in the type I IFN signaling pathway selected in Paper IV are both downstream of IRF3 (Figure 3). The ISRE is at the very end of the pathway; however, the IFNβ promotor is close in the pathway to IRF3. Our results showed an inhibitory effect of the PPRV C protein on both of these steps, further indicating that the C proteins inhibitory activity could be in the nucleus of the host cell.

When the C and the V protein expression plasmids were co-transfected, inhibition on the type I IFN signaling pathway were not cumulative, compared to transfection of the proteins singly (Figure 12A and B). Combined expression of the V and C protein of MeV did likewise not cause a gain of inhibition, except for certain isolates of MeV and with three times the concentration of C protein compared to the concentration of V protein (Fontana *et al.*, 2008).

4.6.2 Type II interferon

In addition to the modulation of type I IFN, the modulation of the type II IFN signaling pathway by PPRV V and C proteins was studied in Paper IV. Type II IFN is produced mainly by NK cells and T cells and is an important modulator of the morbillivirus infection (Abboud *et al.*, 2016; Chinnakannan *et al.*, 2013; Finke *et al.*, 1995). This investigation was done in the same model system, and by measuring the activity at the IFNγ-activated sequence (GAS).

The V protein of all isolates efficiently inhibited the GAS activity to 6.2-20.1% compared to the control (100%) (Figure 13). Inhibition of the type II IFN signaling pathway by the V proteins of morbilliviruses varies, with the PPRV V protein among the ones that do inhibit the activity (Chinnakannan *et al.*, 2013).

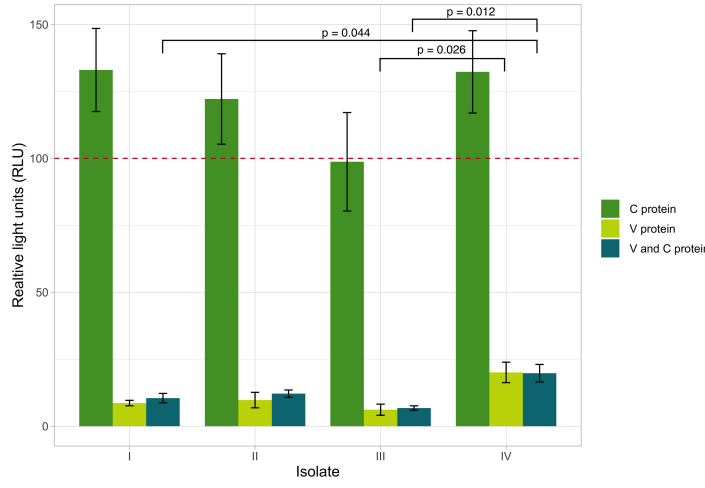

Figure 13. Modulation of the interferon-γ-activated-sequence (GAS) by the V and C proteins of PPRV lineages I-IV, separately and in combination with each other. HEK293T cells were transfected with expression plasmids for PPRV V protein, C protein, or both, together with a GAS-Luc reporter plasmid to measure the gene activity. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. Statistically significant differences ($p < 0.05$) are indicated by their p-values. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

An ANOVA analysis of the results for the V proteins showed statistically significant differences between lineages III and IV ($p = 0.026$).

The results from the C proteins of PPRV, however, stimulated the type II IFN signaling pathway (Figure 13). This stimulation was statistically significant compared to the effect of the V protein and the combination of the C and the V protein, but no difference was detected between the different isolates. To verify if this property is unique to PPRV or an intrinsic property of all morbilliviruses, the same type of experiment was carried out using the C proteins of other selected morbilliviruses: CDV, FeMV and MeV. The C proteins of CDV, FeMV, and MeV likewise stimulated the type II IFN signaling pathway, with the CDV C protein as high as 200% (Figure 14). To further evaluate this stimulation, the experiment was carried out, in technical triplicates and biological duplicates, in a second cell line, A549 cells. This was done to exclude that the type II IFN stimulation was only reproducible in the specific cell line HEK293T. The stimulation was not as strong in this second cell line, but all studied isolates stimulated the pathway to some degree (Figure 15).

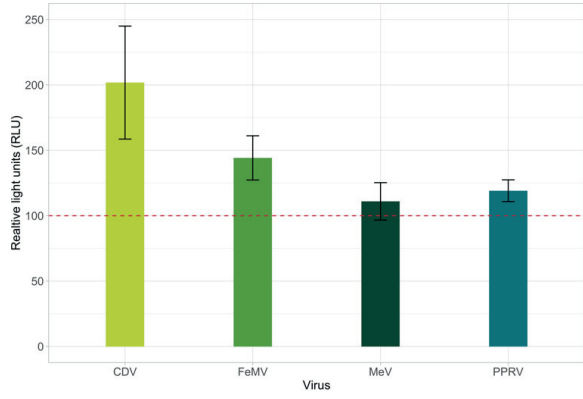

Figure 14. Modulation of the interferon--γ-activated-sequence (GAS) by the C protein from selected morbilliviruses. The results from the PPRV C protein show the mean of all lineages. HEK293T cells were transfected with expression plasmids for C protein of each virus together with a GAS-Luc reporter plasmid to measure the gene activity. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

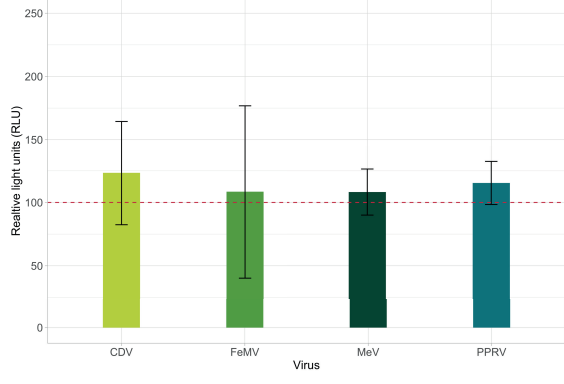

Figure 15. Modulation of the interferon-γ-activated-sequence (GAS) by the C protein from selected morbilliviruses in A549 cell line. The results from the PPRV C protein show the mean of lineages used in the experiment (I and IV). The bars represent the mean of technical triplicates and biological duplicates, and the error bar indicates SE. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

Co-transfection, in HEK293T cells, of the C and the V protein of PPRV resulted in similar inhibition as the V protein on its own (Figure 13). An ANOVA analysis of the results from the combination of C and V proteins showed a statistically significant difference between lineage I and IV ($p = 0.044$) and lineage III and IV ($p = 0.012$).

The stimulatory effect of the C protein was abolished by the presence of the V protein. During a natural infection with PPRV, it is possible that the V and C

proteins are expressed at different time points of the infection cycle, or in different types of cells. All morbilliviruses are dual-tropic, meaning that they infect both epithelial cells and lymphocytic cells. The ability to infect two different cell types also demands the capability to bind to receptors available on both cell types. For morbilliviruses, these are the SLAM/CD150 receptor on lymphocytic cells and nectin-4 on epithelial cells (Prajapati *et al.*, 2019). The virus must also be able to control these different cell types, and a recent study found that lymphocytic cell-adapted viruses expressed less V protein than phosphoprotein (from which gene the V protein derived via post-transcriptional mRNA editing) (Donohue *et al.*, 2019). Donohue *et al.* did not include possible changes in expression of the MeV C protein in different cell lines in their results, and a possible shift in the P/V-C ratio may change the immunomodulatory abilities of PPRV. The V protein is a strong inhibitor of both types I and type II IFN, but the C protein could possibly induce a more pro-inflammatory response due to its effect on the type II IFN signaling pathway.

A similar difference in the modulation of interferon response has been reported for the NS5 protein of the *Flavivirus* Zika virus (ZIKV) (Chaudhary *et al.*, 2017a). The ZIKV NS5 protein is a strong inhibitor of type I IFN, but activates the type II IFN pathway, and additionally, IFNγ actually increases the replication of ZIKV. The ZIKV NS5 was not shown to influence the STAT1 protein stability or phosphorylation, but does induce degradation of STAT2 (Chaudhary *et al.*, 2017b). STAT2 is needed in the type I, but not in the type II, IFN signaling pathway (Figure 6). Reducing the amount of available STAT2, increases the homodimerization of STAT1, that is part of the type II IFN signaling pathway.

Using Western blot analysis, we evaluated the expression and phosphorylation of STAT1 in presence of PPRV C protein and during stimulation of IFNγ (Table 8, and Paper IV, Figure 5). The C proteins of all isolates modulated both the expression and the phosphorylation of the STAT1 protein compared to the control. The expression of the STAT1 protein was inhibited by all proteins and at all time points, except for the C protein from lineage I at 6 hours post stimulation. The inhibition of expression was stabilized to around 50% for all isolates 24 hours post stimulation (Table 8). The phosphorylation of the STAT1 protein at position 701 is needed for the homodimerization and translocation of STAT1 into the nucleus (Wenta *et al.*, 2008). The phosphorylation was initially stimulated by one of the isolates, ICV89. This isolate is one of the more pathogenic isolates; therefore, its immunomodulation mechanism might differ from that of more attenuated isolates. The three other isolates all inhibited the phosphorylation compared to the control. Whether the PPRV C protein uses the same mechanism as ZIKV

NS5 (by increasing the STAT1 homodimerization) or has another mechanism to modulate the type II IFN response is not clear from these results and needs to be studied further.

Table 8. *Modulation of STAT1 expression and STAT1 phosphorylation by PPRV C protein lineages I–IV. Results of Western blot analysis for STAT1 expression and STAT1 phosphorylation with volumes normalized to the total protein levels and expressed as fold change relative to cells transfected with an empty vector backbone (positive control).*

	Hours post-stimulation	I	II	III	IV
STAT1	2	0.80	0.54	0.84	0.97
STAT1	6	1.10	0.23	0.30	0.34
STAT1	24	0.44	0.46	0.45	0.40
STAT1-P-Tyr	2	1.59	0.64	0.86	1.02
STAT1-P-Tyr	6	1.12	0.57	0.53	0.54
STAT1-P-Tyr	24	0.58	0.83	0.95	0.57

5 Concluding remarks

This thesis has focused on different aspects of the peste-des-petits-ruminants virus. First, it focused on the virus in the field, studying its epidemiology in domestic small ruminants and improving diagnostic methods. The second focus was the virus in the host, for which the virus-host interactions were studied in the interferons signaling pathways. The main findings from this thesis were:

- PPRV was circulating in the domestic sheep and goat population in Tanzania, along with other diseases with similar clinical signs, such as foot-and-mouth disease, contagious caprine pleuropneumonia, bluetongue, and bovine viral diarrhea, which can complicate both clinical outcome and diagnosis.

- Being female and an increasing age was identified as risk factors for PPRV seropositivity in domestic sheep and goats in Tanzania. Interaction with wild ruminants was not identified as a risk factor, suggesting that the eradication program could focus on the domestic small ruminant population in Africa for control and vaccination.

- During initial sample collection, transport of samples was identified as an obstacle for reliable serological diagnosis, because an unbroken cold chain is hard to maintain from remote sample areas to the laboratory. For this reason, an alternative method of sample transport on filter paper was evaluated and optimized for a commercial PPRV cELISA. This transport method a viable alternative for transport of serum samples for serological diagnosis.

- After identification of sample transport as an obstacle, a protocol was developed for field-adapted full genome sequencing was also developed. This protocol uses the highly portable miniPCR thermocycler and the

MinION sequencer. The protocol performed well in our hands, and using the comprehensive protocol and bioinformatic pipeline included is predicted and designed to perform well (for any who uses it) in the field. The resulting viral sequences from the protocol produced a phylogenetic tree with better resolution than when only the genetic marker was used.

- The PPRV C and V proteins are both strong inhibitors of the type I IFN signaling pathway. The V protein additionally inhibits the type II IFN signaling pathway, whereas the C protein stimulates the type II pathway.

- The IFN modulation did not statistically significantly differ between wild-type isolates and the vaccine strain Nigeria 75/1.

The findings in this thesis could be of help in the control and eradication of PPRV, by providing improved sample transport and diagnostic methods as well as increased knowledge on the viral-host interactions.

6 Future perspectives

Peste-des-petits-ruminants virus is currently the focus of a global control and eradication program. This thesis explored different aspects of PPRV in the hopes of contributing to this eradication. One of the most important aspects of this program is the vaccine, both its effectiveness in producing a protective immunity and the strategy of its dissemination. The vaccine is an attenuated live virus vaccine, distributed only once to achieve immunity. One of the main clinical signs following an infection with PPRV is a severe immunosuppression, and the viral proteins responsible for this are the C and V proteins. In a comparison of the immunomodulatory abilities of four PPRV isolates, the attenuated vaccine strain—Nigeria 75/1—suppressed the interferon signaling pathways as much as did the wild type isolates. This immunosuppression is not at all desirable in a vaccine, as it can lead to secondary infections or exacerbate other underlying diseases in an animal with compromised general condition. The creation of a variant of PPRV, with silenced C and V proteins, should be constructed and evaluated as a candidate for a less immunosuppressive vaccine, but with improved immunogenicity. A vaccine with less negative effects on the animals could increase the community engagement in the eradication program, especially in the current social climate, with a growing mistrust in vaccines.

The C and V proteins were shown to have an opposite effect on the type II interferon signaling pathway. The biological importance of this phenomenon remains to be clarified. It is possible that the two proteins are expressed at different time points in the infection of a host. The V protein has been shown to decrease its expression during adaptation to epithelial cells (Donohue *et al.*, 2019); however, if or how the C protein changes its expression is still unknown. Revealing the expression levels, and their pattern over time, and pinpointing the mechanism of modulation of type II IFN of the C and V proteins of PPRV could provide important knowledge of how the virus spreads within the host and how the interferon modulation changes over time.

An argument has been made for achieving an eradication as quickly and effectively as possible. Not only for the benefit of an early eradication itself, but also to invest time and effort while the political and financial interests, and the community engagement are high (Cameron, 2019).

To eradicate PPRV as quickly as possible, the first step is to gather information on the virus distribution and the epidemiology. The role of wildlife in the PPR epidemiology is still unclear. Cases of PPR in African wildlife are described as spillovers from domestic animals, and they do not appear to transfer the virus further. Asian wildlife has, however, succumbed to the disease on several occasions (Aziz-Ul-Rahman *et al.*, 2019). The importance of wildlife needs to be clarified to identify populations in need of vaccinations for the eradication of PPRV. It remains to be shown if this is a property within the virus or within the host species present on different continents.

If there is a property, such as receptor specificity or immunomodulatory capabilities, of the specific viral strains isolated from wild ruminants, this needs to be taken in consideration during surveillance, vaccination and control of PPR. Every case of PPR identified within a wildlife species, and possible outbreaks in domestic animals within the same area, should be sampled and the virus isolate characterized to identify changes within the genome, as these changes are indicative of an expanded or otherwise altered host range.

With a field-adapted, highly mobile, sequencing method this would be easier to accomplish. A single genome sequence on its own does not hold much value. For this reason, a data repository for all the produced PPRV sequences should be created and be freely available to all striving to help in the eradication of PPR. Such a source of information would allow for continuous analysis of the viral evolution and make it possible to react to changes in the virus. The more information included about the sequences uploaded to the repository, the more valuable they will become.

Not only the specific properties of PPRV isolates collected from wild ruminants could be studied using such a data base. Outbreaks of PPR can differ in severity, with some being almost subclinical, and others with a mortality and morbidity close to 100%. In some outbreaks, the disease is severe in goats and mild in sheep, even when kept in the same herd (Abubakar *et al.*, 2016). If description of clinical signs and general herd information were included, the mechanism for these differences could possibly be understood. Following identification of characteristics in isolates from wild ruminants or highly virulent strains, these changes in the genome need to be further evaluated using cell model systems or experimental infections to verify the findings.

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Popular science summary

Sheep and goats are the most important source of food and income for many people around the world, especially among vulnerable poor groups in Africa and Asia. The animals provide milk, hides, meat, and sometimes act as a payment for, for example, children's schooling or doctor's visits. Sheep and goats are relatively inexpensive to buy and keep. They can be kept close to home and they survive under harsh conditions. For poverty reduction and food security, it is of the utmost importance that the animals are healthy, especially when the animals are the individual’s or family's main source of food and income.

Peste des petits ruminants (PPR) is a viral disease that mainly affects sheep and goats, but also wild small ruminants such as antelopes or ibex. The clinical signs are high fever, eye and nose discharges, pneumonia, cough, diarrhea, painful mouth ulcers, dehydration, impaired immune system, and in some cases death. The proportion of infected animals that die varies between disease outbreaks, but up to 90% of the animals that fall ill can die.

The closely related virus, rinderpest virus, became the second virus ever to be eradicated in 2011 (the first being smallpox virus in the 1980s). Rinderpest virus and peste-des-petits-ruminants virus (PPRV) share many characteristics, and the health and survival of small ruminants is an important part of poverty reduction. For these reasons, a program is now ongoing to control and eradicate PPRV as well. In this dissertation, various aspects of PPRV have been studied with the aim of contributing to the eradication program.

The first study in the dissertation examined which sheep and goats are at risk of being infected by PPRV. This study was done in Tanzania, a country with a large sheep and goat population, but also many wild ruminants. The country, in addition, has plenty of opportunities for domestic and wildlife to meet. The study showed that females and older animals had a higher risk of being infected. However, contact with wildlife was not proven to be a risk factor. This is important information, as even wild animals would otherwise need to be vaccinated against PPRV to succeed with the eradication.

During the study, blood samples were transported from the animals to a laboratory for analysis, sometimes long distances. As it was difficult to transport the samples and keep them cold, a new, well-functioning mode of transport was developed. The blood was dropped on a special paper that was then allowed to dry and could be transported to the laboratory with preserved quality for the analyzes. The method was published and is described in Paper II of the dissertation.

To eradicate a viral disease, knowledge is needed about how the infection spreads and how the virus interacts with the host animal. Effective monitoring of the virus evolution aids in the control of it. An important source of information for building such knowledge is the virus's genetic code. Paper III therefore describes a method for mapping the whole genome sequence of different variants of PPRV. The method is adapted for fieldwork close to disease outbreaks or for work in less well-equipped laboratories.

In an outbreak of PPR, the clinical signs that the animals exhibit vary, which may be due to factors in the animals themselves, or caused by changes in the virus. With more knowledge of the viral genetic code, the variation in symptoms can be more easily investigated. In Paper IV, the two proteins of PPRV, which are the viral main tools for controlling the host immune response, were investigated. Their effect on the immune system was studied and different variants of PPRV were compared. The PPRV proteins are both strong inhibitors of the immune response and this inhibition did not vary between the vaccine strain and the wild type strains of the virus.

Populärvetenskaplig sammanfattning

Får och getter är den viktigaste källan till mat och inkomst för många människor världen över, framförallt bland utsatta fattiga grupper i Afrika och Asien. Djuren ger mjölk, hudar, kött, och fungerar ibland som betalning för till exempel barnens skolgång eller läkarbesök. Får och getter är relativt billiga att köpa in och att hålla. De kan hållas nära hemmet och de överlever under tuffa förhållanden. För fattigdomsbekämpning och tryggad livsmedelsförsörjning är det av högsta vikt att djuren är friska, framförallt i de fall djuren är individens eller familjens främsta källa till mat och inkomst.

Peste des petits ruminants (PPR) är en virussjukdom som främst drabbar får och getter, men även vilda små idisslare som antiloper eller stenbockar. Symtomen är hög feber, ögon- och nosflöde, lunginflammation, hosta, diarré, smärtsamma sår i munnen, uttorkning, nedsatt immunförsvar, och i vissa fall död. Hur många av de smittade djuren som dör varierar mellan sjukdomsutbrott men det kan vara upp till 90 % av de djur som insjuknar.

Det närbesläktade viruset boskapspestvirus blev 2011 det andra viruset någonsin att bli utrotat (det första var smittkoppsvirus på 1980-talet). Då boskapspestvirus och peste-des-petits-ruminants-virus (PPRV) delar många egenskaper, och för att små idisslares hälsa och överlevnad är en viktig del fattigdomsbekämpning, pågår nu ett program för att kontrollera och utrota även PPRV. I denna avhandling har olika aspekter av PPRV studerats med målet att bidra till utrotningsprogrammet.

I den första studien i avhandlingen undersöktes vilka får och getter som löper risk att smittas av PPRV. Denna studie gjordes i Tanzania, ett land med en stor får- och getpopulation, men också med många vilda idisslare. I landet finns också gott om möjligheter för tama och vilda djur att mötas. Studien visade att hondjur och äldre djur hade en högre risk att ha smittas. Vi kunde dock inte visa att kontakt med vilda djur är en risk. Detta är viktig information, eftersom även vilda djur annars skulle behöva vaccineras mot PPRV för att lyckas med utrotningen. Under studien transporterades blodprover från djuren till ett

laboratorium för analys, ibland långa sträckor. Då det var svårt att transportera proverna kylda utvecklades ett nytt, välfungerande transportsätt. Blodet droppades på speciella papper som sedan fick torka och kunde transporteras till laboratoriet med bibehållen kvalitet för analyserna. Den utvecklade metoden publicerades och återfinns som studie II i avhandlingen.

För att utrota en virussjukdom behövs kunskap om hur smittan sprids och hur viruset interagerar med värddjuret. En viktig informationskälla för att bygga sådan kunskap är virusets arvsmassa. Om virusets utveckling kan följas på ett effektivt sätt underlättas kontrollen av viruset. I studie III beskrivs därför en metod för att kartlägga hela arvsmassan hos olika varianter av PPRV. Metoden är anpassad för fältarbete vid sjukdomsutbrott eller för arbete i mindre välutrustade laboratorium.

Vid utbrott av PPR varierar de symtom som djuren uppvisar, detta kan bero på faktorer hos djuren själva, eller orsakas av förändringar i viruset. Med mer kunskap om virusets arvsmassa kan variationen i symtom lättare utredas. I studie IV undersöktes de två proteiner hos PPRV som är virusets huvudansvariga verktyg för att styra värddjurets immunförsvar. Deras påverkan på olika delar av immunförsvaret studerades och olika varianter av PPRV jämfördes. Båda proteiner är starkt immunhämmande och graden av hämning skiljde sig inte mellan vaccinstammen och virus isolerade från PPR utbrott.

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I

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RESEARCH ARTICLE

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Seroprevalence and risk factors for peste des petits ruminants and selected differential diagnosis in sheep and goats in Tanzania

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ABSTRACT

Introduction: Livestock husbandry is critical for food security and poverty reduction in a low-income country like Tanzania. Infectious disease is one of the major constraints reducing the productivity in this sector. Peste des petits ruminants (PPR) is one of the most important diseases affecting small ruminants, but other infectious diseases may also be present.

Objective: The objective of this study was to determine the seroprevalence and risk factors for exposure to PPR, contagious caprine pleuropneumonia (CCPP), foot-and-mouth disease (FMD), bluetongue (BT), and bovine viral diarrhoea (BVD) in sheep and goats in Tanzania.

Methods: Serum samples were collected in 2014 and 2015, and analysed using enzyme-linked immunosorbent assays to detect antibodies to the five pathogens.

Results and discussion: This is the first description of seroprevalence of FMD and BT among small ruminants in Tanzania. Risk factor analysis identified sex (female) (OR for 2014: PPR: 2.49, CCPP: 3.11, FMD: 2.98, BT: 12.4, OR for 2015: PPR: 14.1, CCPP: 1.10, FMD: 2.67, BT: 1.90, BVD: 4.73) and increasing age (>2 years) (OR for 2014: PPR: 14.9, CCPP: 2.34, FMD: 7.52, BT: 126, OR for 2015: PPR: 8.13, CCPP: 1.11, FMD: 2.98, BT: 7.83, BVD: 4.74) as risk factors for exposure to these diseases.

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Introduction

Small ruminants play an important role in food security and livelihood resilience in many parts of the world [1], but there are several constraints reducing the productivity in this sector [2,3]. Infectious disease is considered a major restriction causing direct losses, such as death and decreased production, and indirect losses, such as export constraints [3].

Peste des petits ruminants (PPR) is one of the most important diseases affecting small ruminants worldwide [4,5]. PPR is caused by peste des petits ruminants virus (PPRV), a highly contagious virus that gives rise to disease in sheep, goats, and camels and has also been reported in wild ruminants [6]. Clinical signs of PPR include pyrexia (40–41°C), ocular and nasal discharges, lesions in the oral and nasal mucus membranes, dyspnoea, cough, pneumonia, diarrhoea, and severe dehydration [7]. Morbidity and case fatality rates vary and, depending on factors such as immune status, age, species, and presence of other co-infections, they can be as high as 90–100% [8].

Clinical presentation of PPR can be difficult to differentiate from other diseases affecting small ruminants [7]. Differential diagnoses include contagious caprine pleuropneumonia (CCPP), foot-and-mouth disease (FMD), and bluetongue (BT) [9]. CCPP is caused by the bacterium *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) [10], FMD is caused by foot-and-mouth disease virus (FMDV) [11], and BT is caused by bluetongue virus (BTV) and is spread by the vector *Culicoides* mosquitos [12]. Infection with bovine viral diarrhoea virus (BVDV), or the closely related border disease virus (BDV), is generally not considered a differential diagnosis of PPR as these viruses mostly cause reproductive disease in small ruminants [13–15]. However, co-infections with PPRV and BVDV, BDV, or BTV are believed to exacerbate the clinical signs of PPR [16,17].

PPR, CCPP, FMD, and BT are among the 10 most important diseases in sheep and goats worldwide in terms of lost livestock units [5]. For PPR, 6 of the 10 most affected countries during 2006–2009 were African countries [5]. Tanzania, located on the east coast of Africa, is a low-income country with 28.2% of the population living below the national poverty line [18]. Of the total population, 68.4% live in rural areas and three of five rural households earn, on

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average, 22% of their income from livestock husbandry [19]. Poorer households tend to keep small livestock, such as chicken, sheep and goats, whereas wealthier households keep large livestock [19]. Small ruminants are kept by 52% of Tanzanian households, with an estimated number of 15 million goats and 6 million sheep [20]. PPR was first confirmed in Tanzania in 2008 [21], but a retrospective study on samples collected in the northern districts found antibodies to PPRV were probably already present in 2004 [22]. The disease has since spread to the southern parts of the country and is now considered endemic in the domestic, small ruminant population in the whole country [23–25]. CCPP, FMD, BT, and BVD are endemic in Tanzania [24], however studies on FMD and BT have only been performed on large ruminants.

All of the diseases in question (PPR, CCPP, FMD, BT, and BVD) have been described in wildlife [26–30]. Wild ruminants have been shown to carry PPRV and several species can develop clinical signs of PPR [28,31,32]. Whether interaction or proximity between livestock and wildlife in general, and wild ruminants in particular, is an important risk factor for exposure to PPRV has not yet been determined.

The objective of this study was to estimate the seroprevalence of, and determine possible risk factors for exposure to, PPR, CCPP, FMD, BT, and BVD in small domestic ruminants in selected areas of Tanzania.

Materials and methods

Study area and study design

This study was carried out with the aim to understand the epidemiology of PPR at the wildlife–livestock interface in Tanzania. Thus, the study area was in parts of the country with such an interface (shared pastures, shared water, and regular proximity) and in regions where PPR had previously been described [21,33]. Tanzania is divided into 26 administrative regions, subdivided into districts, and further into wards [34]. Four districts were purposively selected for this study: Ngorongoro in the northern Arusha region, and Ulanga, Kilombero, and Mvomero in the south-eastern Morogoro region (Figure 1). Wards in

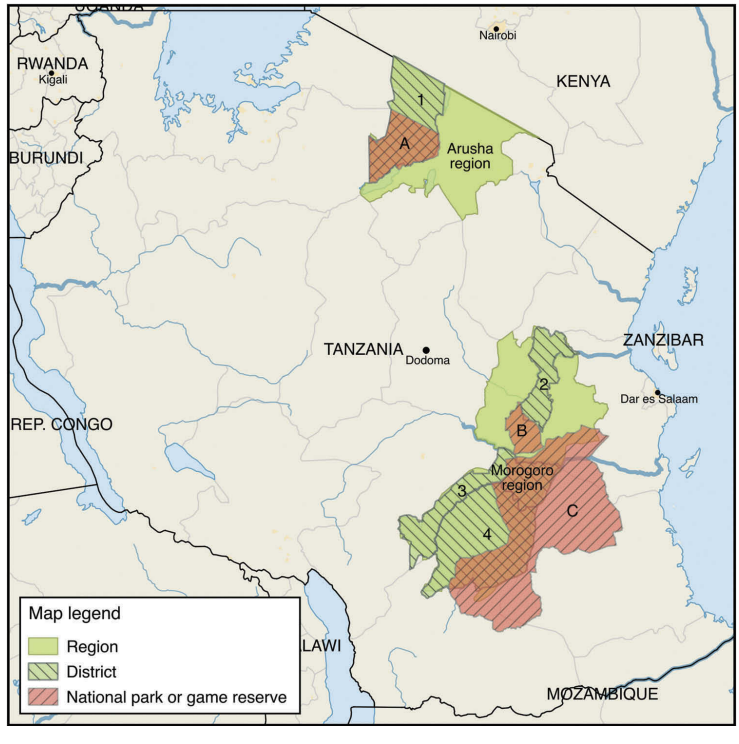

Figure 1. Geographical map of sampling area. Striped green areas indicate visited districts (1 = Ngorongoro, 2 = Mvomero, 3 = Kilombero, 4 = Ulanga). Striped red areas indicate parks or game reserves, i.e. areas with a higher concentration of wildlife (A = Ngorongoro Conservation Area, including Ngorongoro National Park, B = Mikumi National Park, C = Selous Game Reserve).

the districts, located outside, bordering, or within parks or reserves (with a wildlife–livestock interface) were purposively selected, after which 50% were then randomly assigned to the study. In collaboration with local extension officers, wards were replaced with neighbouring wards when those selected did not have enough animals or were inaccessible.

A confidence interval of 95%, a margin of error of 5%, an infinite population, an assumed true overall prevalence of 50% to obtain maximum sample size, and the sensitivity (94.5%) and specificity (99.4%) of the PPR competitive enzyme-linked immunosorbent assay (cELISA) [35] were used [36,37] in calculations of the sample size (PPR cELISA was used to calculate sample size as PPR was the main focus of the study). This gave the needed sample size of 435 samples for each of the two years when herds (containing sheep and/or goats) were visited. To reach the estimated sample size and to assure an even dispersion of the samples in the selected area, we aimed to sample 3 villages in each ward, 2–3 herds per village and 12–20 animals per herd, depending on herd size. If herds were smaller than 12 animals, all of the animals in the herd were sampled.

The study was conducted during two successive years: 2014 (Ngorongoro, Mvomero, and Ulanga) and 2015 (Ulanga and Kilombero). No herd was visited and sampled in both 2014 and 2015. Animals of all ages (2014) were sampled as previously described [38]. However, according to the interview study from this first visit, 43.7% of the sampled animals had been vaccinated against PPRV, possibly resulting in biased prevalence estimates. Therefore, in 2015, young animals (3–12 months) were selected to avoid false positive results due to vaccination or maternal antibodies. If herds did not include enough animals within this age range, older animals were sampled to reach the goal of 12–20 animals per herd.

Ethical consideration

Sampling was done in collaboration with Tanzania District Veterinary Office, and a local veterinarian or veterinary assistant was present at all sampling sites. Ethical approval was sought and received from the Swedish University of Agricultural Sciences Research Animal Council (SLU ua 2017.1.1.1–1881).

Sample and data collection

Herds of pastoralists or traditional farmers were visited during September–October 2014 and June–July 2015. Oral consent to sample animals was obtained from the herd owners prior to sample collection. Blood was collected from the jugular vein using sterile needles and vacutainer tubes without additives (BD vacutainer, Plymouth, UK). Blood samples were left to coagulate and separate in a vertical position in a cool box. After separation, the serum was transferred to 2-ml cryotubes and stored at −45°C until analysis.

A pre-prepared questionnaire in English was used for epidemiological data collection at the sampling sites. Interviews were performed in Swahili by a local translator. The questionnaires differed between the two years, due to preliminary results from 2014 and extension of the study for the sample collection in 2015. The 2014 questionnaire included open-ended questions regarding the size of herd, type of animals in the herd, if and when animals were vaccinated against PPR, if the animals interacted with wildlife, and if so, which wildlife species. Interaction with wildlife was specified as physical proximity or shared pastures. The 2015 questionnaire was modified to include information about vaccinations against PPR, CCPP, and FMD, and interaction of the herd with other domestic herds of sheep, goats, or cattle and wildlife. This questionnaire included open-ended questions regarding size of the herd, type of animals in it, if and when animals were vaccinated against PPR, CCPP, and FMD, how often the herd interacted with other domestic herds, latest introduction of new animals into the herd, and how often the herd interacted with wildlife.

Laboratory analysis

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to analyse the presence of antibodies to the selected pathogens: *ID screen PPR competition ELISA* (detects anti-PPRV nucleoprotein antibodies [35], sensitivity 94.5%, specificity 99.4%; ID. Vet, Grabels, France), *IDEXX CCPP Ab test* (uses monoclonal antibody '4.52' against *Mycoplasma* sp. Type F38 [39], no information for sensitivity, specificity 99.6%; IDEXX, Hoofddorp, The Netherlands), *ID screen FMD NSP competition* (detects anti-FMDV 3ABC non-structural protein antibodies, sensitivity 100%, specificity 99.4%; ID. Vet, Grabels, France), *Bluetongue Virus (BTV) Antibody Test Kit* (detects anti-BTV VP7 protein antibodies, sensitivity 83%, specificity 100%; IDEXX, Hoofddorp, The Netherlands), and *BVDV p80 Ab Test Kit* (detects anti-BVDV p80 antibodies sensitivity 100%, specificity 99.2%; IDEXX, Hoofddorp, The Netherlands). The *BVDV p80 Ab Test Kit* detects antibodies to both BVDV and BDV, without the ability to differentiate between the two. All kits were used and interpreted according to the manufacturers' instructions. For PPR, BT, and BVD, there were three different outcomes for the ELISA: positive, negative, or doubtful. In the statistical analysis a doubtful result was considered as negative.

Statistical analysis

The true prevalence was calculated based on the apparent prevalence and the sensitivity and specificity of the diagnostic tests used, in accordance with [40]. Individual animal results were analysed for possible risk factors for seropositivity, as an indirect measure of exposure: sex, species, age group, and, in the case of PPR and CCPP, vaccination was also included in the analysis, given that the diagnostic test used could not differentiate between infected and vaccinated animals. Age of animal and date of when vaccination had been performed according to the owners were taken into consideration when classifying animals as vaccinated or not. A confidence interval (95%) for the positive proportion was calculated using the score method with continuity correction [41]. To minimize vaccination as a confounder, the animals that owners reported to be vaccinated were excluded from the results in the univariable analysis. All statistical analyses were performed in *R*, Version 3.2.2 [42], and each of the pathogens was analysed separately. Association between risk factors and outcome (i.e. seropositivity to one of the pathogens) was analysed using the command *oddsratio* from the *fmsb* package, with corrections for difference in proportions. A p-value < .05 was considered as significant. Risk factors with a p-value < .2 in the univariable analysis were analysed in a generalized linear mixed-effect model, using the *glmer* command from the *lme4* package [43]. Risk factors were included as fixed effects, and herd was included as a random effect to account for potential clustering. At herd level in 2014, the risk factors included the district and whether owners reported their animals being in proximity to wildlife, whereas in 2015 they included district, reports of proximity to wildlife, interaction with other domestic herds, and introduction of new animals in the last 12 months. The proportion of positive animals in herds and all risk factors were added to the generalized linear mixed-effect model without previous univaribale analysis. Again, herd was included as a random effect. Interactions between risk factors were tested for in all models.

Results

Descriptive analysis

Of 957 animals, 476 animals (from 39 different herds) were sampled in 2014 and 481 animals (from 46 different herds) in 2015. In 2014, 50% of the animals were goats and in 2015 67.2% of the animals were goats. The remainder were sheep. The sex distribution was 73.5% female (including both goats and sheep) in 2014 and 64.9% were female in 2015. In 2014, 17.9% of sampled animals were < 1 year (52.3% females), 26.1% were 1–2 years (58.9% females), and 56% were > 2 years (87.1% females). In 2015, the age distribution was: 53.3% < 1 year (54.3% females), 26.4% 1–2 years (71.4% females), and 20.1% > 2 years (83.3% females).

Seroprevalence

Antibodies to the pathogens were detected in all visited districts, with some exceptions. BVDV was not detected in the 2014 Ulanga samples, nor was CCPP detected either year in this district. No samples from Ngorongoro were analysed for BT or BVD.

The true prevalence for PPR was estimated at 49.3% (95% CI 44.5;54.0) in 2014 and 10.0% (95% CI 7.1;12.8) in 2015. The true prevalence of FMD was 39.0% (95% CI 33.8;44.3) in 2014 and 14.1% (95% CI 10.9;17.2) in 2015. The highest seroprevalence was for BT for both years: 98.9% (95% CI 90.1;100) in 2014 and 74.5 % (95% CI 68.4;80.6) in 2015. The lowest was for BVD: 3.9% (95% CI 0;8.0) in 2014 and 1.7% (95% CI 0.1;3.4) in 2015. It was not possible to calculate the true prevalence of CCPP because there was no information for sensitivity of the ELISA kit [44]. Observed prevalence for the pathogens is given in Tables 1 and 2.

Risk factor analysis

Univariable analysis showed a significant difference between male and female animals, with females at higher risk of being seropositive for the tested pathogens, except BVD and CCPP in 2015 (Tables 3-7). Goats were found to be at higher risk than the sheep for seropositivity against CCPP in both years (OR 57.2 in 2014 and OR 9.68 in 2015), and FMD (OR 1.94) and BT (OR 1.64) in 2015 (Tables 4-6). Multivariate analysis identified sex (female) as a significant risk factor for all pathogens, except CCPP and BT in 2015 (Tables 3-7). Increased seropositivity in animals older than 2 years was significant for all pathogens in both years, except for CCPP in 2015.

Analysis at herd level showed a significant association between the Mvomero region (visited in 2014) and seropositivity for PPR and FMD. For FMD, an association with the region Kilombero was significant in 2015 (Tables 3 and 5). Proximity to wildlife was not identified as a risk factor for any of the pathogens for either of the years (Tables 3-7). Rather, proximity to wildlife was identified in 2015 to have a negative association with seropositivity for CCPP. Interaction with other domestic herds was identified to have the same association for CCPP (Table 4). Interaction with other domestic herds was a significant risk factor for being seropositive for FMD and BT in 2015 (Tables 5 and 6).

Table 1. Seroprevalence at individual animal level according to sex, species, age group, and vaccination status (PPR) from the first round of a repeated cross-sectional study of small ruminants carried out in Tanzania in 2014.

		PPR		CCPP		FMD		BT		BVD	
Variable		Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)
	Total	476 (223)	46.8 (42.8; 51.4)	323 (47)	14.6 (11.0; 19.0)	340 (134)	39.4 (34.2; 44.8)	106 (87)	82.1 (73.2; 88.6)	106 (5)	4.7 (1.7; 11.2)
Sex	Female	350 (190)	54.3 (48.9; 59.6)	230 (41)	17.8 (13.2; 23.5)	264 (118)	44.7 (38.6; 50.9)	76 (71)	93.4 (84.6; 97.5)	76 (5)	6.6 (2.5; 15.4)
	Male	125 (32)	25.6 (18.4; 34.3)	92 (6)	6.5 (2.7; 14.2)	75 (16)	21.3 (13.0; 32.6)	30 (16)	53.3 (34.6; 71.2)	30 (0)	0 (0; 14.1)
Species	Sheep	238 (108)	45.5 (39.0; 52.0)	159 (2)	1.3 (0.2; 5.0)	182 (64)	35.2 (28.4; 42.7)	60 (48)	80.0 (67.3; 88.8)	60 (2)	3.3 (0.6; 12.5)
	Goat	238 (115)	48.3 (41.8; 54.8)	164 (45)	27.4 (20.9; 35.0)	158 (70)	44.3 (36.5; 52.4)	46 (39)	84.8 (70.5; 93.2)	46 (3)	6.5 (1.7; 18.9)
Age group	< 1 year	85 (15)	17.6 (10.5; 27.7)	60 (0)	0 (0; 7.5)	60 (8)	13.3 (6.3; 25.1)	17 (6)	35.3 (15.3; 61.4)	17 (0)	0 (0; 22.9)
	1–2 years	124 (24)	19.4 (13.1; 27.7)	82 (13)	15.9 (9.1; 26.0)	69 (13)	18.8 (10.8; 30.4)	17 (10)	58.8 (33.4; 80.6)	17 (0)	0 (0; 22.9)
	> 2 years	262 (180)	68.7 (62.7; 74.2)	178 (34)	19.1 (13.8; 25.8)	207 (111)	53.6 (46.6; 60.5)	70 (69)	98.6 (91.3; 99.9)	70 (5)	7.1 (2.6; 16.5)
Vaccination	Yes	208 (131)	63.0 (56.0; 69.5)	–	–	–	–	–	–	–	–
	No	253 (86)	34.0 (28.3; 40.2)	–	–	–	–	–	–	–	–

Table 2. Seroprevalence at individual animal level according to sex, species, age group, and vaccination status (PPR and CCPP) from the second round of a repeated cross-sectional study of small ruminants carried out in Tanzania in 2015.

		PPR		CCPP		FMD		BT		BVD	
Variable		Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)	Analysed (positive)	% Positive (95% CI)
	Total	481 (48)	10.0 (7.5; 13.1)	340 (64)	18.8 (14.9; 23.5)	480 (70)	14.6 (11.6; 18.2)	359 (222)	61.8 (56.5; 66.8)	357 (9)	2.5 (1.2; 4.9)
Sex	Female	312 (45)	14.4 (10.8; 18.9)	217 (42)	19.4 (14.5; 25.4)	312 (57)	18.3 (14.3; 23.1)	223 (156)	70.0 (63.5; 75.8)	223 (8)	3.6 (1.7; 7.2)
	Male	169 (3)	1.8 (0.5; 5.5)	123 (22)	17.9 (11.8; 26.1)	168 (13)	7.7 (4.3; 13.1)	120 (66)	55.0 (45.7; 64.0)	128 (1)	0.8 (0.04; 4.9)
Species	Sheep	158 (13)	8.2 (4.6; 13.9)	92 (3)	3.3 (0.9; 10.0)	157 (15)	9.6 (5.7; 15.6)	96 (51)	53.1 (42.7; 63.3)	97 (4)	4.1 (1.3; 10.8)
	Goat	323 (35)	10.8 (7.7; 14.8)	248 (61)	24.6 (19.5; 30.5)	379 (57)	15.0 (11.6; 19.1)	263 (171)	65.0 (58.9; 70.7)	260 (5)	1.9 (0.7; 4.7)
Age group	< 1 year	255 (9)	3.5 (1.7; 6.8)	180 (32)	17.8 (12.7; 24.3)	255 (30)	11.8 (8.2; 16.6)	187 (89)	47.6 (40.3; 55.0)	188 (3)	1.6 (0.4; 5.0)
	1–2 years	126 (16)	12.7 (7.6; 20.1)	90 (19)	21.1 (13.5; 31.2)	126 (13)	10.3 (5.8; 17.3)	96 (66)	68.8 (58.4; 77.7)	96 (1)	1.0 (0.05; 6.4)
	> 2 years	96 (22)	22.9 (15.2; 32.8)	67 (13)	19.4 (11.1; 31.2)	95 (27)	28.4 (19.9; 38.7)	73 (64)	87.7 (77.4; 93.9)	70 (5)	7.1 (2.6; 16.5)
Vaccination	Yes	81 (19)	23.5 (15.1; 34.5)	42 (0)	0 (0; 10.4)	–	–	–	–	–	–
	No	372 (21)	5.6 (3.6; 8.6)	268 (61)	22.8 (18.0; 28.4)	–	–	–	–	–	–

Table 3. Univariable and multivariable analyses for risk factors associated with PPR seropositivity at individual animal level and herd level.

		2014				2015		
Univariable		OR	95% CI	*p*-Value		OR	95% CI	*p*-Value
Sex	Male	2.49	1.37;4.54	**.002**	Male	14.1	1.87;106	**<.001**
	Female				Female			
Species	Sheep	1.01	0.60;1.70	.964	Sheep	1.38	0.49;3.88	.535
	Goat				Goat			
Vaccination	No	3.30	2.25;4.85	**<.001**	No	5.12	2.69;10.0	**<.001**
	Yes				Yes			
Age group	< 1 year	Baseline		**<.001**	< 1 year	Baseline		**<.001**
	1–2 years	1.12	0.56;2.29		1–2 years	3.98	1.70;9.27	
	> 2 years	14.9	8.10;27.5		> 2 years	8.13	3.59;18.4	
Multivariate								
Sex	Male	Baseline		**.002**	Male	Baseline		**.006**
	Female	2.78	1.48;5.40		Female	6.18	1.95;28.2	
Vaccination					No	Baseline		.774
					Yes	1.22	0.31;4.93	
Age group					< 1 year	Baseline		
					1–2 years	3.51	1.11;12.4	**.037**
					> 2 years	17.6	3.78;113	**<.001**
Vaccination *Age group	Yes*< 1 year	5.14	1.43;19.3	**.010**				
	Yes*1–2 years	–	–	–				
	Yes*> 2 years	0.86	0.08;10.2	**.010**				
Herd level								
District	Ulanga	Baseline			Kilombero	Baseline		
	Mvomero	4.68	2.34;10.5	**<.001**	Ulanga	1.10	0.33;3.72	.874
	Ngorongoro	2.21	0.74;7.27	.155				
Interaction with wildlife		0.59	0.21;1.59	.285	Interaction with wildlife	0.94	0.29;2.75	.910
					Interaction with domestic herds	1.65	0.42;7.71	.476
					Introduction of new animals	1.24	0.31;4.90	.740

Serological results from a repeated cross-sectional study of small ruminants carried out in Tanzania. Factors with $p < .2$ in univariabale analysis were used in multivariate analysis. *p*-Values <.05 were considered significant and are in bold. Interaction between vaccination and age group in samples from 2014 are marked with *.

Table 4. Univariable and multivariable analyses for risk factors associated with CCPP seropositivity at individual animal level and herd level.

		2014				2015		
Univariable		OR	95% CI	*p*-Value		OR	95% CI	*p*-Value
Sex	Male	3.11	1.22;7.60	**.010**	Male	1.10	0.62;1.95	.740
	Female				Female			
Species	Sheep	57.2	7.78;420	**<.001**	Sheep	9.68	2.96;31.7	**<.001**
	Goat				Goat			
Age group	< 2 years	2.34	1.18;4.63	**.013**	< 1 year	Baseline		.801
	> 2 years				1–2 years	1.24	0.66;2.33	
					> 2 years	1.11	0.54;2.28	
Multivariate								
Sex	Male	Baseline		**.029**				
	Female	4.46	1.24;19.0					
Species	Sheep	Baseline	–	**<.001**	Sheep	Baseline		**.021**
	Goat	81.9	17.4;726		Goat	9.21	1.70;84.0	
Age group	< 2 years	Baseline	–	**.012**				
	> 2 years	5.17	1.54;21.1					
Herd level								
Interaction with wildlife		0.60	0.06;4.44	.598	Interaction with wildlife	0.008	<0.01;0.16	**.006**
					Interaction with domestic herds	0.045	<0.01;0.48	**.016**
					Introduction of new animals	4.24	0.14;303	.414

Serological results from a repeated cross-sectional study of small ruminants carried out in Tanzania. Factors with $p < .2$ in univariabale analysis were used in multivariate analysis. *p*-Values <.05 were considered significant and are in bold text.

An interaction was found between the variables *age group* and *vaccination against PPR* in the samples from 2014. Effect of vaccination against PPR differed among the age groups.

Discussion

In this study, we investigated the seroprevalence of PPR and some of its differential diagnoses in selected areas in Tanzania. Commercial ELISA tests were used to detect antibodies in serum samples from sheep and goats. The serological results were used further to calculate risk factors for exposure to PPRV, Mccp, FMDV, BTV, and BVDV. In Tanzania, and other east African countries, small ruminant production is an important livelihood for a significant proportion of the population [19]. This important position of small ruminants is one of the reasons behind the joint Food and Agriculture Organization (FAO) and World Organization for Animal Health programme to control and eradicate PPR and control small ruminant diseases [4]. PPRV is quickly increasing its spread

Table 5. Univariable and multivariable analysis for risk factors associated with FMD seropositivity at individual animal level and herd level.

		2014				2015		
Univariable		OR	95% CI	*p*-Value		OR	95% CI	*p*-Value
Sex	Male	2.98	1.63;5.45	**<.001**	Male	2.67	1.41;5.03	**.002**
	Female				Female			
Species	Sheep	1.47	0.95;2.27	.086	Sheep	1.94	1.06;3.56	**.030**
	Goat				Goat			
Age group	< 1 year	Baseline		**<.001**	< 1 year	Baseline		**<.001**
	1–2 years	1.51	0.58;3.93		1–2 years	0.86	0.43:1.72	
	> 2 years	7.52	3.40;16.6		> 2 years	2.98	1.66;5.35	
Multivariate								
Sex	Male	Baseline		**.003**	Male	Baseline		**.001**
	Female	3.77	1.58;9.48		Female	4.70	1.91;13.1	
Species	Sheep	Baseline	–	.099	Sheep	Baseline		**.008**
	Goat	1.81	0.90;3.68		Goat	4.19	1.54;13.0	
Age group	< 1 year	Baseline			< 1 year	Baseline		
	1–2 years	1.51	0.4;5.56	.534	1–2 years	1.21	0.46;3.20	.698
	> 2 years	8.73	2.82;30.5	**<.001**	> 2 years	9.10	3.10;30.8	**<.001**
Herd level								
District	Ulanga	Baseline			Ulanga	Baseline		**.044**
	Mvomero	25.1	11.1;73.8	**<.001**	Kilombero	6.15	1.02;44.7	
	Ngorongoro	2.36	0.43;12.2	.298				
Interaction with wildlife		1.13	0.36;3.47	.816	Interaction with wildlife	1.52	0.28;8.97	.612
					Interaction with domestic herds	20.7	3.10;262	**.005**
					Introduction of new animals	0.13	0.01;1.18	**.067**

Serological results from a repeated cross-sectional study of small ruminants carried out in Tanzania. Factors with $p < .2$ in univariabale analysis were used in multivariate analysis. *p*-Values <.05 were considered significant and are in bold text.

Table 6. Univariable and multivariable analyses for risk factors associated with BT seropositivity at individual animal level and herd level.

		2014				2015		
Univariable		OR	95% CI	*p*-Value		OR	95% CI	*p*-Value
Sex	Male	12.4	3.90;39.5	**<.001**	Male	1.90	1.22;2.95	**.004**
	Female				Female			
Species	Sheep	1.39	0.50;3.88	.527	Sheep	1.64	1.02;2.64	**.040**
	Goat				Goat			
Age group	< 1 year	Baseline		**<.001**	< 1 year	Baseline		**<.001**
	1–2 years	2.62	0.65;10.5		1–2 years	2.42	1.44;4.07	
	> 2 years	126	13.9;1153		> 2 years	7.83	3.68;16.7	
Multivariate								
Sex	Male	Baseline	–	**.030**	Male	Baseline		.437
	Female	7.49	1.29;63.9		Female	1.26	0.70;2.27	
Species					Sheep	Baseline		**.023**
					Goat	2.32	1.14;4.93	
Age group	< 1 year	Baseline	–	–	< 1 year	Baseline		
	1–2 years	3.34	0.42;155	.319	1–2 years	3.04	1.53;6.32	**.002**
	> 2 years	183	15.2;23,216	**.001**	> 2 years	18.4	6.61;61.4	**<.001**
Herd level								
District	Ulanga	Baseline		.356				
	Mvomero	2.42	0.30;21.9					
Interaction with wildlife		0.57	0.03;7.61	.636	Interaction with wildlife	1.18	0.48;2.94	.698
					Interaction with domestic herds	3.85	1.55;10.5	**.004**
					Introduction of new animals	0.99	0.26;3.65	.983

Serological results from a repeated cross-sectional study of small ruminants carried out in Tanzania. Factors with $p < .2$ in univariabale analysis were used in multivariate analysis. *p*-Values <.05 were considered significant and are in bold text.

Table 7. Univariable analysis for risk factor associated with BVD seropositivity at individual animal level.

		2014				2015		
Univariable		OR	95% CI	*p*-Value		OR	95% CI	*p*-Value
Sex	Male	–	–	.150	Male	4.73	0.58;38.2	.110
	Female				Female			
Species	Sheep	2.02	0.32;12.6	.445	Sheep	0.46	0.12;1.73	.239
	Goat				Goat			
Age group	< 1 year	–	–	.279	< 1 year	Baseline		**.023**
	1–2 years				1–2 years	0.65	0.07;6.33	
	> 2 years				> 2 years	4.74	1.10;20.4	

Serological results from a repeated cross-sectional study of small ruminants carried out in Tanzania. There were no positive male animals or age groups <1 and 1–2 years in samples from 2014, so it was not possible to obtain OR for the risk factor 'age group' or 'sex'. Multivariate analysis was not possible due to an insufficient number of seropositive animals. *p*-Values <.05 were considered significant and are in bold text.

across the world and is now threatening the most southern countries of Africa, with Tanzania currently being its southern border on the east coast [25]. To stop the spread further south, it is important to understand the prevalence and epidemiology of both PPR and its most common differential diagnosis, as the clinical presentation can be difficult to diagnose [4].

The calculated true seroprevalence for PPR was 49.3% in 2014 and 10.0% in 2015. A vaccination campaign had been carried out in the Morogoro and Mtwara region prior to our sample collection [45], which may have influenced the 2014 results. Therefore, we aimed to sample animals aged 3–12 months in 2015, as animals in this age group would not have been alive during the vaccination campaign. As expected for an endemic disease, where survival of infection results in lifelong immunity, age was identified as a risk factor for exposure (Table 3). Age and vaccination bias of sampled animals could be the reasons for the difference in seroprevalence in 2014 and 2015. In addition, we did not visit the same areas both years; the differences in seroprevalence could therefore have been due to geographical differences. Previous studies in northern Tanzania found an overall seroprevalence of 45.5% in 2008 [46] and 22.1% in 2008–2009 [21]. In southern Tanzania, in the Mtwara region bordering Mozambique, 31% of sampled small ruminants had antibodies to PPRV [47]. A recent study analysing samples from 14 different regions of Tanzania described an overall seroprevalence of 27.1%, with regions varying from 2.4% (Kagera) to 72.8% (Morogoro), demonstrating the varying level of seroprevalence within the country [23].

Sex has previously been described as a risk factor for PPR; mostly females are identified to be at higher risk [21,48,49]. However, some studies found the opposite association [50–52]. Our results suggest that females had a higher risk of being seropositive for PPRV in both 2014 and 2015. Previous studies on risk factors for PPR have suggested that females are kept longer by their owners (to be used in reproduction), and therefore have a longer risk period for PPRV exposure [48]. In addition, females are more likely to be vaccinated, which may bias the results. The stress associated with pregnancy and milk production may also predispose females to infection [48,49]. Differences between the studies, such as management systems or breed of sheep and goats, may also influence the results. In our study, we found that the age group >2 years was mainly composed of females. This age group had the highest proportion of seropositive individuals; the result might be due to a selection bias. However, the multivariable analysis did not find an interaction between these two variables, indicating that this cannot be the entire explanation.

The true prevalence for CCPP was not possible to calculate because there was no available information on sensitivity for the ELISA test used [44]; however, the apparent prevalence was 14.6% in 2014 and 18.8% in 2015. Previously, a prevalence of 51.2% (in 2007) and 33.7% (in 2009) had been described in southern Tanzania [51].

Goats were identified to be at higher risk than sheep for seropositivity towards CCPP in both years, due to CCPP having a higher affinity for goats. Sheep can develop clinical signs following infection by CCPP, but the infection can also be subclinical [53].

The calculated true prevalence for FMD was 39.0% in 2014 and 14.1% in 2015 for both sheep and goats. To the best of our knowledge, no previous reports of seroprevalence of FMD in small ruminants in Tanzania are available. FMDV causes a less severe disease in small ruminants compared with large ruminants [54]; however, the oral lesions sometimes seen even in small ruminants make FMD an important differential diagnosis of PPR, especially in light of the attempt to eradicate PPR [7]. A study in neighbouring Uganda found a seroprevalence of 14% in goats and 22% in sheep [55]. Seroprevalence of FMD in buffalo and cattle in Tanzania is high. Mkama et al. [56] found an overall prevalence of 76.3% (248 of 330) for buffalo and cattle, with the buffalos from western Tanzania having a 100% seroprevalence (29 of 29). Antibodies to FMDV decrease faster in sheep than cattle [57], which could be one explanation for the difference in seroprevalence between small and large ruminants. As for PPR, our study identified age as a risk factor for FMDV exposure. Age is a documented risk factor for FMDV exposure in cattle, both in endemic and epidemic settings [58,59]. A higher age gives a longer time to be exposed in the endemic setting, and the higher mortality seen in younger animals leaves the older seropositive animals to be sampled [59,60]. Also in line with the results for PPR, female animals were identified to be at a higher risk than males for FMDV exposure. Similar explanations in PPR can be applied to FMD as well, with the exception of vaccination. None of the owners reported that their animals had been vaccinated against FMD.

The calculated true prevalence for BT was 98.9% in 2014 and 74.5% in 2015. No previous studies have been done on seroprevalence of BT in domestic animals in Tanzania. As with FMD, possible oral lesions caused by BTV makes it an important differential diagnosis of PPR [12]. Free-living wild buffalos from eight different areas in Tanzania were sampled between 1987 and 1989 and analysed for antibodies to a selection of pathogens, including BT [61]. An overall prevalence of 91.6% was found, with six of eight areas having a 100% prevalence [61]. A similar

study was performed on wildlife in Zimbabwe with samples collected between 1989 and 1995 [62]. Most samples came from buffaloes, followed by different species of antelopes, and also white and black rhinoceroses. An overall prevalence of antibodies to BTV of 44.1% was found [62]. Domestic cattle were sampled in western Sudan and serological evidence of BTV infection was found in 19.4% of them (58 of 299) [63]. Our results are more in line with those from [59] and [60]. A high seroprevalence is expected from a virus that often gives a subclinical or unapparent disease in ruminants and is spread very efficiently by its vector [12]. Risk factors identified for exposure to BTV included age and sex (Table 6), as for the other pathogens in this study. Age as a risk factor for exposure to BTV is in agreement with a risk factor analysis in cattle in western Sudan [63]. In 2015, multivariate analysis identified goats as being at higher risk for exposure to BTV than sheep. In 2015, 73% of samples analysed came from goats, which may have biased the result.

The calculated true prevalence for BVD was 3.9% in 2014 and 1.7% in 2015. This is lower than what has previously been described for domestic animals in Tanzania. In Tanzanian samples collected between 1985 and 1987 from cattle, sheep, and goats, evidence of BVD exposure was described in 34.0% of cattle, 32.1% of sheep, and 24.9% of goats [64]. In wild buffaloes, mainly from northern parts of Tanzania, 16.9% had antibodies to BVDV [61]. Five cattle herds in the Kafue flats of Zambia were tested for antibodies to a selection of pathogens, and 76.2% were positive for BVDV [65]. A more recent study was performed in western Kenya; calves aged 3–7 days were tested for antibodies to BVDV and an adjusted seroprevalence of 19.8% was identified [66]. Seroprevalence for BVD varies significantly between the different studies, with our study having the lowest prevalence. Dissimilarities in the studies include differences in production of animals sampled, method of analysis, year of sampling, and study design, which makes comparisons difficult. Univariable analysis of our serological results from 2015 identified age (> 2 years) as a risk factor for exposure to BVD. Because of the low number of seropositive animals (9 out of 357), further studies are warranted before making any definite conclusions on risk factors for exposure. Multivariate analysis could not be performed with the BVD results due to too few positive samples.

Correlation between seropositivity for the studied pathogens, except BVD, was analysed at herd level; a generalized linear mixed-effect model was used to identify risk factors affecting the entire herd. In this study no difference was found, for any of the pathogens, between herds with proximity to wildlife and those without. PPRV has long been known to cause disease in wildlife [28]. Clinical signs are yet to be described in wild ruminants in sub-Saharan Africa, but have been reported in wild ruminants in Asia and in the Middle East [67]. Antibodies have been described in wild buffaloes, Grant's gazelle, wildebeest, and impala in Tanzania [32,68]. Recently, a Grant's gazelle without clinical signs of PPR in northern Tanzania tested positive on real-time reverse transcription polymerase chain reaction [32]. The gazelle was sampled in an area with an ongoing outbreak of PPR among domestic animals. The same study found a 63% seroprevalence in 46 sampled wild ruminants [32]. Although it is probable that PPR transmits between domestic and wild animals [32,69,70], our results do not support the hypothesis of wildlife as an important risk factor for exposure for domestic animals in an endemic setting. For the closely related rinderpest virus, the well-accepted hypothesis was that infection in wildlife was not self-sustaining, but rather a case of spillover from domestic animals [71,72]. The same hypothesis has been suggested for PPRV [32,73], and our results seem to be in agreement with this.

For FMD, contact with wildlife has been described as an important risk factor for infection in domestic animals in sub-Saharan Africa [74]. However, among wildlife species, only the African buffalo has been identified as a long-term maintenance host [74]. Small ruminants are highly susceptible to FMDV infection, but they are not as efficient as cattle in maintaining the infection within the population [11]. Years of experience with FMD in southern Africa have been unable to reveal small ruminants as an important part of the maintenance or transmission of the disease [75]. Our results did not identify proximity to wildlife as a risk factor for FMD in domestic small ruminants in these areas of Tanzania.

Bluetongue virus is endemic in both the domestic and wild populations of many African countries [12]. Various wildlife species, both in Africa and in Europe, have been discussed as possible reservoirs [62,76,77]. The epidemiology of BTV differs from the other viruses studied here, as it is spread through its vector, the *Culicoides* mosquito, not through direct contact. In parts of Europe where BT is endemic, studies suggest that wild ruminants, mainly red deer, play a role in the epidemiology [76]. Our results did not indicate proximity to wildlife as an important risk factor for small ruminants to be exposed to BTV in the studied area. However, we did identify interaction with other domestic herds as a risk factor, in agreement with a previous study of sheep and goats in Iran [78]. Possibly the vector is attracted by the increased number of animals in the same location.

For CCPP in 2015, proximity to wildlife had a statistically significant negative association, as did interaction with other domestic herds (Table 4). The

ELISA used for detection of CCPP is specific for antibodies against Mccp [44]; however, cross-protection between different subspecies of mycoplasmas cannot be excluded [53,79,80]. It is possible that other members of the *Mycoplasma mycoides* cluster are circulating in the studied areas and producing cross-protection against CCPP.

Limitations of this study include none of the ELISAs used being able to differentiate between vaccinated and naturally infected animals, and all questionnaire data being collected by a local translator. Information regarding vaccination status of the animals was acquired from the owners. Owners could, for a variety of reasons, provide incorrect information; for example, they do not remember, or a previous owner had the animals vaccinated. To minimize this bias during the sample collection in 2015, we targeted animals 3–12 months of age, animals the owners were more likely to have the correct information about. Further, we used the information from the questionnaires to study whether interaction with wildlife was a possible risk factor for exposure to the studied pathogens. Owners were asked how often the animals had contact with wildlife. The question could, however, have been misunderstood or interpreted in a different way than what we intended. Answers given to the question were, for example: 'never', 'during dry season', and 'everyday'. The interaction between wildlife and livestock can be measured using several methods, with the usage of a questionnaire and local knowledge being a fast and practical method to get preliminary data [81]. The method is not, however, as precise as others, and this insecurity should be considered when interpreting the results of the risk factor analysis.

Conclusion

This study confirmed the presence of antibodies to PPRV, CCPP, FMDV, BTV, and BVDV in sheep and goats in northern and south-eastern Tanzania, indicating a continuous circulation of these pathogens. This is the first description of the presence of antibodies for FMD and BT in small ruminants in Tanzania. Risk factor analysis at individual animal level identified sex (female) and increasing age as two important factors influencing level of exposure to infection. Proximity to wildlife was not identified as a risk factor for any of the pathogens studied.

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Disclosure statement

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Sample Submission Form 2014
SRC-2013-6402 PPRV in Tanzania and Pakistan

Sender name……..

Collection date……

Basic Epidemiological Data of the Herd

(1) Province/Region.....

(2) District.....

(3) Town/Village…….……

(4) Farmer name & telephone.....

(5) Estimated date when first PPR case was observed at this farm ..... / ..... /.....

(6) Detail of animals: (number of sick and dead animals since the start of the outbreak be given)

Species	Age Group (0-1 year)				Age Group (> 1 years)		
	Total Animals	Affected	Died	Aborted	Total Animals	Affected	Died
Sheep							
Goat							
Wildlife (species)							

(7) Type of Farming: [ ] Household [ ] Dairy production [ ] Meat production [ ] Individual seller at live animal market [ ] Others (please mention below)

……
……

(8) Clinical signs at outbreak: [ ] Abortion [ ] Diarrhea [ ] Pneumonia [ ] Oral mucosal lesions [ ] Nasal and ocular discharges [ ] High Temperature

Others signs or comments:

……
……
……
……

Data of Samples Collected

Sample Identification Code per animal	**Age**	**Breed**	**Sex**	**Vaccinated against PPRV?** Y/N/unknown Date of vacc.	**Blood**	**Serum**	**Nasal swab**	**Oral swab**	**Ocular swab**	**Tissues as specified**

Sample Submission Form 2015
SRC-2013-6402 PPRV in Tanzania and Pakistan

Sender name……..

Collection date……..

Basic Epidemiological Data of the Herd

(1) Province/Region.....

(2) District.....

(3) Town/Village……..……

(4) Farmer name & telephone.....

(5) How often do animals come in contact with other domestic herds?……

(6) How often do animals come in contact with wildlife?……..

(7) Latest introduction of new animals to herd……

(8) Last vaccination of herd against PPR ……...……CCPP…… FMD……

(9) Last de-worming treatment of herd ……..…all animals treated?……..…….

(10) Last antibiotic treatment of herd…….. all animals treated?.....

(11) Estimated date when first PPR case was observed at this farm ..... / ..... /.....

(12) Detail of animals: (number of sick and dead animals since the start of the outbreak be given)

Species	Age Group (0-1 year)				Age Group (> 1 years)		
	Total Animals	Affected	Died	Aborted	Total Animals	Affected	Died
Sheep							
Goat							

(13) Type of Farming: [ ] Household [ ] Dairy production [ ] Meat production [ ] Individual seller at live animal market [ ] Others (please mention below)

……

(14) Clinical signs at outbreak: [ ] Abortion [ ] Diarrhea [ ] Pneumonia [ ] Oral mucosal lesions
[ ] Nasal and ocular discharges [ ] High Temperature

Others signs or comments:

……

Data of Samples Collected

Animal **Clinical signs** **Samples**

Sample ID Code/animal	Age	Breed	Sex	Temp.	Diarrhea	Nasal/ocular discharge or lesions	Cough	Blood	Nasal swab	FTA card	Filter strip

Animal **Clinical signs** **Samples**

II

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Filter paper is a simple and cost-effective transport medium for serological diagnosis of Peste des petits ruminants

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ABSTRACT

Peste des petits ruminants (PPR) is a highly contagious disease caused by peste-des-petits-ruminants virus. Following the successful eradication of the related rinderpest virus, a program to control and eradicate PPR was launched by the FAO and OIE. PPR is today present in many tropical countries where maintaining the cold chain for sample transportation is one of the major barriers for timely processing. Transport of samples on filter paper is a simple and cost-effective method, however validation and optimization is required to fully adapt this approach. The objective of this study was to evaluate and validate the use of filter paper in serological diagnosis of PPR. Blood samples (serum and filter paper) were collected from sheep and goats in both Tanzania and Pakistan and analysed using a PPRV-specific cELISA. The positive proportion was 10.7% in Tanzania and 80% in Pakistan when performing the analysis on serum. These results were then considered as reference and compared to the results from the filter papers analysed by the same cELISA. According to the statistical analysis the cut-off for a positive results for samples stored on filter paper was adjusted from < 50 % competition percentage to < 84% in Tanzania and to < 69% in Pakistan.

These results demonstrate that filter papers are an acceptable and cost-effective transport method of whole blood samples for later use in serological analysis.

1. Introduction

Peste des petits ruminants (PPR) is a highly contagious and deadly disease caused by the peste-des-petits-ruminants virus (PPRV) (Gibbs et al., 1979; ICTV, 2016). The main hosts are sheep and goats, however disease in wild small ruminants and camels has also been reported (Abubakar et al., 2011; Aguilar et al., 2018; Mahapatra et al., 2015; Khalafalla et al., 2010). Clinical signs related to PPR include a high fever, ocular and nasal discharge, necrotic lesions in mucous membranes, pneumonia, diarrhoea, immunosuppression, and a high case fatality rate (up to 90%) in an epidemic setting (Torsson et al., 2016). Following the successful eradication of a related morbillivirus, rinderpest virus (Roeder et al., 2013; Gibbs et al., 1979), the Food and Agricultural Organization of the United Nations (FAO) and the World Animal Health Organization (OIE) have launched a program to control and eradicate PPR (FAO and OIE, 2015).

Our understanding on the PPRV epidemiology has increased significantly in the last years, however continued monitoring and strict biosecurity measures would underline the success of the eradication program. Peste-des-petits-ruminants virus is currently present in north, central, east, and west Africa, the Middle East and parts of Asia (OIE, 2016). Many of these are tropical countries where maintaining the cold chain for sample transportation is one of the major barriers for timely processing of samples (FAO, 2013). Filter paper as a mode of sample collection and transport has been suggested previously (Michaud et al., 2007; Matheus et al., 2015; Randriamparany et al., 2016). For PPR, filter paper has been proposed for long-term storage followed by detection and genotyping using PCR (Michaud et al., 2007), but has not yet been studied in serological diagnosis. Transport of samples on filter paper is a simple and cost-effective method, however more robust validation and optimization is required to fully adapt this simple and cost-effective approach (Hopkins et al., 1998). The objective of this study

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was to evaluate the use of filter paper, in comparison to serum, in serological diagnosis of PPR using the OIE suggested competitive enzyme linked immunosorbent assay (cELISA) (Libeau et al., 1995). We use two different kinds of filter paper, the Nobuto Filter Strips and, an even less expensive alternative, chromatography paper. The filter papers were optimized and validated on clinical samples.

2. Materials and methods

2.1. Sample collection

2.1.1. Tanzania

Sample collection was performed during June-July in 2015 in two districts (Kilombero and Ulanga) in the Morogoro region (Torsson et al., 2017). This region was selected due to previous reports of presence of PPR (Kgotlele et al., 2014; Misinzo et al., 2015). Ethical approval was received from the Research Animal Council at the Swedish University of Agricultural Sciences (SLU ua 2017.1.1.1-1881). Blood samples were collected from domestic sheep and goats, between the ages 3–12 month, from herds of different sizes. Each animal was sampled in triplicate: serum in collection tubes using a vacutainer system (BD Biosciences), and two different types of filter paper: Nobuto Filter Strips (NFS) (Advantec, Dublin, CA, United States) and a chromatography paper (CP) (grade FN 100, weight 195 g/m^2, thickness 0.35 mm, capillary rise 115) (Sartorius AG, Goettingen, Germany). The CP was delivered in sheets (260*410 mm) and prior to sample collection the sheet was cut into pieces similar in size to the Nobuto Filter strips, approximately 10*50 mm.

Blood was collected from the jugular vein using sterile needles and vacutainer tubes without additives (BD vacutainer, Plymouth, UK). Tubes were then opened and the two different filter papers were inserted and allowed to soak in whole blood until about half of the paper was saturated with blood. Filter papers were left to dry in a standing position, away from direct sunlight and at ambient temperature (at sampling this was around 29–35 °C). When dry, filter papers were stored separately, wrapped in a paper envelope, at room temperature (20–28 °C). Whole blood was left to coagulate and separate in a vertical position in a cool box. After separation, the serum was transferred to cryotubes and stored at −45 °C until analysis.

2.1.2. Pakistan

Sample collection was performed during October-November in 2015 in the Punjab province. Blood was collected from goats from the jugular vein using sterile needles and vacutainer tubes without additives (BD vacutainer, Plymouth, UK). In Pakistan only the Nobuto filter strips were used for sample collection. Filter strips were inserted into the vacutainer tube and the narrow part of the strip was allowed to saturate with whole blood. Filter strips were dried in a standing position in room temperature away from direct sunlight. When dry, the filter papers were labelled and sent to National Veterinary Laboratory (NVL), Islamabad, via post. The corresponding serum samples were transported to NVL on ice.

2.2. Preparation of filter papers and serological analysis

All samples were analysed with *ID screen PPR competition ELISA* (sensitivity 94.5%, specificity 99.4%; ID.Vet, Grabels, France), which is based on the recombinant nucleoprotein of PPRV (Libeau et al., 1995). Serum was analysed according to manufacturer's instructions. Briefly the analysis was performed as following: to a 96-well plate, pre-coated with recombinant PPRV nucleoprotein, 25 µl of sample was added and incubated for 45 min at 37 °C. The plate was then washed 3 times before adding the conjugate (100 µl/well), followed by 30 min of incubation at 21 °C. The wash was repeated and 100 µl/well of substrate solution was added and incubated for 15 min at room temperature. As a last step, 100 µl/well of stop solution was added to stop the reaction. Plates were read at 450 nm.

The dried filter papers were prepared as following before use in the cELISA: From each filter paper a piece of approximately 75 mm^2 was cut. This piece was then cut into 5–8 smaller pieces and added to 150 µl of ELISA dilution buffer and incubated for 1 h in room temperature. After incubation, 50 µl of this solution was used in each well, which was otherwise performed in the same way as the serum samples.

2.3. Statistical analysis

The results from the serological assays performed on the filter papers were compared to the assays performed on serum, which was considered as the gold standard. A few serological results were between competition percentages 50–60, which according to the manufacturer are considered as doubtful results. These results were considered as negative in the statistical analyses. All statistical analyses were done in the statistical software *R,* version 3.2.2. (R Core Team, 2015). Cohen's Kappa was used to calculate agreement between serum and filter paper assays. Agreement of results was further studied using Bland-Altman plots, with the results from analysis on serum samples considered as the reference. The dots in the Bland-Altman plot represents the difference between the measurements (serum vs. the filter paper of each individual sample) on the y-axis and the average of the measurements on the x-axis (Bland and Altman, 1999). In other words, the difference of the measurements is plotted against the mean of the measurements and the coloured lines represents the mean difference. If the two tests were to give the same results, all the dots would be centred around 0 on the y-axis (marked by the black horizontal line) and the coloured line would overlap with the black line. Plots were produced using the *blandr*-package (Datta, 2017). Receiver operating characteristic curve (ROC-curve) and Precision-Recall curve (PRC) was produced by the *precrec*-package (Saito and Rehmsmeier, 2017). The *pROC*-package was used to find the adjusted cut-off, including sensitivity and specificity, for filter paper assays (Robin et al., 2011). Sensitivity and specificity were given the same weight when evaluating the adjusted cut-off value.

3. Results

In Tanzania, 32 sheep and 164 goats were sampled, and in Pakistan, 60 goats were sampled. Of the 196 animals sampled in Tanzania, 21 animals (10.7%) were seropositive for PPRV antibodies according to the ELISA on serum samples. When performing the ELISA using whole blood stored on either NBS or CP, 11 samples (5.6%) were positive when using the suggested < 50 % cut-off (Table 1A and B). In Pakistan, 48 animals (80%) were seropositive according to assay on serum samples, and 40 animals (66.7%) were positive on assay on NFS (Table 1C).

The Bland-Altman plots indicate a systematic difference between the methods that is comparable all over the test result interval (Fig. 1A–C). To find an adjusted cut-off for the analysis on the different kinds of filter papers a ROC-curve analysis was used (Figs. 2 and 3). The ROC-curve for the NFS in Tanzania had an area under the curve (AUC) of 0.988, where an AUC of 1 indicates a perfect fit. The adjusted cut-off value for a positive sample was calculated to be < 84.6 % (instead of < 50 %) (Fig. 2A), which would give a sensitivity of 95.2% (95% CI 85.7;100) and a specificity of 97.2% (95% CI 94.4;99.4) for the NFS saturated with whole blood. Cohen's kappa for the NFS first using the < 50 % cut-off was 0.66 (0.47-0.85). When adjusting the cut-off to 84.6 %, Cohen's kappa was improved to 0.85 (0.74-0.97) (Table 2). The ROC-curve for the CP had an AUC of 0.983 and an adjusted cut-off of < 84.3%, sensitivity of 90.5% (95% CI 76.2;100), and specificity of 99.4% (95% CI 98.3;100) (Fig. 2A). Cohen's kappa for the cut-off < 50 % was 0.62 (0.42-0.82) and on the adjusted cut-off (< 84.3%) was improved to 0.92 (0.83–1) (Table 2). Due to the data being unbalanced (with a higher ratio of negative results compared to positive results) the performance of the filter papers were further evaluated using a PRC

Table 1
Summary of serological assay on (A) serum samples and whole blood dried on Nobuto Filter Strips (NFS) from samples collected in Tanzania, (B) serum samples and whole blood dried on chromatography paper (CP) from samples collected in Tanzania, and (C) serum samples and whole blood dried on NFS from samples collected in Pakistan. Samples were considered as positive when competition percentage in the cELISA was < 50 %.

A

NFS	Serum		
	Positive	Negative	Total
Positive	11	0	11
Negative	10	175	185
Total	21	175	196

B

CP	Serum		
	Positive	Negative	Total
Positive	11	0	11
Negative	10	175	185
Total	21	175	196

C

NFS	Serum		
	Positive	Negative	Total
Positive	40	0	40
Negative	8	12	20
Total	48	12	60

(Fig. 2B). The PRC gives the precision (equal to the positive predictive value, PPV) for all values of recall (equal to the sensitivity of the test). A perfect test would have a precision and recall of 1 and form a 90° angle in the right upper corner of the graph and an AUC of 1. Our test shows an excellent performance level both on NFS (AUC 0.938) and CP (AUC 0.925) (Saito and Rehmsmeier, 2015).

The results from the samples from Pakistan were analysed accordingly. The ROC-curve had an AUC of 0.996 and a new cut-off of < 69.0% (Fig. 3A). This new cut-off had a sensitivity of 97.9% (95% CI 93.8;100) and a specificity of 100% (95% CI 100;100). The un-adjusted Cohen's kappa was calculated to be 0.77 (0.58-0.96), and with the adjusted cut-off (< 69%) improved to 0.95 (0.85–1) (Table 3). The results in Pakistan were also unbalanced, however with a higher ratio of positive animals compared to negative results, giving both very high precision and recall in the PRC (AUC 0.999) (Fig. 3B).

4. Discussion

Since it was first described in 1942 (Gargadennec and Lalanne, 1942), Peste des petits ruminants (PPR) has spread to most of Africa and Asia. These areas hold the majority of the world's sheep and goat population, with an estimated 1.7 billion animals at risk (FAO and OIE, 2015). One of the major obstacles for the control and eradication of PPR is the transport of samples from remote areas to laboratory facilities for testing. Especially the cold chain can be difficult to maintain, which is important to preserve the sample and prevent the proteins to degrade. Here we used filter papers as a highly cost-effective method for transport of whole blood samples. Furthermore we have optimized the use of these papers in a cELISA for serological diagnosis of PPR (Libeau et al., 1995). The filter papers performed well in the analysis when compared to the results from corresponding serum samples tested in the same cELISA. The AUC of the ROC curve was > 0.98 in all three examples

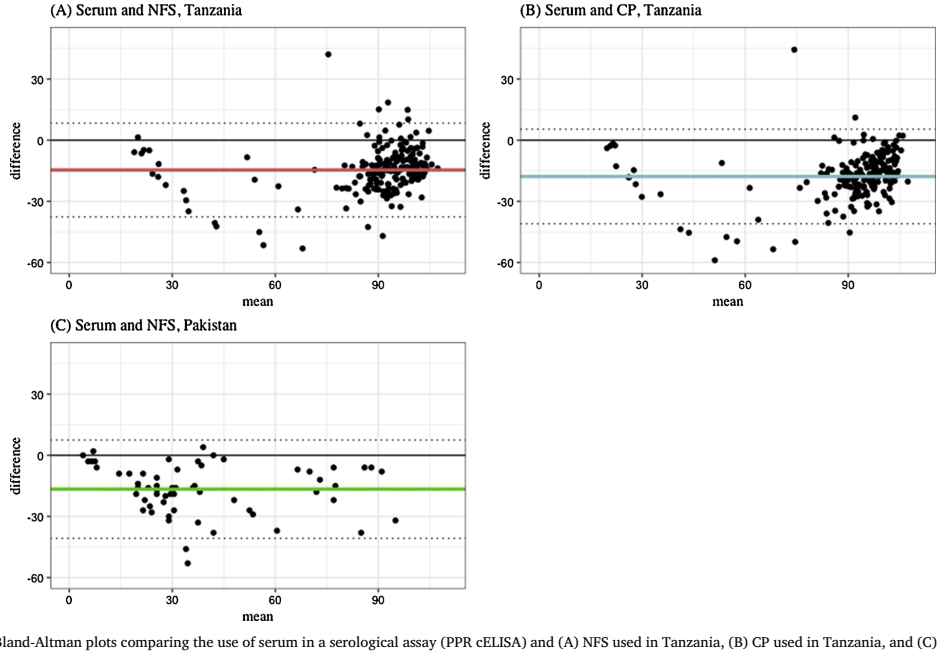

Fig. 1. Bland-Altman plots comparing the use of serum in a serological assay (PPR cELISA) and (A) NFS used in Tanzania, (B) CP used in Tanzania, and (C) NFS used in Pakistan. Coloured line represents the mean bias (A: -14.65, B: -17.78 C: -16.63), dotted line represents the highest and lowest limit of agreement (2 *sd* from the mean bias).

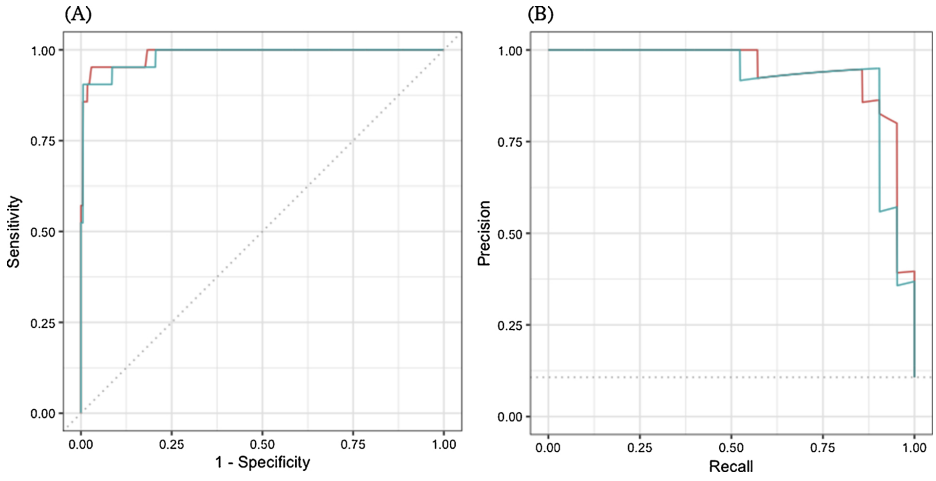

Fig. 2. (A) Receiver operating characteristic curve and (B) Precision Recall curve for samples collected in Tanzania with the results from serological assay (PPR cELISA) performed on serum as the true positive and negative (cut-off < 50%). Red line represent samples stored on Nobuto filter strips (NFS), and blue line represents samples stored on chromatography paper (CP) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Figs. 2A and 3 A), which is considered as an excellent accuracy of the test (Fawcett, 2006). ROC curves can however be misleading when used on unbalanced data (data with a higher proportion of either negative or positive results), and PRC was therefore produced as a complement (Figs. 2B and 3 B). In a PRC it is possible to see the precision (equal to the positive predictive value, PPV) for all values of recall (equal to the sensitivity of the test). The sensitivity values are plotted along the x-axis and the PPV along the y-axis. A perfect test would have a precision and recall of 1 and form a 90° angle in the right upper corner of the graph, and an AUC of 1, our three tests scored an AUC between 0.925–0.999. Additionally, Cohen's kappa was used to measure the agreement between results from analysis performed on serum and analysis performed on filter papers. Before adjusting the cut-off, Cohen's kappa for NFS was 0.66 in Tanzania, 0.77 in Pakistan, and for CP in Tanzania it was 0.62. These values are considered as a moderate to substantial agreement (McHugh, 2012). When adjusting the cut-off and re-calculating Cohen's kappa values they were raised to 0.85, 0.95, and 0.92, respectively, which are considered a near perfect agreement (a value of 1 is seen as a perfect agreement) (McHugh, 2012). This demonstrates how important it is to adjust the cut-off when using filter papers instead of serum in the analysis.

Sensitivity and specificity were over 90% in all examples when the cut-off value for a positive result was adjusted. The NFS gave a higher sensitivity and specificity in both countries (Tanzania: 95.2% and 97.2%, Pakistan: 97.9% and 100%) compared to the CP used in Tanzania (90.5% and 99.4%). The NFS have been optimized for

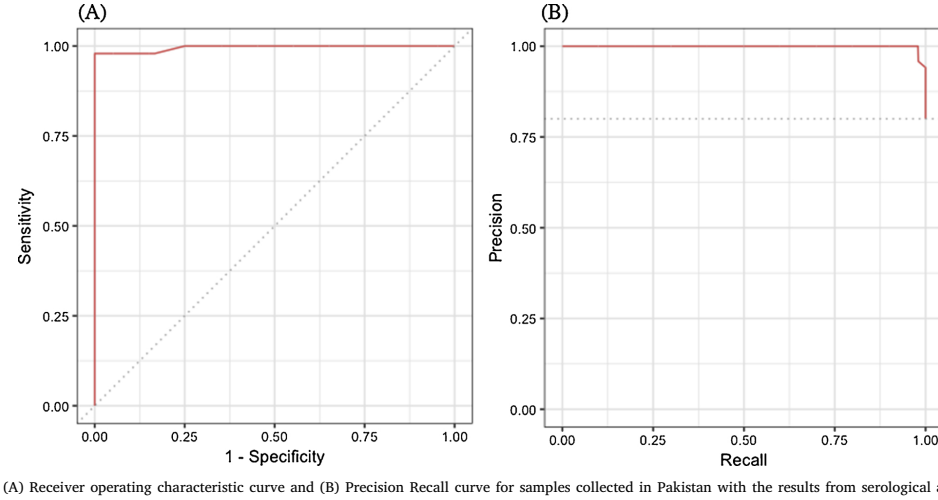

Fig. 3. (A) Receiver operating characteristic curve and (B) Precision Recall curve for samples collected in Pakistan with the results from serological assay (PPR cELISA) performed on serum as the true positive and negative (cut-off < 50%).

Table 2
Results from serological assay (cELISA) from samples collected in Tanzania on serum, Nobuto filter strips (NFS), and chromatography paper (CP) using both the cut-off suggested by the manufacturer (< 50 % = positive for PPRV antibodies) and the adjusted cut-off. Cohen's kappa comparing the NFS and CP to the results from serum using both cut-offs.

	Serum < 50 %	NFS < 50 %	NFS < 84.6 %	CP < 50 %	CP < 84.3 %
Positive	21 (10.7 %)	11 (5.6 %)	25 (12.5 %)	11 (5.6 %)	20 (10.2 %)
Negative	175 (89.3 %)	185 (94.4 %)	175 (87.5 %)	185 (94.4 %)	176 (89.8 %)
Total	196	196	196	196	196
Cohen's kappa		0.66 (0.47-0.85)	0.85 (0.74-0.97)	0.66 (0.47-0.85)	0.92 (0.83-1)

Table 3
Results from serological assay (cELISA) from samples collected in Pakistan on serum and Nobuto filter strips (NFS) using both the cut-off suggested by the manufacturer (< 50 % = positive for PPRV antibodies) and the adjusted cut-off. Cohen's kappa comparing the NFS to the results from serum using both cut-offs.

	Serum	NFS (< 50 %)	NFS (< 69 %)
Positive	48 (80.0 %)	40 (66.7 %)	47 (78.3 %)
Negative	12 (20.0 %)	20 (33.3 %)	13 (21. 7 %)
Total	60	60	60
Cohen's kappa		0.67 (0.46-0.87)	0.95 (0.85-1)

transport and storage of clinical samples and is expected to perform better, however the CP is much cheaper (around 30 times less per sample when using the CP in 10*50 mm pieces). The CP still performed satisfactory, so therefore we consider it a possible option in circumstances where budget is an issue.

The adjusted cut-off differed between the two countries: in Tanzania (NFS) it was adjusted to 84.6 % and in Pakistan to 69.0%. This is due to the higher positive proportion in Pakistan, 80.0% versus 10.7% in Tanzania. Previous studies in Tanzania have found a similar or slightly higher prevalence, however, performed in animals of all ages (Kgotlele et al., 2016; Torsson et al., 2017; Muse et al., 2012). In Pakistan, previous studies have found a similar prevalence or slightly lower (Zahur et al., 2008, 2011; Abubakar et al., 2017). In a high prevalent area, the sensitivity of a test needs to be higher, to avoid a large number of false negatives. To increase the sensitivity in the used ELISA the cut-off was lower in the high prevalent area (Pakistan) compared to the low prevalent area (Tanzania). The final decision on the adjusted cut-off must depend on the aim of the test and where the test is going to be used. When used for a screening purpose a higher cut-off is suggested to detect potentially infected animals as early as possible, which is desirable during the eradication program. As the prevalence in the population decreases so will also the positive predictive value of the test. To increase the positive predictive value one could target the sampling to risk groups, use a test with a higher specificity, or use an additional test and interpret the results in series. The optimum cut-off also depends on the frequency distribution of the test variable in the healthy and diseased populations (Thrusfield, 2007). Moreover, even for a certain diagnostic test, the cut-off value is not universal and should be determined for each region and for each disease condition (Pfeiffer, 2010; Habibzadeh et al., 2016).

These results demonstrate that filter papers are an acceptable and cost-effective transport method of whole blood samples for later use in serological analysis. Here we dipped the filter paper in blood collected in a serum tube. In a field setting we would recommend to take the blood samples using only an injection needle, letting the blood drip down on the filter paper (excluding the need of a serum tube), which is then left to dry away from direct sunlight. Filter papers could be used, and perform well, in low-income and large countries, where access to laboratory facilities is limited or available only at a great distance and where the cold chain is difficult to maintain. It is however important to adjust the cut-off value for a positive result when using filter papers, and as we have shown here by performing the study in two different countries, in a low prevalent setting the cut-off may need to be adjusted to a higher degree than in a high prevalent setting.

Conflict of interest

The authors declare that they have no conflict of interest.

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III

Field-adapted full genome sequencing of peste-des-petits-ruminants virus using Nanopore sequencing

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Abstract

Peste-des-petits-ruminants virus (PPRV) is currently the focus of a control and eradication program. Full genome sequencing has the opportunity to become a powerful tool in the eradication program by improving molecular epidemiology and the study of viral evolution. PPRV is prevalent in many resource-constrained areas, with long distances to laboratory facilities, which can lack the correct equipment for high-throughput sequencing. Here we present a protocol for full, or near full, genome sequencing of PPRV. The use of a portable miniPCR and MinION brings the laboratory to the field and makes the production of a full genome possible within 24 hours of sampling. The protocol has been successfully used on virus isolates from cell cultures and field isolates from tissue samples of naturally infected goats.

Keywords: peste-des-petits-ruminants virus, eradication, molecular epidemiology; full genome sequencing, minion, miniPCR

Introduction

With the development of new and portable sequencing equipment, it is now possible to perform—directly in the field or in very basic laboratories—sequencing that was previously limited to well-equipped laboratories (Krehenwinkel *et al.*, 2019; Rambo-Martin *et al.*, 2019; Faria *et al.*, 2016; Gardy *et al.*, 2015). With a small thermocycler such as the miniPCR (Amplyus, Cambridge, United States), the hand-held MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom), and portable computational resources, full genome sequencing and advanced molecular epidemiology can be performed in almost any setting (Krehenwinkel *et al.*, 2019; Rambo-Martin *et al.*, 2019; Faria *et al.*, 2016; Gardy *et al.*, 2015). This is highly advantageous for the diagnosis and control of viral diseases. This approach enables rapid sequencing-based technologies in resource constrained environments, in addition to bringing the laboratory analysis closer to the disease outbreak and reducing the time from diagnosis to full genome and epidemiological investigations.

Peste des petits ruminants (PPR) is a highly contagious and deadly disease in small ruminants (Parida *et al.*, 2015). The cause is the peste-des-petits-ruminants virus (PPRV), a single-stranded negative-sense RNA virus belonging to the genus *Morbillivirus* (Gibbs *et al.*, 1979). Other morbilliviruses include canine distemper virus, measles virus, feline morbillivirus, marine morbilliviruses, and the now eradicated rinderpest virus (RPV) (Woo *et al.*, 2012). PPR has a large socioeconomic impact, as small ruminants are mainly kept by vulnerable populations that depend on their animals for income and livelihood. Due to this, the Food and Agriculture Organization of the UN (FAO) and the World Animal Health Organization (OIE) have launched a control and eradication program for PPRV to eliminate the disease by 2030 (FAO & OIE, 2015). To reach this goal, accurate and well-functioning diagnostic and epidemiological tools need to be in place (FAO, 2013). The Global Strategy for Control and Eradication of PPR (FAO & OIE, 2015) highlights that countries in stage 2 in the program (out of four stages), have to strengthen laboratory capacity with molecular methods able to better characterize the collected virus isolates (FAO & OIE, 2015). Use of the full genome to characterize isolates, rather than only a partial sequence or genetic marker, ensures detection of important changes within the genome (Ladner *et al.*, 2014).

PPRV is widely distributed in Africa and Asia. In many of these areas, an efficient transport of samples, with prompt delivery and unbroken cold chain to a laboratory with the correct equipment, is hard to achieve (OIE, 2019; FAO, 2013). A broken cold chain during sample transport risks degradation of the sensitive

nucleic acid of single-stranded RNA viruses. Analyses performed as close as possible to the sample collection site avoids these long transports (Wohl *et al.*, 2016). More accessible, less expensive, and more timely full genome sequencing will lead to better comprehensive surveillance and detection in the control of a disease such as PPR. The implementation of these mobile methodologies for molecular epidemiology will also increase the chances for a successful eradication.

Here we have developed a protocol for a quick, on-site, field-adapted full genome sequencing of veterinary significant virus diseases, with PPRV as an important example. The protocol uses the highly portable miniPCR thermocycler and the MinION sequencer.

Material and methods

The full wet lab protocol is available at
DOI: dx.doi.org/10.17504/protocols.io.pnxdmfn

Samples

A selection of samples of different origins and quality was used to verify the protocol. These included: i) viral RNA collected from a cell-culture grown virus (Vero-SLAM cell line), isolate Nigeria 75/1, kindly provided by Dr. Siamak Zohari, National Veterinary Institute (SVA), Uppsala, Sweden; ii) RNA from field samples representing all currently known lineages of PPRV (cultured on the CV-1-SLAM cell line), kindly provided by Dr. William G. Dundon, International Atomic Energy Agency (IAEA), Vienna, Austria, (KP789375 (Dundon *et al.*, 2015b), KR781450, KR781449 (Adombi *et al.*, 2017) and KM463083 (Dundon *et al.*, 2015a)); and, iii) two field isolates (tissue) collected by Tebogo Kgotlele and Prof. Gerald Misinzo from an outbreak in goats in Dakawa, Morogoro region, Tanzania, in 2013 (Kgotlele *et al.*, 2014).

Primer design

Two sets of multiplex full-genome primers were designed using Primal Scheme (http://primal.zibraproject.org) (Quick *et al.*, 2017). One primer set had an amplicon length of 800 base pairs (bp) and an overlap of 100; the other primer set had an amplicon length of 600 bp and an overlap of 40. Primers were designed using eight full genome sequences representing all known lineages available at the NCBI GenBank (Table 1). Primers, for the 600-bp and 800-bp amplicons, are available in the supplementary material (Tables S1 and S2).

RNA extraction, cDNA synthesis and PCR amplification

QIAamp Viral RNA Mini kit (Qiagen) was used according to the manufacturer's instructions to extract RNA from tissue samples from Tanzania (sample type iii). However, other samples shared with us were extracted RNA. cDNA synthesis was performed using Superscript IV First-Strand Synthesis System (Invitrogen) with 11 µl of RNA, according to the manufacturer's instructions. PCR amplification was performed using the Q5 Hot Start High Fidelity Polymerase (New England BioLabs) according to the protocol in (Quick *et al.*, 2017). The protocol divided the multiplex primers into pool 1 with 12 primer pairs, and pool 2 with 13 primer pairs, and was run on the miniPCR thermocycler. The amplicons were then purified using AMPure XP magnetic beads (Beckman Coulter) or HighPrep PCR Clean-up System (MagBio Genomics Inc.) with a 1.8x bead ratio and quantified using Qubit 1.0 Fluorometer dsDNA HS assay (Thermo Fisher Scientific). When needed, the amplification was verified with 1% agarose gel electrophoresis, 6-7 V/cm, 50-60 minutes.

Table 1. *Complete genomes used to generate the multiplex primers with the Primal scheme.*

Accession no.	Lineage	Country	Year
EU267273.1	I	Cote d'Ivoire	1989
KR781451.1	II	Cote d'Ivoire	2009
KR828814.1	II	Nigeria	2012
X74443.2	II	Nigeria	1975
KJ867540.1	III	Ethiopia	1994
KJ867543.1*	III	Uganda	2012
KJ867541.1	IV	Ethiopia	2010
KR828813.1	IV	Nigeria	2013

*First genome in file

Nanopore library preparation and sequencing

The library was prepared using the SQK-LSK109 Ligation Sequencing Kit and EXP-NBD104 Native Barcode expansion according to (Quick *et al.*, 2017) and with modifications according to (Hu & Schwessinger, 2018). The purified PCR amplicons were repaired and A-tailed using the NEBNext Ultra II End Repair/dA-Tailing module (New England BioLabs). Native barcodes and adaptors were ligated to amplicons using Blunt/TA Ligase Master Mix (New England BioLabs). The library was then sequenced on a MinION Flowcell R9.4. for 10 hours.

Data analysis

The reproducible protocol is available at GitHub github.com/Ackia/Field_Seq. In short, raw reads were basecalled using GUPPY (version 3.1.5.) (Oxford Nanopore Technologies). Read-set composition and quality were assessed using plots produced by PycoQC (Leger & Leonardi, 2019). Demultiplexed read-sets were checked for purity using Kraken 2, and results were visualised in Pavian (Wood *et al.*, 2019; Breitwieser & Salzberg, 2016). The read-sets were aligned to a suitable reference genome (RefSeq

assembly accession: GCF_000866445.1) using minimap2 (Li, 2018). The resulting alignment file was sorted and converted into an index bam-file for further processing with samtools (Li *et al.*, 2009). The consensus sequence was extracted using UGENE (Hahne & Ivanek, 2016; Okonechnikov *et al.*, 2012).

Phylogeny

Phylogenies were created using three approaches. First, the commonly used marker-gene fragment from the nucleocapsid protein was extracted from the consensus sequences previously extracted with UGENE. The fragments were then aligned using MAFFT (Katoh *et al.*, 2002), and a tree was constructed with Mrbayes (Ronquist *et al.*, 2012; Huelsenbeck & Ronquist, 2001) with the HKY85 substitution model and standard settings in the UGENE implementation. Following this, the WGS sequence of the genomes was aligned using MAFFT and the tree constructed the same way. Finally, DENDROSCOPE was used to calculate and create a tanglegram comparing the two trees (Huson *et al.*, 2007). Four of the isolates kindly provided by Dr. Dundon was previously published (KP789375, KR781450, KR781449, and KM463083). These full genome sequences, produced with the Sanger sequencing technique, were compared to the genomes created with this method in a separate tanglegram using the same approach as described above.

Results

Gel electrophoresis following PCR amplification of Nigeria 75/1 virus cultured on Vero-SLAM cells showed two bands—one very clear at 800 bp, and a second, slightly larger and weaker band at approximately 2400 bp (Figure 1). These longer amplicons are not seen on the gel electrophoresis image for the Tanzanian field samples. However, a strong band is seen at 800 bp. For the samples cultured on CV-1 cells, the gel electrophoresis image shows a narrow band at 800 bp, together with a wide selection of bands of all sizes.

Sequencing of the Nigeria 75/1 isolate produced 741,787 raw reads for the 800-bp primer set and 629,875 raw reads for the 600-bp primer set. The 800-bp primers gave a genome coverage of 98.4 % and an average coverage of 4602 reads, whereas the 600-bp primers produced a genome coverage of 99.5 %, with an average coverage of 4586 reads (Table 2). Following this first evaluation of the primer sets, we found that the 800-bp primer set gave more even coverage of the PPRV genome, including a higher coverage of the ends of the genome. On the basis of this result, we decided to continue working with only the 800-bp amplicon primer set for further samples.

The Nigeria 75/1 isolate, the first trial sample, was run in duplicate to see if the results were reproducible and equal between samples within one run.

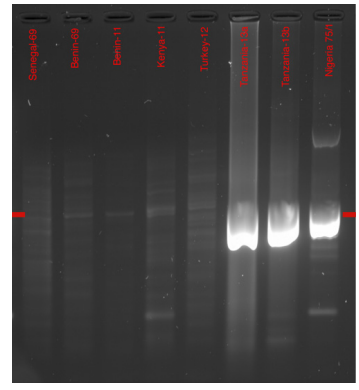

Figure 1. Gel electrophoresis of purified 800-bp PCR amplicons. The red marker indicates the 800-bp size marker.

They were highly comparable and are presented as a mean of the duplicates (Table 2). A total of 672,805 reads was mapped to the PPRV genome to give a coverage (above 50x) of 98.4% of the full genome (Table 2). For the isolates cultured on CV-1 cells, the protocol was run using the 800-bp multiplex primers. The total number of raw reads varied between 354,531 and 1,123,782; however, most reads did not map against the PPRV reference genome (Table 2). Despite this, an average of 69.4% of the genome was covered above 50x.

For the two field isolates from Tanzania, the sequencing results were 947,742 and 1,418,713 raw reads, respectively, out of which 771,053 and 1,197,778 reads mapped to the PPRV reference genome (Table 2). For these isolates, 91.9% and 93.5% of the genome had a coverage above x50.

Phylogenetic trees were produced using both the full genome sequences and the 255 nucleotides at the 3' end of the nucleoprotein gene. These 255 nucleotides are what are currently used for phylogenetic analysis and separation into lineages I-IV (Kwiatek *et al.*, 2007). The sequences for the four previously published isolates were all produced using the Sanger sequencing technique: Senegal -69 KP789375, Benin -69 KR781450, Benin -11 KR781449, and Kenya -11 KM463083. These full genomes were compared to the sequences produced in this study (Figure 2). The placement of the isolates within the phylogenetic trees did not change, when using either the genetic marker or the full genome. However, a slight change was seen in the branches, removing one node and adding another one in the tree for the full genomes (Figure 2). A phylogenetic tree comparing the usage of only the phylogenetic marker to the full genome of sequences in this study, produced a tree with higher resolution for the full genomes, but did not change the placement of the isolates (Figure 3).

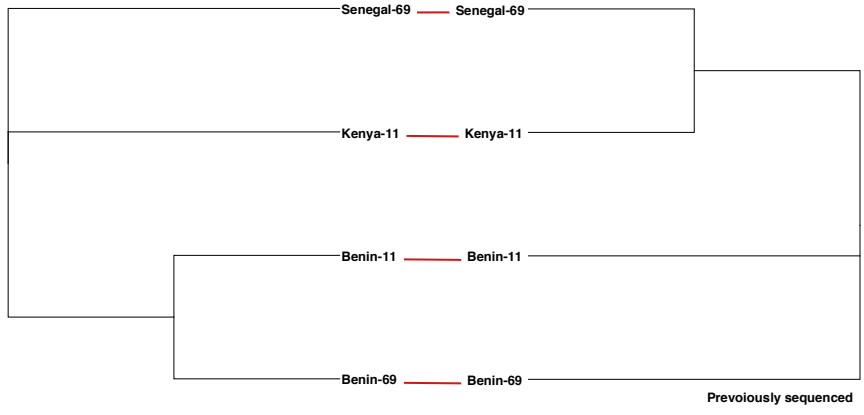

Figure 2. Tanglegram comparing the phylogenetic trees produced using the full genomes from our study (left) with the exact same isolate previously sequenced using the Sanger sequencing method (right) Isolates: Senegal -69 KP789375, Benin -69 KR781450, Benin -11 KR781449, and Kenya -11 KM463083.

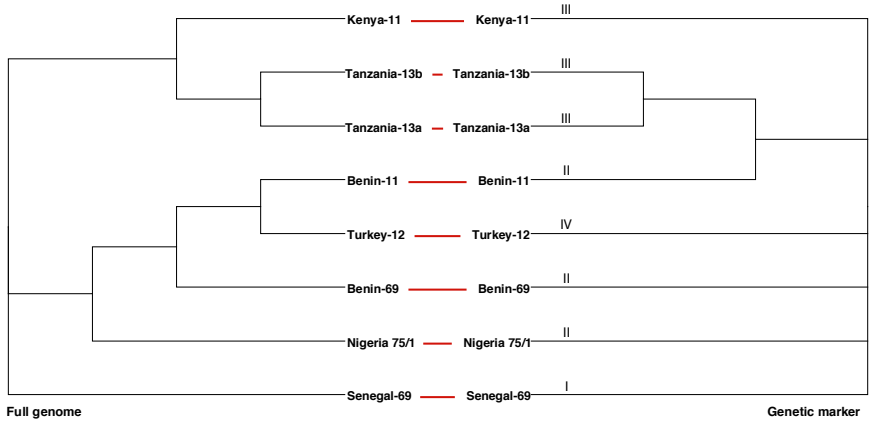

Figure 3. Tanglegram comparing phylogenetic trees built using either the full genome produced in our study (left) or the extracted, commonly used genetic marker, partial sequence of the nucleoprotein gene (right). Roman numerals on the right hand tree indicate the previously reported lineage.

Table 2. *Results from sequencing using the Oxford Nanopore MinION sequencer.*

Sample	Raw reads	Total bp	N50 length (bp)	Reads mapped to PPRV	Average coverage	Genome coverage > 50x (%)	Genome coverage > 25x (%)	Source
Nigeria 75/1* (800 bp)	741,787	660,217,802	870	672805	4601	98.6	99.4	Cultured on Vero-SLAM
Nigeria 75/1 (600 bp)	629,875	500,972,391	630	597110	4586	99.5	99.5	Cultured on Vero-SLAM
Senegal -69	721,283	483,015,988	753	10196	416	49.6	71.8	Cultured on CV-1**
Benin -69	945,266	619,883,689	826	35716	554	78.9	87.5	Cultured on CV-1**
Benin -11	354,531	221,621,251	779	47828	460	66.4	79.2	Cultured on CV-1**
Kenya -11	1,123,782	662,242,080	736	178526	2311	85.0	88.8	Cultured on CV-1**
Turkey-12	776,693	500,690,835	748	11554	493	67	79.8	Cultured on CV-1**
Tanzania -13a	947,742	707,688,820	782	771053	4340	91.2	93.0	Field isolate
Tanzania -13b	1,418,713	1,089,046,940	780	1197778	4506	93.5	93.5	Field isolate

*Mean from duplicate runs

** Stably transfected with plasmid expressing the goat SLAM receptor

Discussion

Here we have presented a protocol for full genome sequencing of the peste-des-petits-ruminants virus (PPRV) using the miniPCR thermocycler and Oxford Nanopore MinION. Both are suitable for use in the field or a minimally equipped laboratory facility. PPRV is currently the target of a control and eradication program, launched by the FAO and OIE in 2015, with a goal of eradication by 2030 (FAO & OIE, 2015). The success of this program depends on vaccination campaigns and the ability to trace the source of an outbreak (FAO & OIE, 2015). PPRV often occurs in areas that lack infrastructure and laboratory facilities (OIE, 2019), making it difficult to reach a quick diagnosis or do adequate epidemiological investigations. Moreover, long transports of samples increase the risk of degrading the sensitive viral nucleic acid in the sample, leading to false negative results (Parida *et al.*, 2015). By bringing the laboratory closer to the outbreak, these risks are minimized and the time from recognizing clinical signs to a molecular epidemiological investigation is significantly reduced.

The proposed protocol does not require an expert laboratory- or sequencing technician, but it does need a basic understanding of contamination avoidance and handling of laboratory equipment. We estimate that, assuming previous training in basic pipetting skills, this protocol can easily be performed following one full run-through auscultation. The loading of reagents to the MinION flow cell requires the most practice, which can be done on used flow cells, or this single step can be performed by more experienced personnel. The time needed to run the full protocol, from the purification of RNA to analysed sequences, is around 22-24 hours (Figure 4).

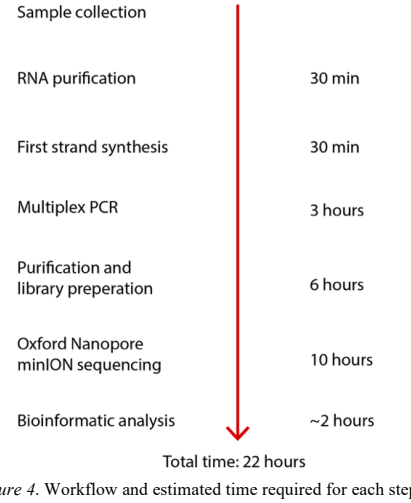

Figure 4. Workflow and estimated time required for each step of the protocol.

Table 3. *Reagents used within the protocol, with cost calculations based on prices stated on suppliers' homepages in September 2019*

Reagent	Product number	Source	Cost/Unit	Cost/Sample (USD)
RNA extraction			variable	
SuperScript IV First-Strand Synthesis System	18091050	ThermoFisher Scientific	USD 2978 (200 reactions)	14.89
Multiplex primers		SigmaAldrich	variable, our 800-bp primers cost USD 158	0.02
Q5 Hot Start High-Fidelity DNA polymerase	M0493L	New England Biolabs	USD 532 (500 reactions)	1.10
dNTPs (10 µM each)	R0192	ThermoFisher Scientific	USD 88 (1 ml)	0.13
HighPrep™ PCR Clean-up System	AC-60050	MagBio Genomics	USD 526 (50 ml)	1.40
Qubit dsDNA HS assay kit	Q32854	ThermoFisher Scientific	USD 289 (500 reactions)	1.73
NEBNext Ultra II End Repair/dA-Tailing Module	E7546L	New England Biolabs	USD 795 (96 reactions)	4.10
Native Barcoding Expansion 1-12	EXP-PBC001	Oxford Nanopore	USD 288	4
Blunt/TA Ligase Master Mix	M0367L	New England Biolabs	USD 520 (250 reactions)	20.80
Ligation Sequencing Kit (incl. FlowCell priming Kit)	SQK-LSK109	Oxford Nanopore	USD 599	8.30
MinION Flow Cell	R9.4.1	Oxford Nanopore	USD 500-900 /flow cell, depending on the quantity ordered*	42
Total				USD 98.5

*Possible to wash up to 5 times, then USD 8.4/sample and total USD 81/sample (including the cost of Flow Cell Wash kit)

The protocol here does not include the time for RNA purification. In a field setting, either a spin column protocol using a small battery-driven centrifuge would be a good option or a magnetic bead-based system (as the latter is also needed in other steps of the protocol). Table 3 gives a full list of reagents and cost calculation. With our protocol, a full genome is possible to produce for under USD 100 per sample. Washing and reusing the flow cells reduces the cost even further, to around USD 80 per sample.

With good quality virus isolates, this protocol performed well and yielded a full genome with a mean coverage of around 4500 reads. To standardize the quality assessment of the many new high-throughput sequences being produced, Ladner et al. (2014) suggests five standard sequenced viral genomes could be placed in (Ladner *et al.*, 2014). For molecular epidemiology, they suggest the standard "Coding complete", which means 90-99% of the genome is sequenced with no gaps, all open reading frames (ORFs) are complete, and the coverage is 100x. The sequences produced using our method more than meet these requirements when the virus isolates are of good quality.

In the isolates cultured on CV-1 cells, we did not get equally good coverage over the full genome as we did for the Nigeria 75/1 and Tanzanian isolates (Table 2). The majority of the reads from the CV-1 samples instead mapped against the human genome. We suspect this is due to the low concentration of viral RNA, degradation of the viral genomes in the samples, and that the human sequences were mistakenly interpreted as such but in fact had originated from the CV-1 cells (African Green monkey kidney cells). Even though this is not a perfect result, it shows how this protocol works with degraded and damaged samples. Despite the reduced coverage of the genome, we were able to extract

49.6-85.0% of the full genomes in these five samples with an average coverage well above 100x for them (Table 2).

By comparing the consensus sequences produced by the described protocol with previously published sequences produced using the Sanger sequencing technique, we were able to evaluate the performance of the protocol in phylogenetic analysis (Figure 2). We found that the isolates retained their placement within the tree, but altered the branching slightly. The sequences did not distinguish the isolates from Kenya and Senegal as separate nodes. However, a new node was added between the two isolates from Benin (Figure 2). Slight changes in sequence composition, as well as inherent differences in sequencing methodology, might explain this minor change in the phylogeny. Our protocol did not produce an equally high coverage of the ends of the genome which can affect the phylogenetic analysis slightly (Shrivastava *et al.*, 2018). This change in coverage does not critically change placement within the tree (Ladner *et al.*, 2014).

A common practice is to use only the genetic marker, the partial nucleoprotein sequence, to study the phylogeny of a PPRV isolate. This increases the risk of missing important changes in the genome outside of the marker, but these changes could be important indications in the transmission routes and the virus evolution (Ladner *et al.*, 2014). A comparison of the full genome sequences with the genetic marker extracted from the same, placed isolates on the same branch (Figure 3). Nevertheless, the full genomes provide a much higher resolution to the phylogenetic tree and enable use of advanced phylogenies such as those produced by alignments with VIRULIGN (Libin *et al.*, 2018). The isolates used to verify our protocol are from very different timepoints and geographic regions. If the sequences had belonged to an ongoing outbreak within the same area, this improved resolution of the phylogenetic tree could help determine the start and transmission route of the outbreak. It would also have made it possible to track the outbreak in real-time using tools such as Nextstrain (Hadfield *et al.*, 2018; Wohl *et al.*, 2016). For such analyses during outbreaks, the viruses need to be thoroughly sequenced. With our protocol, the production of complete genomes from PPRV field isolates are simplified and will hopefully lead to more full genomes being produced and published.

The use of full genome sequencing for epidemiology and disease surveillance is dependent on the sharing of data and the uploading of the sequences to freely available databases. A genome sequence viewed in isolation can only give limited information (Gardy *et al.*, 2015). Currently, there are 74 complete PPRV genomes available in the NCBI GenBank. Only two are isolated from a wild ruminant: a Dorcas gazelle from a zoological collection in the United Arab Emirates in 1986 (Muniraju *et al.*, 2014; Furley *et al.*, 1987), and a Capra Ibex in China in 2015 (Zhu *et al.*, 2016). One of the questions in PPR epidemiology is the role of wild ruminants in the spread of the disease. Identified cases in African wildlife are so far considered to be spill-overs from domestic animals, but outbreaks of PPR have occurred several times in Asian wildlife (Aguilar *et al.*, 2018). With more full genome sequences available, this question could be solved.

In conclusion, we have presented a field-adapted, easy to follow, protocol for full genome sequencing of PPRV using the miniPCR thermocycler and the MinION sequencer. With high-quality isolates the protocol produces a near-complete genome for less than USD 100 per sample. We hereby hope to increase the number of complete genomes available for PPRV. More genomes would allow evaluation of the virus evolution and more precise molecular epidemiological investigations. In addition, they would provide a basis for vaccine and drug development (Faria *et al.*, 2016).

Supplementary Materials

Table S1: Primer sequences for 800-base pair amplicons, Table S2: Primer sequences for 600-base pair amplicons.

Author Contributions

Conceptualization, E.T. and O.K.L.; formal analysis, E.T., T.K. and O.K.L; writing—original draft preparation, E.T.; writing—review and editing, O.K.L, J.J.W., M.B.; visualization, O.K.L.; supervision, M.B., G.M., J.J.W.; funding acquisition, E.T., J.J.W. and O.K.L.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. *Primer sequences for 800 base pair amplicons*

Primer	Sequence	Pool	Tm	GC%	Start	End	Amplicon size
PPRV_1_LEFT	CCAAACAAAGTTGGGTAAGGATAGATCT	1	61.22	39.29	1	29	740
PPRV_1_RIGHT	AAAGCGGAATTCCCCAATCACC	1	61.47	50.00	740	718	
PPRV_2_LEFT	CCTCCATACTGGCACAAGTTTGG	2	61.68	52.17	609	632	792
PPRV_2_RIGHT	ATGCATGTGACTCTCCCTCTCC	2	61.55	54.55	1401	1379	
PPRV_3_LEFT	TCACAGCAGAGGAAGCCAAACT	1	62.14	50.00	1263	1285	762
PPRV_3_RIGHT	GATTTCGCCTGATCCGATTGCT	1	61.38	50.00	2025	2003	
PPRV_4_LEFT	AGAGATACCCTTGAAAGCTGGAGG	2	61.67	50.00	1893	1917	770
PPRV_4_RIGHT	GGCAACTCCTGGATCGTCTTTG	2	61.51	54.55	2663	2641	
PPRV_5_LEFT	ACGGTGACAGAGTGTTCATCGA	1	61.65	50.00	2526	2548	816
PPRV_5_RIGHT	GGTGCATTACGGTTGCTTAGCA	1	61.77	50.00	3342	3320	
PPRV_6_LEFT	CAAGCAAGCTCAACATTGATCACA	2	60.56	41.67	3214	3238	792
PPRV_6_RIGHT	ATATTGAAGGCGACGGCATTGG	2	61.64	50.00	4006	3984	
PPRV_7_LEFT	CGCTGTCTTTGTTAACACCTTGGA	1	61.74	45.83	3789	3813	734
PPRV_7_RIGHT	GGGAGCCTTGGAGTGTGTTTTT	1	61.28	50.00	4523	4501	
PPRV_8_LEFT	TCAGTACCCCAAGAATTCCGTGT	2	61.58	47.83	4370	4393	822
PPRV_8_RIGHT	TTTTTGTGGCGGGGTTGGATTC	2	62.18	50.00	5192	5170	
PPRV_9_LEFT	GAAGACACCCAACCACCGAAAC	1	61.89	54.55	4971	4993	816
PPRV_9_RIGHT	ACAGAGCATCCTCGACAGGTTT	1	61.67	50.00	5787	5765	
PPRV_10_LEFT	CGAGCCAACAAACCCTGGTTAT	2	61.14	50.00	5658	5680	787
PPRV_10_RIGHT	CATTCTTGTGCCCCGATGTTGT	2	61.72	50.00	6445	6423	
PPRV_11_LEFT	AAAGGCCCGAGTCACCTATGTG	1	62.32	54.55	6307	6329	812
PPRV_11_RIGHT	TGAGCCCTGGGTTGATCTTAGG	1	61.49	54.55	7119	7097	
PPRV_12_LEFT	CCCTTCAGCGGCAATTTGTACA	2	61.71	50.00	6983	7005	770
PPRV_12_RIGHT	GGAGGATTCATACACCACCGGA	2	61.55	54.55	7753	7731	
PPRV_13_LEFT	TCATCGGTGATGAAGTCGGCAT	1	62.11	50.00	7617	7639	807
PPRV_13_RIGHT	CTACCCAATTGGCCTCGTTGTC	1	61.51	54.55	8424	8402	
PPRV_14_LEFT	ACTTAATCTAGCCGGGCCTACTC	2	61.32	52.17	8293	8316	752
PPRV_14_RIGHT	GCCAGGGGAAACACTCTATCCT	2	61.49	54.55	9045	9023	
PPRV_15_LEFT	ATGTTTCCAGGAGCGATCATGC	1	61.33	50.00	8913	8935	774
PPRV_15_RIGHT	TGCCGTTGACTCTTCTGAGACA	1	61.59	50.00	9687	9665	
PPRV_16_LEFT	TCTGTTTTATATCAGGGACGGCTG	2	60.44	45.83	9565	9589	765
PPRV_16_RIGHT	TCCCCAGTTAAGTGTATATCGTGGG	2	61.68	48.00	10330	10305	
PPRV_17_LEFT	GGTGCATTCTTGGATCACTGCT	1	61.20	50.00	10190	10212	831
PPRV_17_RIGHT	CGCCATTCCATTGTCTCGGAAA	1	61.51	50.00	11021	10999	
PPRV_18_LEFT	AGAGATAAAAGAGACAGGGCGACT	2	61.12	45.83	10897	10921	788
PPRV_18_RIGHT	CTGCTCTAGATGCCTCCCTCTT	2	61.02	54.55	11685	11663	
PPRV_19_LEFT	GCCCATGAGAGCGGAGTAAGAA	1	62.12	54.55	11561	11583	728
PPRV_19_RIGHT	GCAGAATAAGGGTCACTTGCCC	1	61.52	54.55	12289	12267	
PPRV_20_LEFT	AGCCGACCTGAAGAGGATGATA	2	60.42	50.00	12169	12191	749
PPRV_20_RIGHT	GCACACGAAGAGCTGAAGTCTC	2	61.49	54.55	12918	12896	
PPRV_21_LEFT	TGGCTATCTTATCAAACGCCATGAG	1	61.26	44.00	12787	12812	797
PPRV_21_RIGHT	AAAGCTGACTTGTTGACCAGGT	1	60.36	45.45	13584	13562	
PPRV_22_LEFT	CGAATGAGCTATGTACAAACCCCTT	2	61.15	44.00	13452	13477	818
PPRV_22_RIGHT	AGGATCAGTGTTCCATGAGGCC	2	62.08	54.55	14270	14248	
PPRV_23_LEFT	CCGATGTGTATTGCAGCTCCAA	1	61.26	50.00	14133	14155	723
PPRV_23_RIGHT	CCCAGGTTACTTCAGGTTTGCC	1	61.40	54.55	14856	14834	

Primer	Sequence	Pool	Tm	GC%	Start	End	Amplicon size
PPRV_24_LEFT	GCAGAGCCGGACAAAGAGAAAT	2	61.20	50.00	14736	14758	762
PPRV_24_RIGHT	TGAAATGAGCCAGCTCCCTGTA	2	61.42	50.00	15498	15476	
PPRV_25_LEFT	AGTAATTTCATTTCAACTGAGTGTTACCTT	1	60.20	30.00	15128	15158	749
PPRV_25_RIGHT	TTTCATGGTGGAGGAGAAGGGG	1	61.70	54.55	15877	15855	

Table S2 *Primer sequences for 600 base pair amplicons*

Primer	Sequence	Pool	Tm	GC%
PPRV_1_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	61.21	39.28
PPRV_1_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.43	52.17
PPRV_2_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.45	50.0
PPRV_2_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.50	50.0
PPRV_3_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.61	45.45
PPRV_3_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.20	50.0
PPRV_4_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	62.76	54.54
PPRV_4_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.63	50.0
PPRV_5_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	62.95	54.54
PPRV_5_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.53	50.0
PPRV_6_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.06	50.0
PPRV_6_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.55	50.0
PPRV_7_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	62.25	54.54
PPRV_7_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.58	54.54
PPRV_8_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.92	45.83
PPRV_8_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	60.95	50.0
PPRV_9_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	62.93	54.54
PPRV_9_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.27	50.0
PPRV_10_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.18	43.47
PPRV_10_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.89	54.54
PPRV_11_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	61.83	50.0
PPRV_11_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.51	50.0
PPRV_12_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.49	50.0
PPRV_12_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.77	54.54
PPRV_13_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.35	45.45
PPRV_13_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.54	54.54
PPRV_14_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.13	40.74
PPRV_14_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.58	54.54
PPRV_15_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.85	50.0
PPRV_15_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.45	50.0
PPRV_16_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.61	54.54
PPRV_16_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.62	50.0
PPRV_17_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	61.80	54.54
PPRV_17_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.66	50.0
PPRV_18_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.46	54.54
PPRV_18_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.48	54.54
PPRV_19_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	61.63	46.15
PPRV_19_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	62.19	54.54
PPRV_20_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.99	50.0
PPRV_20_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.63	54.54
PPRV_21_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	62.22	50.0

Primer	Sequence	Pool	Tm	GC%
PPRV_21_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.89	50.0
PPRV_22_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.75	47.82
PPRV_22_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.86	47.82
PPRV_23_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	61.39	50.0
PPRV_23_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.00	50.0
PPRV_24_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.70	50.0
PPRV_24_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.02	54.54
PPRV_25_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	62.10	54.54
PPRV_25_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.50	47.82
PPRV_26_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.98	40.0
PPRV_26_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.60	54.54
PPRV_27_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.56	40.0
PPRV_27_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.48	50.0
PPRV_28_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.33	54.54
PPRV_28_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.31	50.0
PPRV_29_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.91	44.0
PPRV_29_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.25	50.0
PPRV_30_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	61.60	50.0
PPRV_30_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.48	54.54
PPRV_31_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.46	40.74
PPRV_31_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	61.59	54.54
PPRV_32_LEFT	GATTGCCAGTGGAATTTCATGGTG	2	60.23	38.46
PPRV_32_RIGHT	GATTGCCAGTGGAATTTCATGGTG	2	61.55	50.0
PPRV_33_LEFT	GATTGCCAGTGGAATTTCATGGTG	1	60.84	33.33
PPRV_33_RIGHT	GATTGCCAGTGGAATTTCATGGTG	1	60.91	45.83

IV

Peste-des-petits-ruminants virus C protein modulate the type I and type II interferon signaling pathways

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Abstract

The non-structural proteins of morbilliviruses, the C and V proteins, modulate the host interferon (IFN) response to infection. The V protein of peste-des-petits-ruminants virus (PPRV) has been shown to suppress the type I IFN response by interacting with MDA-5 and STAT1. However, the effect of the PPRV V protein on type II IFN and the immunomodulatory effects of the PPRV C protein are not yet clear. Using a luciferase reporter system for selected steps in the type I and II IFN signaling pathways, we show that the C protein of PPRV is a potent inhibitor of type I IFN while the C protein of PPRV, as well as C proteins from other morbilliviruses, on the other hand stimulate the type II IFN pathway. The modulation of the type I and type II IFN pathways could lead to a more beneficial host immune response for the virus.

Keywords: peste-des-petits-ruminants virus, non-structural proteins, immunomodulation, type I interferon , type II interferon

Introduction

Peste des petits ruminants (PPR) is an acute and highly contagious disease that affects small ruminants, particularly domestic sheep and goats, and is caused by the morbillivirus peste-des-petits-ruminants virus (PPRV) (Bailey *et al.*, 2005). The clinical signs associated with PPR include fever, ocular and nasal discharge, pneumonia, diarrhea, severe immunosuppression, and death (Roeder & Obi, 1999). The morbidity and case fatality rates vary from 10% to as high as 80–90% in some outbreaks, and fatality is mainly due to dehydration and secondary infections (Torsson *et al.*, 2017). PPRV, which is currently distributed throughout most parts of the Asian and African continents (OIE, 2019; Banyard *et al.*, 2010), can be divided into four genetic lineages by the nucleotide sequence of the nucleoprotein or fusion protein genes (Munir *et al.*, 2013a). Other closely related viruses in the genus *Morbillivirus* include measles virus (MeV), canine distemper virus (CDV), the now-eradicated rinderpest virus (RPV), and the recently discovered feline morbillivirus (FeMV) (Woo *et al.*, 2012; Anderson *et al.*, 2011). Morbilliviruses belong to the Paramyxoviridae family and are negative-sense single-stranded RNA viruses. The approximately 16 kb genome encodes for six structural proteins: nucleo-, phospho-, matrix, fusion, hemagglutinin, and large protein or polymerase. Within the phosphoprotein (P) gene, two additional non-structural proteins are encoded: the V and C proteins (Munir *et al.*, 2013a). The V protein is a product of post-transcriptional editing of the mRNA transcript, in which a non-templated G is inserted at the editing site (position 751), thereby changing the reading frame (Mahapatra *et al.*, 2003). As a result, P and V have the same N-terminus sequence but different C-terminus sequences. The C protein is produced through leaky scanning with translation initiation at the second AUG codon, which is located 19 nucleotides downstream of the first (Mahapatra *et al.*, 2003). The V and C proteins are considered to be the most important viral proteins for the morbilliviruses' ability to modulate the host immune response during infection (Chinnakannan *et al.*, 2013; Goodbourn & Randall, 2009).

Interferons (IFNs) are a host's first line of defense against a viral infection. IFNs are a group of signaling proteins and, depending on which receptor they bind, can be divided into three groups (I–III) (Randall & Goodbourn, 2008). Type I interferons include several members, although this study focuses on IFNα and IFNβ since they are the most important members for the response to viral infections (Randall & Goodbourn, 2008). IFNγ is the only type II interferon, and it is secreted by natural killer (NK) cells and cytotoxic T-cells (Lee & Ashkar, 2018). Type III interferons comprise IFNλ1–3 and are induced directly in response to a viral infection. Type III IFNs appear to use the same pathways as those used by IFNα/β. However, the receptor for type III has a limited tissue distribution (Randall & Goodbourn, 2008).

Type I IFNs are induced by a virus after pathogen-associated molecular patterns (PAMPs) are recognized

by pattern recognition receptors (PRRs). One type of PRR consists of the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which are located in the cytoplasm (Kato *et al.*, 2006). RLRs recognize intracellular pathogens by identifying pathogen-specific RNA structures, such as double-stranded RNA and uncapped single-stranded RNA with a 5' phosphate. Included in this group of PRRs are the helicases RIG-I and melanoma differentiation-associated protein 5 (MDA-5) (Kato *et al.*, 2006). Both RIG-I and MDA-5 are involved in downstream signaling pathways that lead to the activation of the IFNβ promotor (Seth *et al.*, 2005; Xu *et al.*, 2005). RNA viruses can also be detected by toll-like receptors (TLRs)—transmembrane proteins located in the cell membrane and within lysosomes and endosomes—which are capable of initiating a signaling pathway that activates the IFNβ promotor (Dhanasekaran *et al.*, 2014; Randall & Goodbourn, 2008). Once the IFNβ promotor is activated by either pathway, IFNβ is produced and released from the cell. The secreted IFNβ activates the Jak/STAT pathway, which initiates the expression of IFN-induced genes (Randall & Goodbourn, 2008). Type I IFNs are the first response to a viral infection and, therefore, essential for activating the innate immune response and inducing an antiviral state in the host. The effects include, but are not limited to, the upregulation of up to 300 IFN-stimulated genes; the induction of protein kinase R (PKR), which inhibits cellular translation functions that are essential for the virus; the activation of RNA degradation via OAS and RNase; T-cell activation; the enhancement of MHC II expression; and the recruitment of inflammatory monocytes (Lee & Ashkar, 2018). Another effect of type I IFN induction is the activation of NK cells, which, in turn, can produce IFNγ (type II IFN) (Abboud *et al.*, 2016). Once the NK cell is activated by type I IFN, it can recognize viral products and virus-infected cells through a number of receptors, depending on the virus. IFNγ enhances the antiviral state and facilitates the activation of the adaptive immune response by a variety of means, including activating T-cells, promoting dendritic cell maturation, increasing MHC I and II expression, and inducing nitric oxide production by macrophages (Lee & Ashkar, 2018).

Studies on the immunomodulatory properties of PPRV have shown that the V protein is the major modulator of the immune response, similar to the V proteins of other paramyxoviruses (Bernardo *et al.*, 2017; Ma *et al.*, 2015; Chinnakannan *et al.*, 2013). The exertion of this modulating effect has been suggested to occur at several different steps in the interferon signaling pathway. First, the cysteine-rich C-terminus of the V protein binds to and inhibits MDA-5, thereby inhibiting the MDA-5-mediated induction of the type I IFN signaling pathway (Bernardo *et al.*, 2017; Childs *et al.*, 2009). A similar interaction with RIG-I has also been proposed (Bernardo *et al.*, 2017). Second, the V protein has been shown to inhibit the immune response by interacting with and inhibiting the phosphorylation of STAT1/2 (Ma *et al.*, 2015; Chinnakannan *et al.*, 2013). The C protein of PPRV has not been as well studied as the V protein. While the V protein has been found to bind both MDA-5 and RIG-I, the C protein binds neither of these. However, a PPRV with a mutated V protein but a functioning C protein still inhibits the induction of type I IFN (Bernardo *et al.*, 2017).

This study investigated the effect of the PPRV C protein on the type I and type II interferon pathways. The C protein was assessed both as a separate unit and together with the PPRV V protein. We also compared four PPRV isolates, with different characteristics and originating from the four currently established lineages, for their ability to interfere with the interferon response. The results show that the C protein of all isolates of PPRV, as well as other morbilliviruses, are inhibitors of the type I IFN pathways, but stimulators of the type II IFN pathways.

Materials and Methods

Cell lines and plasmids

HEK293T (human embryonic kidney cells) and A549 (human alveolar epithelial carcinoma cells) cells were purchased from the American Type Cell Culture Collection (ATCC). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 IU/mL), streptomycin (100 µg/mL), and 5% fetal calf serum. A549 cells were maintained in DMEM containing penicillin (200 IU/mL), streptomycin (200 µg/mL), and 5% fetal calf serum. Cells were incubated at 37 °C with 5% CO_2.

Expression plasmids of the C and V proteins of PPRV were designed using four complete PPRV genomes available from NCBI's GenBank: ICV89, Nigeria 75/1, Uganda/2012, and Ethiopia/2010 (Table 1). These isolates derive from the four currently known lineages of PPRV, and all originate from the African continent. The full V protein was produced by adding a G base at the editing site. The expression plasmids were generated by GenScript by inserting the indicated C or V sequence into the vector backbone pCMV-3Tag-1a. Additional morbillivirus C proteins (canine distemper virus: KF914669; feline morbillivirus: KR014147; and measles virus: DQ227319) were designed in the same way.

PathDetect pISRE-Luc Cis-Reporter plasmid was purchased from Agilent. The pGL4[luc2P/GAS-RE/Hygro] reporter plasmid and internal control plasmid pGL4.75[hRluc/CMV] were purchased from Promega. The p-125luc reporter plasmid, which was used to measure activity at the IFNβ promotor region, was kindly provided by Professor Takashi Fujita (Graduate School of Biostudies, Kyoto University).

Table 1. *The peste-des-petits-ruminants virus (PPRV) isolates from which the V and C genes used in this study originated.*

Lineage	Country of origin	Year collected	Accession No.	Comment
I	Côte d'Ivoire	1989	EU267273	Highly virulent isolate
II	Nigeria	1975	KY628761	Vaccine strain
III	Uganda	2012	KJ867543	Field isolate
IV	Ethiopia	2010	KJ867541	Field isolate

Transfections and luciferase reporter assay

Cells were seeded in 24-well plates 24 h before transfection, and the approximate confluency at transfection was 70%. All transfections were conducted with TransIT LT1 (Mirus) according to the manufacturer's instructions using a ratio of 3 µL of transfection reagent per µg of DNA. The cells were transfected with a combination of either three or four plasmids: (a) 2 ng/mL pGL4.75[hRluc/CMV] as an internal transfection control, (b) the luciferase reporter plasmid at a concentration of 200 ng/mL (for pISRE-Luc and p-125luc) or 500 ng/mL (for the pGL4[luc2P/GAS-RE/Hygro]), and (c) the expression plasmid (C, V, or a combination of the two) at 200 ng/mL. Equal concentrations of both expression plasmids were used when C and V were co-transfected, 200 ng/mL each. Twenty-four hours after transfection with reporter plasmids pISRE-Luc or p-125luc, cells were treated with 1 µg/mL poly I:C (Sigma-Aldrich) or left untreated. Poly I:C was transfected using Lipofectamine 3000 according to the manufacturer's instructions. Twenty-four hours after transfection with reporter plasmid pGL4[luc2P/GAS-RE/Hygro] cells were treated with 1000 IU/mL human IFNγ (Sigma-Aldrich) or left untreated. IFNγ was added dropwise to cells; for the mock treatment, Opti-MEM was used. As a control in each experiment, cells were transfected with an empty backbone pcDNA3.1(+) and treated as described above.

A luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega) and a Tecan Infinite M1000 plate reader. The reagents (Luciferase Assay Reagent II and Stop&Glo® Reagent) in the Dual-Luciferase Reporter Assay System were diluted 10× in Milli-Q water; otherwise, the assay was performed according to the manufacturer's instructions. Luciferase activity was normalized by dividing the luciferase value from the reporter plasmid by the luciferase value from the internal control. All the experiments using HEK293T cells were performed in technical triplicate and biological triplicate. The experiments using A549 cells were conducted in technical triplicate and biological duplicate. The mean value of the replicates was used for calculations; values are expressed as relative light units (RLUs) and presented as the fold change relative to the uninhibited control. The activation of the reporter plasmids was calculated by comparing stimulated cells with non-stimulated cells, and the effect of the viral proteins was compared to the control cells.

Western blot

Transfections for Western blots to analyze the expression levels of viral proteins were performed in parallel to transfections for the luciferase assay using a plasmid concentration of 200 ng/mL, and cells were lysed 48 h after transfection. Protease Inhibitor Cocktail (P8340, Sigma-Aldrich) was added upon cell lysis. Transfections to study the phosphorylation of the STAT1 protein were carried out separately. Twenty-four hours after transfection, cells were stimulated with 1000 IU/mL IFNγ and lysed 2, 6, or 24 h after stimulation. The lysates were then separated by SDS-PAGE using Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad Laboratories), and the separated proteins were transferred to a nitrocellulose membrane (Trans-Blot® Turbo™ Transfer Pack, Bio-Rad Laboratories). The membranes were blocked using a blocking buffer containing milk powder at room temperature for 1 h. After that, the membranes were incubated overnight with continuous agitation with primary antibodies raised in rabbits. The primary antibodies targeted the FLAG-Tag (1 µg/mL, ANTI-FLAG, F7425, Sigma-Aldrich) to measure expression levels, unphosphorylated STAT1 (0.5 µg/mL, Anti-STAT1, 06-501, Millipore), or phosphorylated STAT1 (1:3000, Anti-Phospho-STAT1-PTYR701, Sigma-Aldrich). After washing with washing buffer (TBS, Tween 0.1%), the membranes were incubated with secondary peroxidase-conjugated anti-rabbit antibodies (611-1302, Rockland) for 1 h with continuous agitation. After washing, the membranes were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories), visualized by the ChemiDoc Touch Imaging System, and analyzed using Image Lab (version 5.2.1, Bio-Rad Laboratories). β-Actin was used as a loading control (sc-7210, Santa Cruz Biotechnology, 1:1000, 4 °C overnight).

Statistics

All statistics were calculated using RStudio (R Core Team, 2015). Confidence intervals (95%) were calculated using the standard error of the mean of the biological replicates. Possible differences between the four isolates when comparing the four C or V proteins (proteins compared separately) were calculated by one-way analysis of variance (ANOVA) using the *lm* and *anova* functions from the *stats* package. When the *anova* function indicated a statistical significance in the linear model, least-square means were computed to identify the observations with statistical significant differences using the *lsmeans* function and package (Chambers & Hastie, 1992).

Results

The effect of the PPRV C protein on type I interferon expression

The type I IFN-modulating capacities of the PPRV C and V proteins, separately and combined, were studied by measuring the activity at two different steps in the signaling pathway: the interferon-stimulated response element (ISRE) and the IFNβ promotor region. Four isolates with different characteristics and originating from the four currently known lineages of PPRV were also compared and evaluated for differences in modulating abilities. Using the ISRE-Luc reporter plasmid and stimulating by poly I:C gives an indication on the effect on the entire type I IFN signaling pathway. The PPRV C protein inhibited the pathway to 32.6-72.0% compared to control (100%), and the V protein inhibited the pathway to 27.2-32.9% (Figure 1). Co-transfection of the two viral proteins inhibited the pathway to 22.5-52.6%. ANOVA of the results showed no statistically significant difference between the different isolates of the C and V proteins, nor any difference between the inhibition by the C, V, or combination of proteins. To evaluate a step earlier in the type I IFN pathway, the IFNβ-promotor, we used the p125-Luc reporter plasmid. The C proteins of the different PPRV inhibited activation of the IFNβ promotor to 18.4–55.4% compared to control (100%), and the V proteins to 10.6–33.5% (Figure 2). A combination of the two proteins inhibited the pathway to 22.5–33.9%. ANOVA of the results showed no statistically significant difference between the isolates or the C or V proteins separately or a combination of the two.

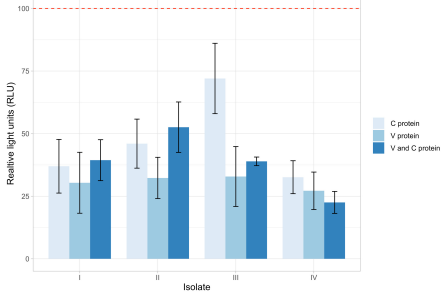

Figure 1. Modulation of the type I IFN signaling pathway, measured at the interferon-stimulated response element (ISRE) by the C and V proteins of PPRV from four different isolates, lineage I-IV. HEK293T cells were transfected with expression plasmids (200 ng/ml) for PPRV C protein, V protein, or both, together with an ISRE–Luc reporter plasmid (200 ng/ml). Twenty-four hours after transfection, cells were stimulated using 1 µg/mL poly I:C to study the entire type I IFN signaling pathway. After an additional 24 h, cells were lysed, and a dual-luciferase reporter assay was performed. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. No statistically significant difference between isolates was found by ANOVA analysis. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

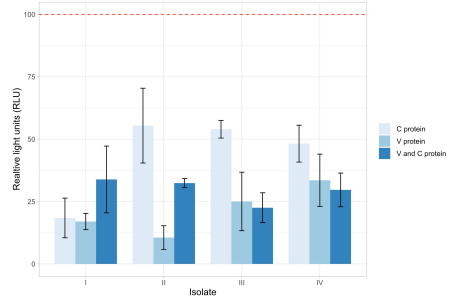

Figure 2. Modulation of the type I IFN signaling pathway, measured at the IFNβ promotor by the C and V proteins of PPRV from four different isolates, lineages I–IV. HEK293T cells were transfected with expression plasmids (200 ng/ml) for PPRV C protein, V protein, or both, together with the p125–Luc reporter plasmid (200 ng/ml). Twenty-four hours after transfection, cells were stimulated using 1 µg/mL poly I:C. After an additional 24 h, cells were lysed, and a dual-luciferase reporter assay was performed. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. No statistically significant difference between isolates was found by ANOVA analysis. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

The effect of PPRV C proteins on type II interferon expression

In addition to the modulation of type I IFN, the modulation of the type II IFN signaling pathway by PPRV C and V proteins was studied by measuring the activity at the IFNγ-activated sequence (GAS). The PPRV V proteins of all studied isolates strongly inhibited the type II IFN pathway, the inhibition left an activity of between 6.2% and 20.1% compared with the control (100%) (Figure 3). ANOVA of the results for the V proteins showed statistically significant differences between V protein from isolates in lineage III and IV ($p = 0.026$) (Figure 3).

All the investigated PPRV C proteins, stimulated the type II IFN pathway. The mean modulation of the pathway among the four isolates varied between 98.7% and 133.0% compared with the control (Figure 3). No statistically significant difference was detected between the four C proteins studied. The same experiment was carried out with expression plasmids carrying the C protein of other closely related morbilliviruses to investigate whether this stimulatory property is specific to PPRV or shared with other morbilliviruses, namely, the canine distemper virus (CDV), feline morbillivirus (FeMV), and measles virus (MeV).

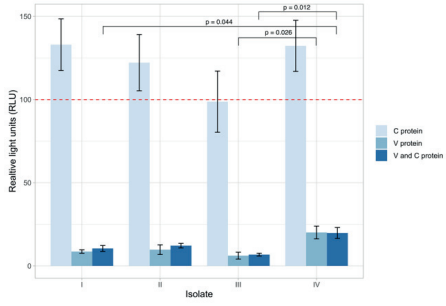

Figure 3. Modulation of the type II IFN signaling pathway, measured at the interferon-γ-activated-sequence (GAS) by the C and V proteins of PPRV from four different isolates, lineages I–IV. HEK293T cells were transfected with expression plasmids (200 ng/ml) for PPRV V protein, C protein, or both, together with a GAS–Luc reporter plasmid (500 ng/ml). Twenty-four hours after transfection, cells were stimulated using 1000 IU/mL IFNγ. After an additional 24 h, cells were lysed, and a dual-luciferase reporter assay was performed. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. Statistically significant differences ($p < 0.05$) found by ANOVA analysis are indicated in figure. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

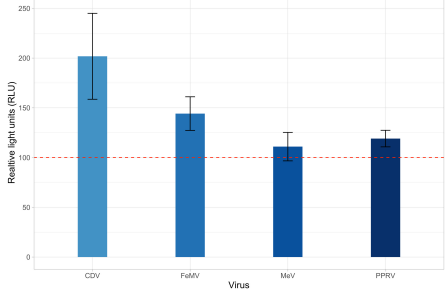

Figure 4. Modulation of the type II IFN signaling pathway, measured at the interferon-γ-activated-sequence (GAS) by the C protein from a selection of morbilliviruses. The results obtained from the canine distemper virus (CDV), feline morbillivirus (FeMV), measles virus (MeV), and PPRV (mean of all four lineages) are shown. HEK293T cells were transfected with expression plasmids for the C protein of each virus (200 ng/ml), together with the GAS–Luc reporter plasmid (500 ng/ml). Twenty-four hours after transfection, cells were stimulated using 1000 IU/mL IFNγ. After an additional 24 h, cells were lysed, and a dual-luciferase reporter assay was performed. The bars represent the mean of technical and biological triplicates, and the error bar indicates SE. Cells transfected with an empty vector backbone and then stimulated were used as a control and set to 100% (dotted line).

The same pattern of stimulation of the pathway was observed, and the C protein of CDV had the strongest stimulatory effects, stimulating the pathway to as high as around 200% (Figure 4). The same experiment was carried out in A549 cells with C proteins from PPRV, CDV, FeMV, and MeV to verify these findings further and confirm that it is not a phenomenon occurring only in the HEK293T cells. The results were similar to the results with HEK293T cells: the type II IFN pathway was stimulated, but it was not as strong as the effect in the HEK293T cell line (data not shown). When the PPRV C and V proteins were co-transfected, the stimulatory effect of the C protein disappeared completely, and only the inhibitory effect of V (6.8–19.8% compared with the control) was observed. ANOVA of the results showed a statistically significant difference ($p < 0.005$) for all PPRV C proteins compared to V proteins separately and V and C protein co-transfected. For the co-transfected viral proteins, a statistical significance was found between isolates from lineage I and IV ($p = 0.044$) and isolates lineage III and IV ($p = 0.012$) (Figure 3).

Modulation of STAT1 phosphorylation by the PPRV C protein

The expression levels of the C and V proteins of the four isolates were compared utilizing antibodies against the FLAG-tag on the vector backbone. All proteins were well expressed in this system and to similar levels between the different isolates (Table 2).

To further evaluate the effect of the PPRV C protein on the IFN type II signaling pathway, we performed transfections with PPRV C proteins of all isolates at a plasmid concentration of 200 ng/mL, with an empty vector backbone used as a control. Twenty-four hours post-transfection, cells were stimulated with IFNγ, and the cells were then lysed 2, 6, or 24 h later. The results were normalized to the total protein levels of the stain free gel, and then compared to the stimulated control lysed at the same time point and presented as a ratio (Figure 5, Table 3). The C protein inhibited the expression of STAT1 at all time points and stabilized at around 50% inhibition at 24 hours post stimulation with IFNγ.

Table 2. *Expression levels of the PPRV C and V proteins of the four different isolates. Expression plasmids (200 ng/ml) encoding the C and V proteins of four different isolates of PPRV were transfected in HEK293T cells and lysed together with a proteinase inhibitor 24 hours post transfection. Expression levels in the cell lysates were measured using Western blot analysis and antibodies against the FLAG-tag on the expression plasmid. Protein volumes are normalized to the total protein levels on the stain free gel using the ImageLab software (BioRad). Values were calculated on biological duplicates.*

Protein	I	II	III	IV
C	1	1.4	0.57	2.3
V	1	0.78	2.2	2.8

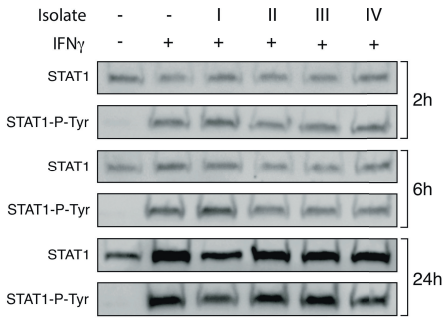

Figure 5. Modulation of STAT1 expression and STAT1 phosphorylation by PPRV C protein from four different isolates, lineages I–IV. Western blot analysis was performed to analyze STAT1 expression and the phosphorylation of STAT1 at the tyrosine701 phosphorylation site. HEK293T cells were transfected with either an empty vector backbone or expression plasmid for one of four different isolates (lineages I–IV) and stimulated with IFNγ 24 h later. Cells were lysed 2, 6, and 24 hours post-stimulation. Western blot analysis was then performed using Anti-STAT1 or Anti-Phospho-STAT1-PTYR701 antibodies. Gels were then visualized using the ImageLAb Software (BioRad). All rows originate from different gels and are here shown cropped together full lengths gels are available in Supplementary Material.

The effect on tyrosine phosphorylation of STAT1 differed among the isolates. The isolate from lineage I initially stimulated phosphorylation, isolates from lineage II and III varied in their inhibition levels over time, and lineage IV did not initially affect phosphorylation but inhibited 50% of the phosphorylation at 6 and 24 h post-stimulation (Figure 5, Table 3).

Discussion

In this paper, the ability of the non-structural C protein of peste-des-petits-ruminants virus (PPRV) to modulate the type I and type II interferon (IFN) signaling pathway was studied. We also compared whether these abilities varied among four isolates representing different characteristics, such as, the highly virulent ICV89 strain from lineage I and the vaccine strain Nigeria 75/1 from lineage II, as well as representatives from the rest of the four currently known lineages of PPRV. It should be noted that the results we generated only reflects the ability of these particular isolates and may not represent all variants in each lineage.

The PPRV C proteins in our study were all potent inhibitors of the type I IFN pathway, IFNβ promotor activity decreased to 18.4–55.4%, and the interferon-stimulated response element (ISRE) decreased to 32.6%–72.0 (Figure 1 and 2). Previous studies on the C proteins of other morbilliviruses have found that the C protein is an inhibitor of the type I IFN pathway, but the strength of this inhibition has varied from weak to very strong among studies (Boxer *et al.*, 2009; Nakatsu *et al.*, 2008b; Nanda & Baron, 2006a; Shaffer *et al.*, 2003). The exact mechanism of how the C protein modulates the type I IFN pathway is not yet clear (Boxer *et al.*, 2009). These results indicate, together with previous studies (Bernardo *et al.*, 2017; Boxer *et al.*, 2009; Shaffer *et al.*, 2003), that this mechanism is located in the earlier part of the signaling pathway, before the induction of the IFNβ promotor.

We confirm that the PPRV V protein has a strong inhibitory effect on the type I IFN signaling pathway (Bernardo *et al.*, 2017; Boxer *et al.*, 2009), with an effect measurable on both the IFNβ promotor (10.6–33.5% activity relative to the control set to 100%) and the downstream ISRE promotor (27.2–32.9% activity) (Figure 1 and 2). Previous studies on the CDV, RPV, and MeV have shown similar results in the ability of their V proteins to inhibit type I IFN (Svitek *et al.*, 2014; Chinnakannan *et al.*, 2013; Nakatsu *et al.*, 2008a). We also compared the inhibitory effect between the four different PPRV isolates, with different characteristics. For example, the ICV89 isolate (lineage I) is a highly virulent strain, whereas Nig75/1 (lineage II) is a vaccine strain (Table 1).

Table 3. *Modulation of STAT1 expression and STAT1 phosphorylation by PPRV C protein isolates from lineages I–IV. Expression plasmids (200 ng/ml) encoding the C proteins of four different isolates of PPRV were transfected in HEK293T cells. 24h post transfection cells were stimulated with IFNγ and lysed together with a proteinase inhibitor 2, 6, or 24 hours post stimulation. Western blot analysis was then performed using Anti-STAT1 or Anti-Phospho-STAT1-PTYR701 antibodies. Protein volumes are normalized to the total protein levels on the stain free gel using the ImageLab software (BioRad) and expressed as fold change relative to cells transfected with an empty vector backbone (positive control).*

	Hours post-stimulation	I	II	III	IV
STAT1	2	0.80	0.54	0.84	0.97
STAT1	6	1.10	0.23	0.30	0.34
STAT1	24	0.44	0.46	0.45	0.40
STAT1-P-Tyr	2	1.59	0.64	0.86	1.02
STAT1-P-Tyr	6	1.12	0.57	0.53	0.54
STAT1-P-Tyr	24	0.58	0.83	0.95	0.57

However, we did not find any statistically significant difference in type I IFN inhibition between the four different V proteins, the attenuated vaccine isolate inhibited the type I IFN signaling pathway equally well as compared to the highly virulent ICV89 strain. Similar results were described previously for MeV, with no relationship found between the type of isolate (attenuated and wild-type isolates) and type I IFN inhibition (Fontana *et al.*, 2008). For the MeV V protein, the importance of a tyrosine residue at amino acid 110 has been linked to its ability to strongly inhibit type I IFN: changing this tyrosine to a cysteine weakened the inhibitory ability (Fontana *et al.*, 2008; Ohno *et al.*, 2004). The V protein isolates from lineages I–III in our study have a tyrosine at position 110, while the lineage IV isolate has a cysteine (amino acid alignment of the PPRV V and C proteins in this study are available in Supplementary Figure 7 and 8). However, in our study, substitution did not lead to a significant loss of inhibitory function. For MeV, a cysteine at position 272 in the V protein was also found to be important for its inhibitory function (Ohno *et al.*, 2004), and all isolates in this study had this particular residue.

The PPRV V proteins were also shown to strongly inhibit the type II IFN response, which was measured by the activity in the IFNγ-activated sequence (GAS) (Figure 3). For MeV, the tyrosine residue at position 110 have been found to be essential for the inhibition of IFNγ (Fontana *et al.*, 2008). Our ANOVA results reveal that the type II interferon inhibitory effect of the isolate from lineage IV (with a cysteine at position 110) was slightly lower than that of the others: lineages I and IV, $p = 0.055$; lineages II and IV, $p = 0.099$; lineages III and IV, $p = 0.026$. Overall, this suggests that the amino acid residue at position 110 in the PPRV V protein is not as important as that in the MeV V protein. Lineage IV, currently the most prevalent lineage, was first described in Asian outbreaks, but its spread is now widening to include a large part of the African continent (Albina *et al.*, 2013). The extended spread of lineage IV could be due to a more attenuated virus variant, which might be exemplified by the cysteine residue in lineage IV. However, the results of this study do not show any clear attenuation of the ability of the V protein of lineage IV to inhibit type I or II IFNs. Studies on the ability of the V protein of morbilliviruses to affect type II IFN have mainly found that the protein inhibits the type II IFN pathway (Chinnakannan *et al.*, 2013; Fontana *et al.*, 2008; Nanda & Baron, 2006b), although one study found no inhibition (Takeuchi *et al.*, 2003). This could be because of different cell lines and methods being used in different studies.

In our system, we found that, rather than inhibiting the activity of the type II IFN pathway, the PPRV C proteins were weak stimulators (Figure 3). To investigate whether this stimulatory effect could be observed for other morbilliviruses, we performed the same experiment with C proteins from CDV, FeMV, and MeV (Figure 4). All morbilliviruses showed the same pattern of stimulation, suggesting that this stimulation is an intrinsic property of all morbillivirus C proteins. The C protein of RPV has previously been shown to have no effect on the type II IFN response in Vero cells (Nanda & Baron, 2006b), and the C protein of MeV has been reported to inhibit the response by 50% relative to the control (Shaffer *et al.*, 2003). Another study found that the MeV C protein did not inhibit GAS gene activity at all, despite the increasing concentrations of the added plasmids (Fontana *et al.*, 2008).

Viruses from different virus families have other proteins that modulate the IFN response. For example, the NS5 protein in the *Flavivirus* Zika virus (ZIKV) has been found to be a strong inhibitor of type I IFNs (Chaudhary *et al.*, 2017). However, similar to the PPRV C protein, NS5 was found to be an activator of type II IFN and, in addition, IFNγ amplified the replication of ZIKV (Chaudhary *et al.*, 2017). The ZIKV NS5 protein did not influence STAT1 stability or phosphorylation but promoted the degradation of STAT2, thereby increasing STAT1–STAT1 homodimerization, which is needed to activate the IFNγ pathway (Chaudhary *et al.*, 2017). This could be a means by which the virus alters the host immune response from an antiviral response to a proinflammatory response that is more advantageous for the virus. To further study the observed stimulation of the type II IFN pathway, we analyzed the effect of different PPRV C proteins on the levels and phosphorylation of STAT1 (Figure 5). The C proteins all modulated both the expression and the phosphorylation of the STAT1 protein compared to the control. The expression of STAT1 was inhibited by all studied isolates, except at 6 hours post stimulation by the isolate from lineage I. This however was transient and all isolates stabilized their inhibition to around 50% 24 hours after stimulation (Table 3). We also studied the phosphorylation of STAT1 at position 701, a phosphorylation needed for the homodimerization of STAT1 (Wenta *et al.*, 2008). The phosphorylation of STAT1 was initially stimulated by one of the PPRV isolates, ICV89 (lineage I). This isolate is one of the more pathogenic isolates; therefore, its immunomodulation mechanism might differ from that of more attenuated isolates. The three other isolates all inhibited the phosphorylation compared to the control. Whether the PPRV C protein uses the same mechanism as ZIKV NS5 (increasing STAT1–STAT1 homodimerization) or has another mechanism to modulate the type II IFN response is not clear from these results and needs to be studied further.

Co-transfection of both viral proteins did not lead to gain of inhibition of the type I IFN signaling pathway (Figures 1 and 2). In a previous study, the co-

transfection of the MeV V and C expression plasmids did not cause a gain of inhibition either, except for two strains of MeV when up to three times the concentration of the C protein was added relative to the concentration of V (Fontana *et al.*, 2008). Our results show that the C protein-induced stimulation of the type II IFN pathway was completely nullified by the presence of the V protein, and the pathway was strongly inhibited (Figure 3). It is possible that, during a natural infection, V and C are expressed at different time points of infection and thereby control different parts of the host immune response. Morbilliviruses are all dual-tropic, meaning that they infect both the epithelial cells and lymphatic tissue (Munir *et al.*, 2013b). This dual tropism means that the virus must be able to adapt to these different cell types during an infection. A recent study investigated the adaptation of MeV to lymphocytic or epithelial cell lines and found that lymphocytic cell-adapted viruses expressed less V protein compared with the phosphoprotein from which it is derived (Donohue *et al.*, 2019). The researchers did not study the possible change in the expression of the C protein, but an alteration in the expressed P/V–C ratio might change the immunomodulatory abilities of PPRV from immunosuppression due to the V protein to a proinflammatory response due to the expression of the C protein.

In conclusion, our results show that the PPRV C protein is a potent inhibitor of type I IFN, equally as potent as the V protein, and no significant difference was observed among the isolates studied here. Co-transfecting the two proteins did not have an additive inhibitory effect. The PPRV V protein is a strong inhibitor of type II IFN in this system, whereas the C protein stimulated type II IFN activity, possibly to induce a proinflammatory response that is more beneficial to the virus. When co-transfected, the V protein's inhibitory effect on the type II IFN pathway was stronger than the stimulatory effect of the C protein, leading to an inhibition of the pathway. Future studies will be needed to evaluate whether the opposing effects of C and V are exerted at different time points or in different cell types during infection to benefit the viruses' replication and spread.

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Competing interest

The authors declare no competing interest.

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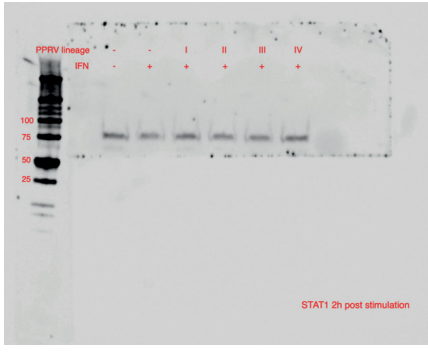
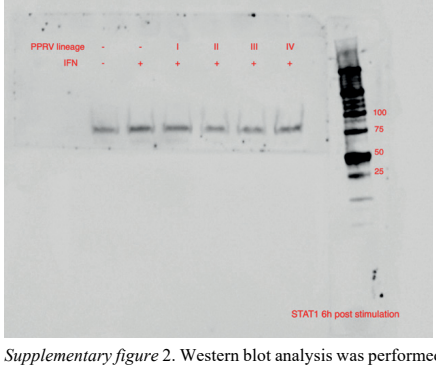
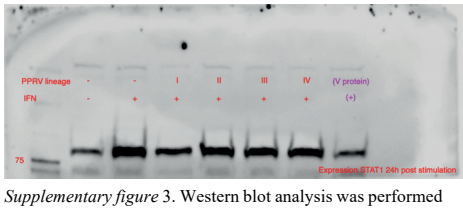
Supplementary materials



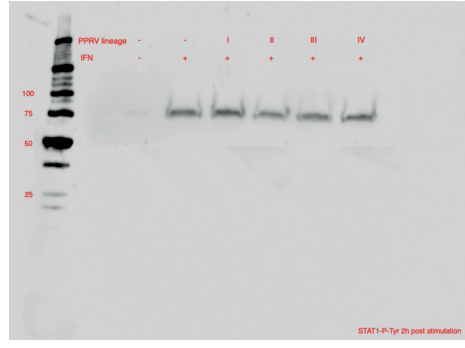
Supplementary figure 1. Western blot analysis was performed to analyze STAT1 expression 2 h post-stimulation using 1000 IU/mL IFNγ. Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories) High: 7000, Low: 0, Gamma: 0.8.



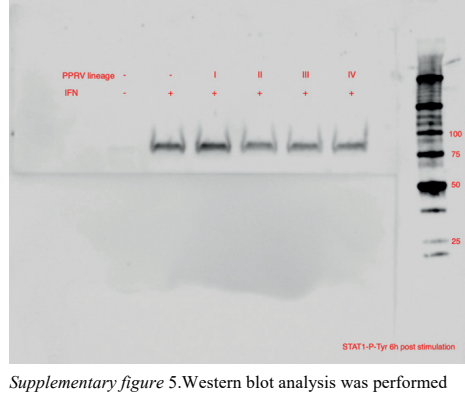
Supplementary figure 2. Western blot analysis was performed to analyze STAT1 expression 6 h post-stimulation using 1000 IU/mL IFNγ. Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories) High: 7000, Low: 0, Gamma: 0.8.



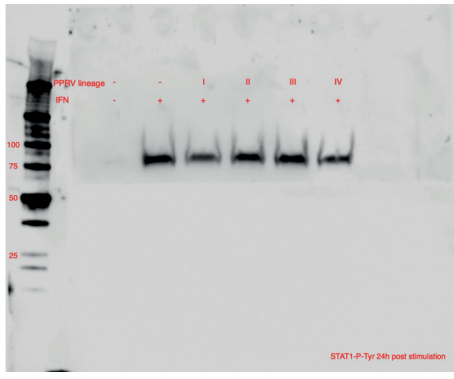
Supplementary figure 3. Western blot analysis was performed to analyze STAT1 expression 24 h post-stimulation using 1000 IU/mL IFNγ. Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories) High: 7000, Low: 0, Gamma: 0.8.



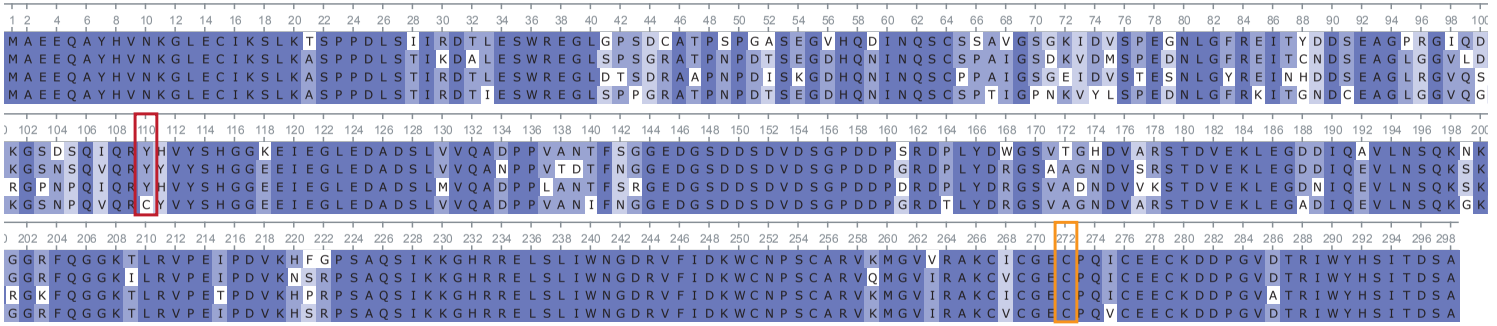
Supplementary figure 4. Western blot analysis was performed to the phosphorylation of STAT1 at the tyrosine701 phosphorylation site 2h post-stimulation using 1000 IU/mL IFNγ. Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories) High: 7000, Low: 0, Gamma: 0.8.



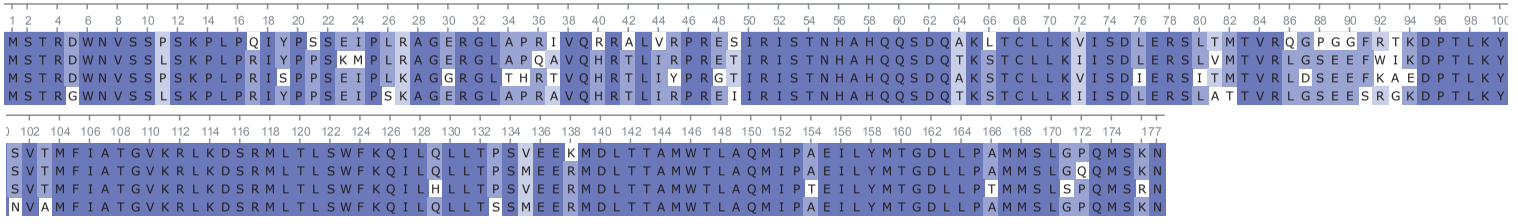
Supplementary figure 5.Western blot analysis was performed to the phosphorylation of STAT1 at the tyrosine701 phosphorylation site 6h post-stimulation using 1000 IU/mL IFNγ. (Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories) High: 7000, Low: 0, Gamma: 0.8.



Supplementary figure 6. Western blot analysis was performed to the phosphorylation of STAT1 at the tyrosine701 phosphorylation site 24h post-stimulation using 1000 IU/mL IFNγ. Settings for images used in ImageLab (version 5.2.1, Bio-Rad Laboratories): High: 7000, Low: 0, Gamma: 0.8.



Supplementary Figure 7. Amino acid alignment of PPRV V proteins. From the top: ICV89 (lineage I), Nigeria 75/1 (lineage II), Uganda/2012 (lineage III), and Ethiopia/2010 (lineage IV). The red square indicates amino acid residue 110 that in measles virus have been shown to be an important determinant of IFN type I modulatory abilities and depend on a tyrosine (Y) at this position. In PPRV, the substitution of tyrosine to cysteine in the Ethiopia/2010 (lineage IV) isolate did not affect the ability to modulate IFN type I. The orange square indicates amino acid residue 272, that is also an important determinant for IFN type I modulation and depend on a cysteine (C) residue. Alignment created using UGENE software.



Supplementary Figure 8. Amino acid alignment of PPRV C proteins. From the top: ICV89 (lineage I), Nigeria 75/1 (lineage II), Uganda/2012 (lineage III), and Ethiopia/2010 (lineage IV). Alignment created using UGENE software.

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Sheep and goats are one of the most important sources of food and income for many people. The disease peste des petits ruminants (PPR) mainly affects domestic sheep and goats, and is caused by the highly contagious PPR virus. This thesis has explored aspects of PPR, among other things, prevalence and risk factors, development of better diagnostic methods, and the virus-host interactions.

Emeli Torsson received her graduate education at the Department of Biomedical Sciences and Veterinary Public Health. She obtained her veterinary degree at the Swedish University of Agricultural Sciences (SLU).

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