ESTABLISHMENT OF LONG-READ NANOPORE SEQUENCING AND PROFICIENT NANOBODIES AGAINST PESTE DES PETITS RUMINANTS VIRUS ON THE ROAD TO DEVELOP DIAGNOSTIC AND THERAPEUTIC TOOLS

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

Peste des petits ruminants virus (PPRV) causes a highly devastating disease of sheep and goats, peste des petits ruminants (PPR), that threatens food security, animal production and the conservation of wild small ruminants. Growing body of evidence suggests that multiple wildlife and atypical host species can be infected with PPRV, posing a serological diagnostic challenge in multi-host environment. Recent studies confirmed that single-domain antigen binding fragments (nanobodies) derived from heavy-chain-only camelid antibodies and nanopore sequencing have proven to be powerful technologies for the development of cost-effective and robust therapeutic and diagnostic tools, respectively. Therefore, the main objective of this study was to generate PPRV-reactive nanobodies in order to set pace for the development of diagnostic and possible therapeutic nanobodies in the future, alongside with establishment of rapid complete genome nanopore sequencing of PPRV. Firstly, a strategy was developed to generate nanobodies against PPRV, whereby an alpaca (*Vicugna pacos*) was immunized with live attenuated vaccine strain (PPRV/Nigeria/75/1) to raise an affinity-matured immune response in the heavy-chain-only antibody classes. An immune nanobody library with approximately 64 million independent transformants was engineered, of which 100% contained an insert with the proper size of nanobody gene. Following phage display and *in vitro* affinity selection (biopanning), nine nanobodies that specifically recognise PPRV were identified on enzyme-linked immunosorbent assay. They showed superb potency in identifying rapidly PPRV, which is likely to open a new perspective in the diagnosis and possible treatment of PPRV infection. Secondly, prior to the full genome sequencing of PPRV, nanopore sequencing protocol was tested for amplification and sequencing of PPRV. With this protocol, there were no DNA fragments and nucleotide sequences in the GC-rich region between matrix (M) and fusion (F) genes at the genome position between 4,444 and 5,526. Thus, a tiling multiplex polymerase chain reaction method was developed to amplify the

missing DNA fragments. Following redesigning of three pairs of overlapping long read primers and cascade of optimization, the GC rich region was successfully amplified and sequenced (accession numbers: MW580394, MW580395 and MW580396). These three pairs of primers targeting the GC-rich region were used along with other 22 pairs of primers in tiling multiplex PCR for complete PPRV genome sequencing. The resulting PCR amplicons were used for nanopore library preparation and ultimate sequencing. This method has resulted into complete genomes of PPRV, with 15,948 nucleotides long for both isolates that were produced within four hours of sequencing (Accession numbers MW960272 and MZ322753). Phylogenetic analysis of the complete genomes revealed a high nucleotide identity between 96.19 and 99.24% with lineage III PPR viruses currently circulating in East Africa indicating a common origin. The nanopore sequencing platform can be deployed to overcome PPR diagnostic and surveillance challenges, unanticipated variations in virus pathogenicity, circulation of disease in wildlife populations and to service remote and nomadic communities with challenging geographical landscapes. However, further investigations are recommended for PPRV reactive nanobodies especially on diagnostic and therapeutic applications. Once validated, these technologies have great potential for use in the field as rapid and cost-effective tools in context of planned PPR Global Control and Eradication Programme.

DECLARATION

I, EDSON KINIMI, do hereby declare to the Senate of Sokoine University of Agriculture, that this thesis is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for a degree award in any other institution.

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Prof. Gerald Misinzo **Date**

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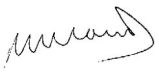
Prof. Serge Muyldermans **Date**



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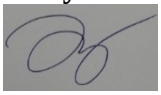

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DEDICATION

This work is dedicated to my mom Paschazia Fundi, my late father Fredinand Kinimi, and my wife Rehema Makaka and my children; Shalom and Covman, in recognition of their love, prayers and support in their own special way during the entire period of study.

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Paper II: Kinimi, E., Muyldermans, S., Vincke, C., Odongo, S., Kock, R., Parida, S., Mahapatra, M. and Misinzo, G. (2021). Development of Nanobodies Targeting Peste des Petits Ruminants Virus: The Prospect in Disease Diagnosis and Therapy. *Animals* 11(8): 206-219.

Paper III: Kinimi, E., Hakizimana, J.N. and Misinzo, G. (2021). Nucleotide amplification and sequencing of the GC-rich region between matrix and fusion protein genes of peste des petits ruminants virus. *Journal of Virological Methods* 300(1): 390-396.

Paper IV: Kinimi, E., Mahapatra, M., Kgotlele, T., Makange M.R., Tennakoon, C., Njeumi, F, Odongo, S., Muyldermans, S., Kock, R., Parida, S., Rweyemamu, M. and Misinzo, G. (2021). Complete genome sequencing of field isolates of peste des petits ruminants virus from Tanzania revealed a high nucleotide identity with lineage III PPR viruses. *Animals* 11(10): 976-995.

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ORGANISATION OF THE THESIS

This PhD thesis has been developed based on published papers format of the Sokoine University of Agriculture. The first chapter consists of general introduction which comprises the background, problem statement, justification of the study, research questions and study objectives. Chapter two contains original published research papers. The third chapter consists of general discussions, conclusions and recommendations. The arrangement of the papers follows the chronological order of the specific objectives.

LIST OF ABBREVIATIONS

AGID	agar gel immunodiffusion
BEAST	Bayesian evolutionary analysis sampling trees
BEAUti	Bayesian evolutionary analysis utility
CAHEC	China Animal Health and Epidemiology Center
CDR	complementarity determining region CDR
C-ELISA	competitive enzyme-linked immunosorbent assay
CIRAD	International Cooperation Centre for Agronomic Research and Development
CITES	Convention on International Trade in Endangered Species
CIE	counter-immunoelectrophoresis
COVID-19	coronavirus disease of 2019
DNA	deoxyribonucleic acid
ESS	effective sample size
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation of the United Nations
GF-TADS	Global Framework for the Progressive Control of Transboundary Animal Diseases
GC	guanine-cytosine content
GEP	Global PPR Control and Eradication Programme
HCAbs	heavy chain-only antibodies
IC-ELISA	immunocapture enzyme-linked immunosorbent assay
IC-LFDs	immunochromatographic lateral flow devices
IgG	immunoglobulin G
LIPS	luciferase immunoprecipitation system
MAbs	monoclonal antibodies

MCC	Bayesian maximum clade credibility
MCMC	Bayesian Markov chain Monte Carlo
NGS	next generation sequencing
OIE	World Organisation for Animal Health
ORF	Open reading frame
PCR	polymerase chain reaction
PPR	peste des petits ruminants
PPRV	peste des petits ruminants virus
RACE-PCR	rapid amplification of cDNA ends by polymerase chain reaction
RNA	ribonucleic acid
RT-LAMP	reverse transcription loop mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
RT-PRA	reverse transcription recombinase polymerase amplification assays
SACIDS	Southern African Centre for Infectious Disease Surveillance
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SUA	Sokoine University of Agriculture
TMRCA	time to the most recent common ancestor
TPI	The Pirbright Institute
UK	United Kingdom
UTR	untranslated intergenic region
VNT	virus neutralization test
VHH	Single domain antigen binding fragment of HCAbs
VUB	Vrije Universiteit Brussel, Brussels, Belgium

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Peste des petits ruminants (PPR) is an economically important transboundary disease of sheep and goats caused by peste des petits ruminants virus (PPRV) (Gibbs *et al*., 1979; Jones *et al*., 2016). Based on the Global PPR Control and Eradication programme (GEP), an initiative of World Animal Health Organization (OIE) and Food and Agriculture Organization of the United Nations (FAO), the eradication of PPR depends primarily on rapid and accurate diagnosis and implementation of prompt vaccination programme (Zhao *et al*., 2021). A plethora of diagnostic tests are available to screen and confirm PPR outbreaks, but they were principally developed for domestic sheep and goats (Kinimi *et al*., 2020). The presence of a wide host range for PPRV infection does present diagnostic challenges in a multi-host environment (Fine *et al*., 2020).

The access to cost-effective sequencing technology, vaccines, diagnostics and therapeutics are limited in many developing countries especially in Africa where PPR is endemic (Britton *et al*., 2019). This highlights the need for rapid, sensitive, reliable and virus-specific diagnostic tests and cost-effective therapeutics to control the spread of PPR in both domestic animals and wild ruminants (Kinimi *et al*., 2020). There is also restrictions in transporting clinical samples across international borders, due to the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora and Nagoya Protocol regulations (Fine *et al.*, 2020). In addition, there is a constrain of RNA degradation due to difficulty in maintaining cold chain in the field and temperature fluctuations in ultralow temperature freezers as a result of frequent power cuts, and this has remained

major challenge in low and middle income countries (Torsson *et al*., 2019). For this reason, confirmatory diagnosis is usually delayed, often for several months after the initial cases, resulting in increased transmission in PPR free zones (Baron *et al*., 2014). Deployment of cost-effective technologies to break the limitations in the development of diagnostic and therapeutic tools is critically important for PPR control and eradication in resource-limited settings.

The development of necessary tools for PPR control, including vaccines, diagnostics and therapeutics, greatly depends on in-depth genomic data (Parida *et al.*, 2015). The sequencing of the N and F genes in 1994 was crucial in the development of diagnostic tests for PPR detection and confirmation (Couacy-Hymann *et al*., 2002; Kwiatek *et al*., 2010). In early, 2005, a complete genome of PPRV was generated for the first time using Sanger sequencing standard methods (Bailey *et al*., 2005). Unfortunately, few complete genomes of PPRV field isolates exist and very few from East Africa, despite of the existence of lineages II, III and IV PPRV in East Africa (Dundon *et al.*, 2020). The PPRV complete genome sequencing is often performed at OIE reference laboratories (Table 1). In order to widen the scope of PPR diagnosis in non-typical hosts and in the field, there is a need to development alternative diagnostic tools to obtain cost-effective epidemiological information. Thus, the deployment of nanopore sequencing and nanobody Ablynx technologies for the development of cost-effective diagnostic and therapeutic tools can be of additional value to the GEP.

Table 1: Complete genomes of PPRV available at the GenBank and European nucleotide archive database from East Africa. Sequencing of East African PPRV field isolates were carried outside the country of origin with exception of Tanzanian field isolates generated in this study

Name of Isolate	Country of origin	Sequencing laboratory (Country)	Sequencing technology	Accession number
KN5/2011	Kenya	CIRAD (Montpellier, France)	Sanger dideoxynucleotide cycle sequencing	KM463083.1
Uganda 2012	Uganda	TPI, (Surrey, United Kingdom)	Illumina	KJ867543.1
B3	Burundi	FAO/IAEA Animal Production and Health (Seibersdorf, Austria)	Sanger dideoxynucleotide cycle sequencing	MK686066.1
Tanzania/ Dakawa	Tanzania	Swedish University of Agricultural Sciences, Uppsala, Sweden		PRJEB35549
Tanzania/ Ngorongoro/ 2016	Tanzania	Sokoine University of Agriculture, Morogoro, Tanzania	Nanopore sequencing	MW960272
Tanzania/	Tanzania	Sokoine University of	Nanopore	MZ322753

Momba/2018	Agriculture, Morogoro, Tanzania	sequencing

1.1.1 Peste des petits ruminants virus genome

Peste des petits ruminants virus belongs to the genus Morbillivirus of the family Paramyxoviridae, alongside rinderpest virus, measles virus, canine distemper virus, phocine distemper virus, cetacean and feline viruses (De Vries *et al*., 2015). Like any member of Morbillivirus, the genome of PPRV consists of six transcriptional units encoding the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H), and large polymerase protein also known as RNA dependent RNA polymerase (L) (Fig. 1). The *P* gene also codes two more non-structural proteins designated by C and V (Mahapatra *et al*., 2003).

The N protein is abundant in PPRV-infected cells because the *N* gene is located in the proximity of genomic promoter and it is the most transcribed gene (Zhang *et al*., 2011). Given its abundance and antigenic stability, the N protein is an ideal candidate in PPR diagnostic development and the most appropriate gene for the molecular characterization of closely related isolates (Choi *et al*., 2005). Most anti-PPRV neutralizing antibodies are directed against the surface glycoprotein H (Rojas *et al*., 2014). As a result, the N and H proteins are ideal targets in diagnostics and vaccine development, respectively.

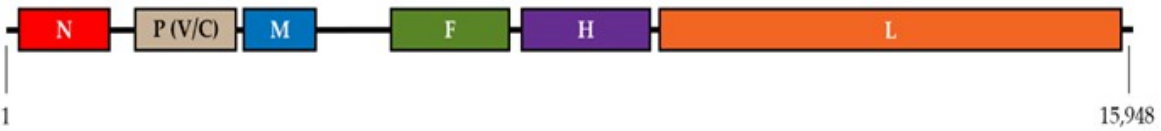

Figure 1: Schematic illustration of peste des petits ruminants virus genome. The viral genome consists of six transcriptional units in order of

nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H), and large polymerase (L). This figure was sketched in this study.

1.1.2 Nanobodies

Nanobody is a single domain antigen binding fragment (~15kDa) of heavy-chain-only camelid antibodies devoid of light chains derived from camels, llamas and alpaca (Fig. 2) (Hamers-Casterman *et al*., 1993). Camelids are members of the Camelidae family in the suborder Tylopoda (Fig. 3). The antigen binding site of these special antibodies is formed by one single domain only, the so called VHH domain or nanobody (Fig. 2). The high production cost of classical monoclonal antibodies, large size and less favourable pharmacokinetics stability, have stimulated the use of nanobodies in diagnostics and therapeutics (Muyldermans, 2020).

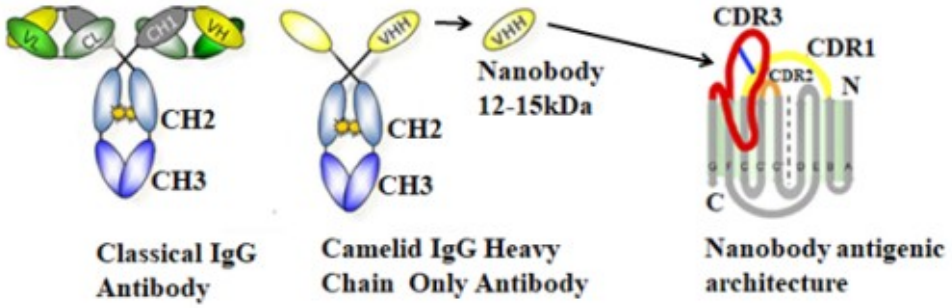

Figure 2: Schematic illustrations of a classical IgG and a Camelidae heavy chain-only antibodies (HCAbs). In HCAbs, antigen binding occurs through a single domain antigen binding fragment known as the VHH domain or nanobody. The VHH comprises three complementarity determining regions (CDRs) in the paratope. This Figure was modified from Rothbauer (Rothbauer *et al*., 2006).

Figure 3: **Images of camelidae species that produce a special antibody devoid of light chain; (a) Lama glama, (b) Lama alpaca, (c) Lama guanoco, (d) Camelus dromedarius, (e) Camelus bactrianus and (f) Lama vicugna. The camelids blood comprise 45% of heavy chain-only antibodies in *Lama* spp and 75% in *Camelus* spp. This image was modified from Muyldermans' presentation (Muyldermans, 2020).**

1.1.3 Production of proficient nanobodies

Immunizing camelid with viral antigens raises an affinity-matured immune response in the special camelid heavy-chain only antibodies (HCAbs) (Vincke *et al*., 2012). The peripheral blood lymphocytes of the immunized camelid are used to clone the VHH from the HCAbs in a phage display vector (Romão *et al*., 2018). A representative aliquot of the library of these antigen-binding fragments is used to retrieve nanobody binders by successive rounds of affinity selection (Fig. 4).

Advances in cloning strategy, high-quality immune nanobody library is generated by employing Golden gate cloning vector (pMECS-GG phagemid), whereby a cytotoxic gene (ccdB) that encodes a lethal protein is substituted by the nanobody gene (Romão *et al*., 2018). In this approach, a considerably large immune library is produced where 100% of the clones possess a phagemid carrying an insert with a length of a nanobody gene. Thus, the nanobody library can be constructed within a week and is more cost-effective than previous standard approaches using classical restriction enzymes and ligations (Chow *et al*., 2019). In addition, nanobodies are expressed at a large scale in bacterial systems with superiority, which is key for their use in diagnostics and therapeutics applications (Huang *et al*., 2010).

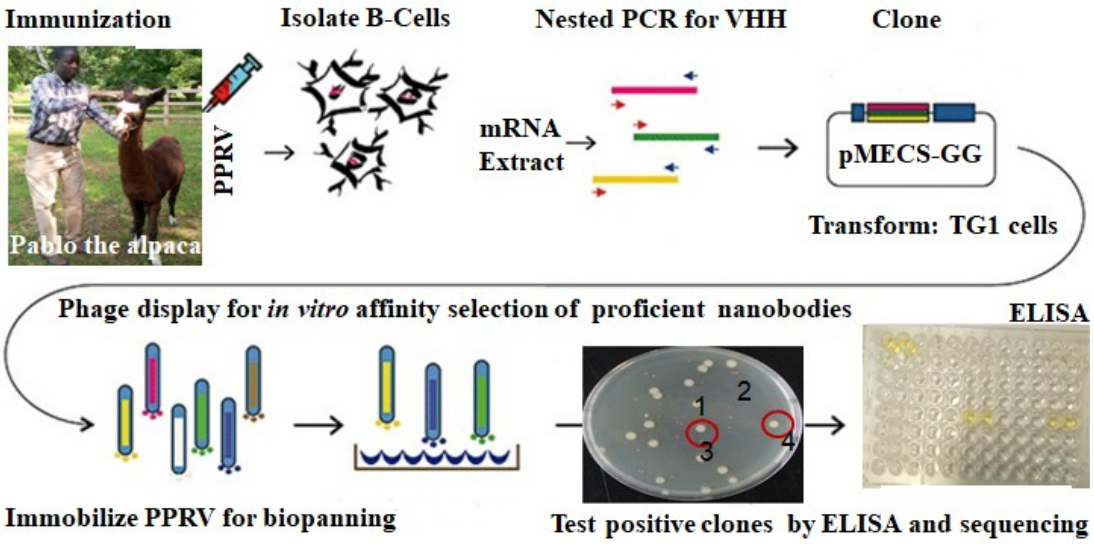

Figure 4: Schematic overview to produce proficient nanobodies. Nanobody gene repertoires from B-cells are engineered in phage display vector pMECS-GG to obtain pMECS-nanobody library after transforming TG1 cells. This is followed by retrieving antigen specific nanobodies from a library through phage display and biopanning. The highest nanobody binders are expressed and tested on enzyme linked-immunosorbent assay against the target antigen(s). This figure was sketched in this study.

1.1.4 Nanopore sequencing of PPRV

Nanopore sequencing is a rapid and relatively cheap next generation sequencing technology uses portable devices that require minimal supporting laboratory infrastructure or technical expertise for sample preparation (Quick *et al*., 2016). With nanopore sequencing, the ARTIC method for enrichment of PCR amplicons has been a robust technique to generate complete genomes of field isolates including Zika virus, Ebola virus and severe acute respiratory syndrome coronavirus 2 (Liu *et al*., 2022). Using this advanced technique, by assessing the long overlaps among multiplex amplicons, the accurately assembled and complete viral genome can be obtained. However, the longest untranslated intergenic region, which is rich in guanine-cytosine (GC) content impedes nucleotides amplification and affects the downstream full genome sequencing (Torsson *et al*., 2020). The GC-rich

region is rich about 1,080 nucleotides long with 66 to 72% GC content (Meyer and Diallo, 1995).

Recent studies have demonstrated that extremities and GC-rich region of the PPRV genomes are usually very difficult to generate by NGS sequencing (Torsson *et al*., 2020). The NGS technologies can exhibit bias when applied to GC-rich regions of DNA, primarily due to reduced sequence complexity but also as a result of PCR bias during nucleotide amplification steps (Benjamini and Speed, 2012). With Illumina, one step more is carried out to complete the extremities of the genome using rapid amplification of cDNA-ends by polymerase chain reaction (RACE PCR) (Eloiflin *et al*., 2019). In order to evade these deficiencies, a PCR method need to be developed to amplify the troublesome GC-rich region of PPRV to allow a single sequencing run.

1.2 Problem statement and Justification of the Study

In many developing countries where PPR is endemic, an access to cost-effective sequencing technology, therapeutics and diagnostics are limited. This is also compounded by the existence of a broad host range, which poses serological diagnostic challenge in atypical animal species. Nevertheless, the development of necessary tools for PPR control, including vaccines, diagnostics and therapeutics, greatly depends on the presence of in-depth genomic data. Unfortunately, few complete genomes of PPRV are available in public databases and very few from East Africa, despite of the existence of lineage II, III and IV PPRV. The establishment of nanobody Ablynx and nanopore sequencing technologies provide an important opportunity for development of cost-effective immunodiagnostic reagents and revolutionize molecular epidemiological surveillance of PPR, respectively.

Therefore, there was need for the production of nanobodies as an alternative immunoreagent to enable the development of cost-effective diagnostic and possibly therapeutic tools for PPR in the future, alongside establishing rapid complete genome nanopore sequencing of PPRV in resource limited settings.

1.3 Research Questions

i. What are potent nanobodies from immune nanobody library for detection of PPRV?
ii. How can nanopore sequencing be used to generate a complete genome of PPRV in a single run for use in advanced molecular epidemiological surveillance of PPR in developing countries?

1.4 Research objectives

1.4.1 Main objective

The general objective of this study was to establish PPRV-reactive nanobodies for use in PPR diagnostic and therapeutic research in the future, alongside establishing rapid complete genome nanopore sequencing of PPRV for advanced molecular epidemiological surveillance.

1.4.2 Specific objectives

i. To establish an immune nanobody library against PPRV through golden gate cloning strategy,
ii. To investigate proficient nanobodies against PPRV from immune nanobody library

iii. To establish rapid complete genome nanopore sequencing of PPRV using tiled multiplex long-read primers.

CHAPTER TWO

PAPER ONE

2.0 PARADIGM SHIFT IN THE DIAGNOSIS OF PESTE DES PETITS RUMINANTS: SCOOPING REVIEW

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REVIEW Open Access

Paradigm shift in the diagnosis of peste des petits ruminants: scoping review

Edson Kinimi[1*], Steven Odongo[2], Serge Muyldermans[3], Richard Kock[4] and Gerald Misinzo[1]

Abstract

Peste des petits ruminants virus causes a highly contagious disease, which poses enormous economic losses in domestic animals and threatens the conservation of wild herbivores. Diagnosis remains a cornerstone to the Peste des petits ruminants Global Control and Eradication Strategy, an initiative of the World Organisation for Animal Health and the Food and Agriculture Organisation. The present review presents the peste des petits ruminants diagnostic landscape, including the practicality of commercially available diagnostic tools, prototype tests and opportunities for new technologies. The most common peste des petits ruminants diagnostic tools include; agar gel immunodiffusion, counter-immunoelectrophoresis, enzyme-linked immunosorbent assays, reverse transcription polymerase chain reaction either gel-based or real-time, reverse transcription loop-mediated isothermal amplification, reverse transcription recombinase polymerase amplification assays, immunochromatographic lateral flow devices, luciferase immunoprecipitation system and pseudotype-based assays. These tests vary in their technical demands, but all require a laboratory with exception of immunochromatographic lateral flow and possibly reverse transcription loop-mediated isothermal amplification and reverse transcription recombinase polymerase amplification assays. Thus, we are proposing an efficient integration of diagnostic tests for rapid and correct identification of peste des petits ruminants in endemic zones and to rapidly confirm outbreaks. Deployment of pen-side tests will improve diagnostic capacity in extremely remote settings and susceptible wildlife ecosystems, where transportation of clinical samples in the optimum cold chain is unreliable.

Keywords: Diagnostics, Nanobodies, Nanopore, *Peste des petits ruminants*

Background

Peste des petits ruminants virus (PPRV) causes an acute and highly contagious infection, which can cause significant socio-economic losses in domestic animals and threatens the conservation of wild herbivores. The PPRV belongs to the genus Morbillivirus of the family *Paramyxoviridae* [1, 2], which includes eradicated *Rinderpest virus, Measles virus, Canine distemper virus, Phocine distemper virus*, Cetacean and Feline morbilliviruses [3]. Rapid field diagnostics against rinderpest became available in final phases of the eradication process, but were never really tested within livestock whilst proving valuable in wildlife environment [4–6]. The rinderpest eradication in 2011 provided the pathway and the possibility of peste des petits ruminants (PPR) eradication given the close phylogeny of these viruses, availability of a reliable and effective vaccine against PPR and sensitive and specific diagnostic tests [7]. PPR most likely emerged in the early part of the twentieth century whilst its presence was masked by ongoing rinderpest epidemics, which also affected small stocks and where immunity was cross protective [8]. Based on phylo-geographical analysis, geographic origins of the most recent common ancestor of PPRV lineages I, II, and III were proposed to originate from Africa whilst lineage IV might have originated from India [9]. PPRV has continued to expand its

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geographic boundaries, reaching regions previously not infected and putting hundreds of millions of both domestic small ruminants and wildlife at risk of infection. However, the occurrence of PPRV in previously uninfected regions, together with the mixing of lineages in endemically infected countries, highlights the dynamic and transboundary nature of this disease [10–12]. The expansion of PPR range has been known for many decades but it has taken a considerable time to raise international interest and to bring PPR to the status of a priority disease for livelihood and food security. PPR was finally included in the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs), an initiative of the World Organisation for Animal Health (OIE) and the Food and Agriculture Organisation (FAO) of the United Nations [13, 14]. The annual global impacts of PPR was in 2017 estimated at between US$1.4 and $2.1 billion [13]. However, it is estimated that an investment of US$ 7.1 billion on global PPR eradication could be recovered within 5 years of successful eradication [13]. Some academics believe that the actual cost of eradication could be much lower than this [15], but unfortunately the tardiness of the response to its expansion, increases the likely cost by the day.

Reported PPR outbreaks and infection studies in captive and wild ruminants have extended the known spectrum of potentially infected species to include most *bovidae* and *suidae*. In 2014, there were warnings of the risk of PPR infection of the saiga antelope (*Saiga tatarica mongolica*), which is a critically endangered species in central Asia [16], and since then there has been no proper actions to protect susceptible wild ruminants population from PPR outbreaks. This was followed by PPR epidemic in the small surviving population of a sub-species of saiga with extinction of more than half of the population [17]. Consequently, the saiga catastrophes emphasized the failure of PPR eradication strategies in considering wildlife and possible virus spill over from livestock. From 2014 to 2016, more than 1000 wild goats (*Capra aegagrus*) and sheep (*Ovis orientalis*) in the northern and central provinces of Iran died from PPRV infection [18]. Transmission of PPRV from infected goats to cattle has also been reported [19], and PPRV antigen has been detected in camels [20] and even companion animals, in particular dogs [21]. Goats and sheep are the maintenance hosts and the other hosts are apparently considered as spill over without any other reservoir populations confirmed.

Sheep and goats are vital for more than 330 million poor subsistence and marginal farmers in Africa and Asia a home to more than 1.7 billion sheep and goats, where over 80% of the world's small ruminants occur and here PPR causes food insecurity and contributes to poverty [13, 22]. The clinical signs elicited by PPRV may vary depending on the breed of the affected animal species and/or the strain of virus [23, 24]. Besides, other factors such as the resilience of the population, nutrition, co-infection and other stressors contribute to the pathogenesis of PPRV infection. The severity of disease depends on the immune status of the animal; for example, newborn animals become susceptible to PPRV infection at three to 4 months of age following natural decline in colostral antibodies. Early and accurate diagnosis of PPRV infection is important for prompt control and this can be facilitated by pen-side diagnostics (Table 1). The availability of simple cost-effective pen-side diagnostics and laboratory based tests would aid in the prevention and control of PPR in low-income countries. However, these tests should be performed in reference with OIE prescribed tests for confirmation of clinical cases using immunocapture enzyme-linked immunosorbent assay (IC-ELISA) and reverse transcription polymerase chain

Table 1 Diagnostic value of commercially available field-deployable diagnostic tools and pen-side prototype tests for PPR diagnosis

Diagnostic tests	Target (s)	Merits	Limitations	Detection limit	References
Immunochromatography lateral flow test	H and N proteins	Very rapid and pen-side test	Less sensitive than PCR	10^3 to 10^4 $TCID_{50}$	[26]
Quantum dots lateral flow	PPRV IgG antibodies	Ultrasensitive and field test	Cannot detect active case	Specificity 99.47%, sensitivity 97.67%	[27]
One-step RT-LAMP	M gene	Rapid and easy to perform	Not a field-level diagnostic	1.41×10^{-4} ng total RNA per assay	[28]
Two-step RT-LAMP	N gene	Rapid and pen-side test	Require six primers	100% specificity and sensitivity	[28–30]
Recombinase polymerase amplification assay	N gene	Rapid compared to RT-LAMP	Less sensitive compared to RT-PCR	Sensitivity 90% and specificity100%	[31, 32]
Oxford nanopore MinION sequencers	Viral genome	Rapid	Prone to high host nucleic acids		[33, 34]

Table 2 OIE diagnostic methods that are recommended (+++) and suitable (++) for confirmation of clinical cases and certifying freedom from peste des petits ruminants

Diagnostic method	Purpose				
	Target	Case confirmation	Population freedom	Immune status	International trade
IC- ELISA	Viral protein	+++			
RT-PCR	Viral genome	+++			
Virus isolation	PPRV	++			
VNT	Antibodies		+++	+++	+++
C-ELISA	Antibodies		++	+++	

reaction (RT-PCR) and for certification of population freedom from infection by competitive enzyme-linked immunosorbent assay (C-ELISA) and virus neutralization test (VNT) [25] (Table 2). Thus, the use of automated assays that do not require supplementary multiple reagents and lateral flow diagnostic strips technologies based on low cost immunoreagents such as nanobodies may accelerate the development of powerful diagnostic assays. In addition, lack of validated tests amongst wildlife species creates uncertainties in the interpretation of surveillance data.

Search strategy and selection criteria

The MEDLINE (PubMed) and Google scholar search machines were used as source of the peer-reviewed articles included in this review. The articles were selected using keywords combined by Boolean operators (peste des petits ruminants OR PPR OR *Peste des petits ruminants virus* OR PPRV OR diagnosis OR diagnostic*AND (PPR diagnosis). All searches on PPR diagnosis were performed in 2 years. Only 142 articles out of 4782 written in English from the first description of PPR in 1942 met inclusion criteria as shown in PRISMA flow diagram (Fig. 1).

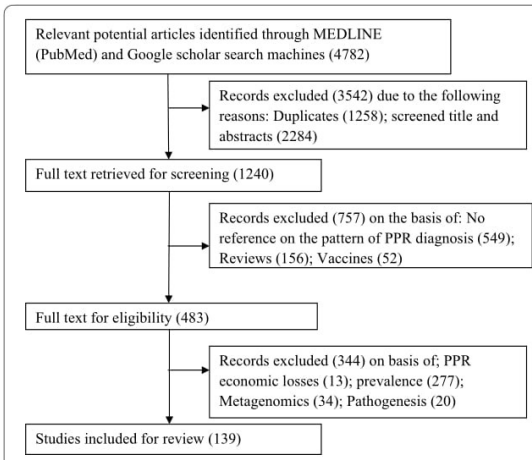

Fig. 1 Flow diagram for the review process

Susceptible animal species and transmission of peste des petits ruminants

PPRV infects domestic as well as wild ruminants with goats and sheep being the most susceptible domestic animals and which also serve as primary hosts. The disease has been reported to be more severe in goats than in sheep, although this claim still lacks scientific proof [35]. Transmission of PPRV occurs through direct contact with infected animals, inhalation of aerosol (expectorate), or contacts with lacrimal secretions, nasal exudates, saliva and faeces.

Studies have shown that both camels and suids are susceptible to PPRV infection and develop clinical disease [36, 37]. The role of wildlife animals and domestic Artiodactyls in the epidemiology of PPR is unknown or insufficiently understood [38]. Infections of various wildlife species including African buffalo (*Syncerus caffer*) and many antelope species occur apparently subclinical [38, 39] but the only confirmed reports of disease in African wildlife have occurred under captive or semi-free range conditions [40, 41]. According to previous studies, animals that recover from PPRV infections develop life-long immunity [42, 43]. Because of its immunosuppressive effect, PPRV infections are usually accompanied by secondary infections thereby complicating clinical diagnosis.

Clinical manifestation

It takes 3–4 days before onset of clinical signs. During this incubation period, PPRV replicates in the draining lymph nodes of the oropharynx followed by spreading (via blood and lymph) to other tissues and organs including the lungs resulting in a primary viral pneumonia. The acute stage of disease is characterised by high

body temperature (39.5 to 41 °C) which may last for 3–5 days [44]. Other signs are depression, anorexia, dry muzzle, excessive salivation, lachrymal discharges and serous nasal discharge, which gradually turn mucopurulent (Fig. 2). The affected animals develop papules in the oral cavity, which become erosive and necrotic. In severe cases, these necrotic lesions occur concurrently with fibrin deposits on the tongue [24, 35, 45]. In the later stages, there is diarrhoea and cough with labored abdominal breathing. Terminally, the animal may become dyspnoeic, progressively lose weight and eventually dies. In mild infections, self-cure occurs after 10–15 days of infection. Protective immunity responsible for self-recovery is attributed to infection-induced antibodies against the haemagglutinin (H) and fusion (F) proteins [46, 47], although most of the neutralizing antibodies are directed against the H protein [48]. Nucleoprotein (N) is the most abundantly transcribed gene in the host cells. For this reason, the H and N proteins are the two most preferred PPRV targets for the development of vaccine and immunodiagnostics, respectively [49, 50].

Epidemiology of peste des petits ruminants

The development of specific and sensitive molecular and serological techniques have improved the diagnostic precision for PPR since not all cases of PPR can be distinguish from rinderpest, pneumonic pasteurellosis and contagious caprine pleuropneumonia, based on clinical sigs [4]. Based on previous similar outbreaks of the disease in Senegal and Guinea in 1871 and 1927, respectively, it was believed that PPR might have been existing much earlier than previously thought [9, 10]. The disease spread subsequently to the neighbouring African countries like Nigeria and Ghana [51]. Until early 1980s, definite outbreaks of PPR were reported from different parts of West Africa [42, 52] and it was regarded as a disease of West African countries. However, it was later realised that the disease spread beyond West Africa with cases being recorded in Sudan [53]. In the northern part of Africa, PPR was reported in Morocco in 2008 and later Egypt, Algeria and Tunisia have also reported PPR [54–56]. Globally, PPR affects about 70 countries in Africa, Asia and the Middle East [57]. Out of 70 countries that have either reported PPR infection to the OIE or are suspected of being infected, more than 60% are in Africa (except southern Africa). Other infected countries are in Asia (South-East Asia, China, South Asia and Central Asia/West Eurasia including Turkey) and the Middle East [13, 14, 57]. PPRV lineages I and II have been found exclusively in western and central Africa; lineage III is common to eastern Africa and the southern part of the Middle East. Lineage IV is found in Southeast Asia, Middle East and North Africa [23, 44, 58–60]. Incursions of PPRV lineages have been also reported, for instance lineage II and IV were found in East Africa and lineage IV in Ethiopia [12, 61–64]. Recent outbreaks of PPR in Bulgaria, Georgia and the Marmara region in Turkey increase the threat to Europe [65–67]. The spread of PPR beyond its usual boundaries is attributed to cross-border movements of animals and animal products, which are being promoted by trade, nomadic lifestyle, tourism and migration of wild animals [9, 59, 68].

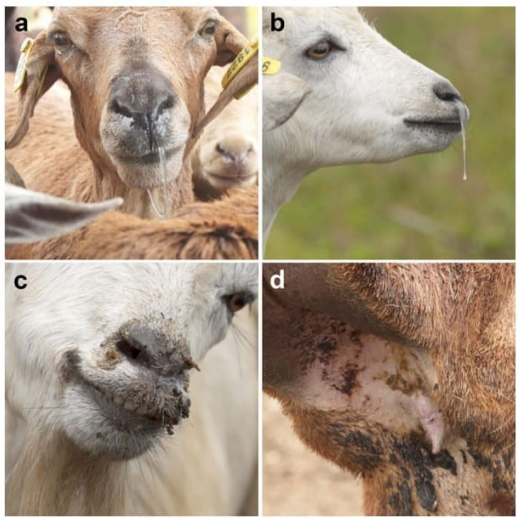

Fig. 2 Clinical signs in goats and sheep confirmed with *peste des petits ruminants virus* infection in a farm located in Tanga, Tanzania. Nasal discharges in **a** a sheep and **b** a goat, **c** dried-up purulent nasal discharges in a goat, and **d** diarrhoea in a sheep

PRRV as a target for diagnostics and vaccine development

Like other members of the genus *Morbillivirus*, PRRV is enveloped, pleomorphic with the particles diameter ranging from 400 to 500 nm [1]. The genome is a linear, non-segmented negative sense single stranded RNA, which is15,948 nucleotides long. There are six genes i.e. 3′-*N, P, M, F, H, L*-5′, which constitute the genome. Each of these genes codes for a distinct structural protein and each of these proteins bear the acronym of the respective gene of origin; nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H) and large polymerase protein (L). Besides coding for the phosphoprotein, the *P* gene also codes for two non-structural proteins designated C and V. The C and V proteins are generated through alternative start codons (leaky scanning) and RNA editing, respectively [69]. It has been shown that the N protein is abundant in infected cells and highly immunogenic. On the other hand, given its abundance and antigenic stability, the N

protein has been a preferred candidate antigen for development of PPRV immunodiagnostics [70, 71]. The P protein is a co-factor for viral replication and transcription in conjunction with the L protein, which is an RNA-dependent RNA polymerase. The M constitutes the inner coat of the viral envelope and acts as a bridge connecting the surface glycoproteins (F and H) to the ribonucleoprotein core. The H protein mediates attachment of virions to the host receptors whilst F protein induces fusion of the viral membrane with the host cell membrane in order to enter host cells [70]. Then on-structural proteins play several functions including viral RNA synthesis, virulence determination and modulation of RNA-dependent RNA polymerase activity and blockade of interferon signaling.

PPRV exists as a single serotype, but is divided into four distinct genetic lineages [72]. The development of necessary tools for PPRV control, including vaccines and diagnostics, greatly relied on detailed insights into the PPRV genome and protein constituents; and on this same basis the virus is now classified into lineages. Structurally, the N protein is divided into four regions I, II, III and IV of amino acids sequences, 1–120, 121–145, 146–398 and 421–525, respectively [49]. Owing to high immunogenicity of regions I and II, these could be targeted for improvement and development of immunoassays. On the other hand, based on H protein mapping of the functional domain, two regions are the most immuno-dominant epitopes (263–368 and 539–609) and are diverse among all the members of Morbilliviruses with significant potential for development of a DIVA vaccine that could differentiate infected from vaccinated animals [47].

Tentative diagnosis

Presumptive diagnosis of PPR is based on clinical signs presented in living animals and postmortem lesions. Such diagnosis in PPRV endemic zones could play an important role in early warning in disease-symptomatic surveillance when coupled to digital diagnostic technology [73–75]. However, definitive laboratory diagnosis of PPR is the key to achieving accurate result because PPRV infections manifest similar clinical picture with other diseases such as bluetongue, contagious caprine pleuropneumonia, capripox and foot-and-mouth disease [4, 23, 76].

Virus isolation

PRRV isolation using primary cells (bovine, ovine and caprine kidney and lung epithelial cells) requires multiple, sequential blind passages and takes up to weeks in culture before the development of any cytopathic effect [42, 77]. The quality of primary cells is not guaranteed due to the presence of endogenous virus and there is considerable batch to batch variation (Table 3). The infection efficiency of PPRV in primary cultures is up to 100–1000 times less than that of the lymphoid cells expressing signaling lymphocyte activation molecule (SLAM) [78, 79]. Thus, non-lymphoid cells expressing this recombinant protein, are used to isolate and propagate PPRV efficiently [79]. However, transformed marmoset B-lymphoblastoid cells (B95a) derived from Epstein-Barr virus, are more sensitive and support better growth of PPRV lineage IV compared to Vero cells. Virus isolation is expensive and time consuming, thus, it cannot be deployed for routine diagnostic, but it can only be used as gold standard for further disease confirmation and in research studies. Thus, establishment of cell lines with high infection efficiency to PPRV will be

Table 3 Progress towards the development of suitable platforms for PPRV isolation, maintenance and production of biosafe antigen

Platform	Strength (s)	Limitation (s)	References
Primary cell culture	Cheap and easily accessible	Variations in batches and low quality due to the presence of endogenous viruses	[53, 77, 80–82]
Vero cells	Easy to maintain in culture	Low infection efficiency compared to lymphoid cells	[42, 81, 83]
Madin-Darby bovine kidney epithelial cell line (MDBK)	Suitable for PPRV isolation.	Requires multiple sequential blind passages for visible cytopathic effect	[84]
MDBK-nectin-4 cell line	Rapid for clinical isolation of PPRV	Only limited to Nectin-4 and high overhead cost	[85, 86]
Baby hamster kidney (BHK-21)	Suitable for growth kinetics of PPRV	PPRV replicates at relatively lower titers in BHK-21 cells	[87, 88]
Vero-SLAM	Highly efficient for PPRV isolation	Prone to fungal and bacterial contaminations	[79, 89, 90]
Vero dog SLAM-L protein (VDS-L)	Produces biosafe antigens in low level biocontainment	Prone to fungal and bacterial contaminations	[91]
Alpine goats	Suitable for in vivo pathological studies	Require high level containment	[92]

of help in confirming viable PPRV in the last phase of global PPRV eradication (Table 3).

Paradigm shift in peste des petits ruminants diagnostic assays

For PPR diagnosis, a plethora of serological and molecular assays have been developed with continuous on-going improvements. The assays detect PPRV antigens, nucleic acid or PPRV induced antibodies [93]. These assays include agar gel immunodiffusion (AGID), counter-immunoelectrophoresis (CIE), enzyme linked-immunosorbent assay (ELISA),reverse transcription polymerase chain reaction (RT-PCR) either gel-based or real time, or reverse transcription loop mediated isothermal amplification (RT-LAMP), reverse transcription recombinase polymerase amplification assays (RT-PRA), immunochromatographic lateral flow devices (IC-LFDs), luciferase immunoprecipitation system (LIPS) and pseudotype-based assays. They vary in their technical demands, but all require a laboratory with exception of IC-LFDs, and possibly RT-LAMP and RT-RPA (Table 4). The AGID and CIE assays are less sensitive at the early stages of infection where antigen levels are below detectable

Table 4 Demonstration of peste des petits ruminants diagnostic spectrum and prototype assays undergoing development

Diagnostic technique	Reliability		References
	Strengths	Limitation (s)	
Tentative diagnosis	Less costly	Unreliable due to presence of PPR related diseases	[73, 109]
Virus culture and isolation	Discerns active infections	High overhead cost	[77, 79, 110]
Virus neutralisation test (VNT)	It is specific and able to discern PPRV exposure	Cannot be used as DIVA test	[53, 111]
Agar gel immunodiffusion (AGID)	Simple and cheap	Low sensitive and is affected by prozone effect	[94, 112]
Counter-immunoelectrophoresis (CIE)	The test is fast, simple and cheap	Not free from prozone effect	[94, 112]
Enzyme-linked immunosorbent assays (ELISA)	Suitable for routine diagnosis on large scale	Low sensitive compared to PCR	[95, 96, 113–115]
Haemagglutination (HA) test	Simple to perform and it is inexpensive	Non-specific	[116–118]
Haemagglutination inhibition (HAI) test	Fast and relatively easy to perform and easy to standardise	Works best with human blood group"O"	[119, 120]
Immuno-peroxidase test	Test is easy to perform	Test is less sensitive compared to RT-PCR	[109]
Fluorescent antibody test (FAT)	The test is highly specific and able to detect active infection	High overhead cost and impracticable in the field setting	[116]
Immunofiltration test	Pen-side test and serves to screen large sample size	Less sensitive compared to ELISA	[105]
Immunochromatographic test	Rapid and does not require instrumentation	Less sensitive compared to IC-ELISA	[26]
Luciferase immunoprecipitation system tests	Highly sensitive for sero-surveillance	Not DIVA test	[121]
Pseudotype-based assays	No need of sophisticated facility	Technically demanding test	[122]
Quantum dots-lateral flow immunoassay strips	Very rapid test and highly sensitive	Limited to previous exposure	[27]
Surface Plasmon resonance-biosensor	Ultrasensitive diagnostic tools	Expensive and technically demanding	[123, 124]
Reverse transcription polymerase chain reaction (PCR)	Highly sensitive and accurate	High maintenance cost	[97, 125]
Reverse transcription loop-mediated isothermal amplification	Highly sensitive, cheap and rapid for pen-side test	Requires many primers	[29]
Microarray	It allows multiple virus screening	Less sensitive compared to PCR	[21, 126]
Reverse transcription recombinase polymerase amplification	Point of care diagnostics following miniaturisation	Sensitivity is low compared to RT-PCR	[32, 127]
Sequencing platforms	Highly accurate for aetiologic agents confirmation	Costly and require expertise	[128–130]
Oxford nanopore MinION sequencers	Rapid and accurate for genomic surveillance in field settings	Requires extra efforts for monitoring signal to noise ratio in base detection	[33, 129]

threshold and could only detect 42.6% of ante-mortem and necropsy specimens [94]. Thus, progress was made to replace them with assays that are more sensitive and specific, such as ELISA, immunochromatographic assays and nucleic acid-based assays [95–98]. ELISA employs an enzyme–substrate reaction for the detection of antigen–antibody interactions. They are suitable for screening large sample sizes and better documentation of evidence-based clinical samples status. Later on, a high sensitive immunocapture enzyme-linked immunosorbent assay (IC-ELISA) was developed based on conventional monoclonal antibodies (MAbs)and it demonstrated diagnostic sensitivity of $10^{0.6}$ $TCID_{50}$ [74, 95, 99]. Again, sandwich-ELISA and dot-ELISA based on conventional antibodies were developed and they have been in use since 2002. Dot-ELISA when was compared for its relative diagnostic sensitivity and specificity with routinely used sandwich-ELISA were 82% and 91% respectively, for the diagnosis of PPR [100]. However, dot-ELISA could serve as simple field test to screen clinical samples from suspected PPR cases. In comparison with commercial IC-ELISA kit, sandwich ELISA exhibited 88.9% and 92.8% relative diagnostic sensitivity and diagnostic specificity, respectively [101]. Although the sensitivity of the dot-ELISA is lower, it can also be used in combination with other assays such as LIPS, pseudotype-based assays and nucleic acid-based tests in laboratories where resources are limited. The gel based RT-PCR assays serve to detect viral nucleic acid with high sensitivity and accuracy regardless of being labor intensive, time-consuming and prone to high risk of cross-contamination. Alternatively, real-time RT-PCR assays detect and quantify PPRV present in clinical samples in real time [102]. The high cost of the equipment and technical demands impede its utility in low-income countries. In low-income countries, potent, inexpensive field-deployable diagnostic tools are prospect for use in the prevention and control measures of PPR. Recently developed lateral flow devices based on conventional antibodies have rejuvenated hopes in the least developed countries for rapid detection of PPRV [6, 26]. Despite the pen-side versatility of some lateral flow devices, their sensitivities were not able to detect PPRV in clinical samples with a low virus load as sensitively when compared to IC-ELISA, LIPS and nucleic acid-based diagnostic tools. Detection of serum antibody is also not effective because all assays based on detection of PPRV antibody could not differentiate infected animals from vaccinated animals. Recombinant antigen-based assays are of value during post vaccination evaluation and in the last phase of eradication where free PPRV diagnostic tools are required [96, 103–108]. On the other hand, a battery of potential field-based diagnostic tools have been developed and introduced for use in the diagnosis of PPR (Table 4).

Recent advances in peste des petits ruminants field-deployable diagnostic assays

Immunochromatographic lateral flow test

A novel pen-side diagnostic tool for diagnosis of PPR was developed at The Pirbright Institute (Pirbright, UK) in 2014. This lateral flow immunochromatographic assay is based on the specificity and affinity of conventional monoclonal antibody (MAb) C77 that was prepared using hybridoma cells technology in a miniPerm bioreactor and purified on a protein G HiTrap column [26]. The C77 MAb recognises the PPRV H protein and has been previously used in a prototype pen-side test for PPRV and rinderpest [6, 26]. In principle, the MAb C77 serves as the antigen fishing reagent on the chromatographic test strip and detection reagent that is labeled with colloidal gold-red. The performance of this diagnostic assay was evaluated in the laboratory and under field conditions on a superficial sample (ocular or nasal swabs). The test showed a sensitivity and specificity of 84% and 95%, respectively, relative to RT-PCR and detected as little as 10^3 $TCID_{50}$of cell culture-grown PPRV. The test could detect PPRV in swabs from animals as early as 4 days post-infection at a time when clinical signs were minimal. The IC-LFD kit is a prospect for field diagnosis of PPR and is being manufactured by Foresite Diagnostics Ltd (Sand Hutton, York, UK). The availability of this field-deployable diagnostic tool in developing countries will improve the diagnostic capacity for PPR. This will lead to early detection, which will significantly reduce the negative impact of PPR. Furthermore, this method could be utilised in the field without the need for expensive equipment, removing the requirement for its operation in a well established laboratory. However, case confirmation is essential during an outbreak of PPR. In such situation, field friendly, rapid and accurate nucleic acid-based diagnostic tools (RT-LAMP, RT-RPA and Oxford nanopore MinION sequencers) could be deployed.

Quantum dots-lateral-flow immunoassay strip

Recently, a fast and ultrasensitive quantum dots lateral flow immunoassay strip was established at the State Key Laboratory of Agricultural Microbiology in China to detect anti-PPRV antibodies. In this assay, N protein of PPRV is immobilised on the detection zone of the test strip and luminescent water-soluble carboxyl-functionalised quantum dots were used as signal output and were conjugated to streptococcal protein G. The performance of the test is extraordinary compared to C-ELISA and the IC-LFD for PPR serum IgG antibody detection [27]. The

test is rapid, sensitive and suitable for on-site, point-of-care diagnosis and post vaccination evaluation of PPRV. This test cannot be used for early detection of active infection where only IgM and viral particles are present in circulation. Alternatively, nucleic acid-based tests or PPRV antigen detection methods could be used to assess during disease outbreaks.

Reverse transcription loop-mediated isothermal amplification assay

A novel inexpensive RT-LAMP provides an isothermal method to amplify viral RNA without the requirement of expensive specific thermal cycler [29, 30]. Moreover, RT-LAMP reagents can be stored at ambient temperature for at least 2 weeks. The RT-LAMP reaction could be performed in an inexpensive water bath, dry bath, or heat block and the reaction results could be directly distinguished through color change or formation of the precipitate by the naked eye or alternatively via agarose gel electrophoresis or real-time turbid meter. Reverse transcription loop mediated isothermal amplification assays have been developed for the diagnosis of PPR based on M and N genes of PPRV with higher sensitivity than RT-PCR [28]. This sensitive, inexpensive and streamlined method can be more readily used in developing countries that do not have access to high technology laboratories. However, in each RT-LAMP assay, primers must be specifically designed to be compatible with the target nucleic acid sequences, which may discourage researchers. In addition, the RT-LAMP assay requires six primers and has unsatisfactory reliability in detection of highly variable viruses. An alternative field deployable recombinase polymerase amplification assay was developed targeting viruses of veterinary importance [32].

Reverse transcription recombinase polymerase amplification assay

In the advancements of the novel point-of-care molecular tests in recent years, RT-RPA assay was developed and coupled to a lateral flow. This assay is used for rapid detection of different viruses and parasites of veterinary and public health importance [32, 127].The assay demonstrated a pen-side usefulness for rapid detection of pathogens such as PPRV, *Foot-and-mouth disease virus*, *Orf virus*, *Bovine viral diarrhoea virus* and *Leishmania spp* [32]. Generally, the assay uses recombinase, single strand binding protein, strand displacing DNA polymerase and a fluorescent probe. Then, the lateral-flow strips are coupled in the detection system. These assays are highly specific for detection of PPRV as there is no cross-reaction with *Foot-and-mouth disease virus* and *Orf virus*, which may cause similar clinical signs to PPRV in small ruminants, indicating the potential of being a novel testing tool for differential diagnosis [32]. Although the sensitivity of RPA is lower than for RT-PCR, some advantages of the RT-RPA assay over RT-PCR assay make it rather attractive. Firstly, reaction mixtures are pre-made pellets and provided in vacuum-sealed pouches, which can be kept at room temperatures for several days. This would save on cold chain costs and facilitate on-site diagnosis of PPR in the field. Secondly, the reaction can be performed in a water bath at a temperature of 37 to 45 °C for a maximum of just 20 min. The RT-RPA assay is rapid compared to RT-LAMP and it does not require expensive equipment and the results are read with the naked eye in less than 25 min. However, virus genetic sequences analysis could not be determined by aforementioned diagnostic tools in field settings to match with the plasticity of RNA viruses including PPRV. The Oxford nanopore MinION sequencers may be of choice in such situation.

Oxford nanopore MinION sequencers

The Oxford nanopore MinION technology brings rapid comprehensive detection, diagnostics, and bio-surveillance of emerging infectious diseases to extremely remote and physical challenging geographical landscapes, completely detached from the traditional physical building. This technology has been used in arbovirus surveillance and during Ebola and Zika outbreaks [34, 129]. This technology is non-PCR-based tool for meta-transcriptomic detection of RNA virus from the clinical samples using Oxford nanopore MinION sequencers. In principle, clinical samples are processed in the Biomeme's bulk nucleic acid extraction developer kit. There are few challenges in applying Oxford nanopore MinION sequencing to diagnosis of infectious diseases, these include; high host's nucleic acid to pathogen ratio and low quality nucleic acid in the sample. It is, therefore, very important to carefully design, develop and optimise diagnostics pipelines before attempting to apply them to clinical samples. This diagnostic platform may provide the capacity for genomic surveillance of PPRV as well as other infectious diseases in resource-limited settings in real time.

Rational integration of diagnostic tools for diagnosis of peste des petits ruminants

Despite of increased development and use of PPRV novel field-deployable diagnostic tools, diagnostics are not being integrated into disease control optimally. A comprehensible integration of diagnostic tools is essential in PPRV endemic areas. The efficient integration of diagnostics may be influenced by a multitude of factors including the existence of co-infections, clinically closely related diseases and asymptomatic cases in both wildlife and domestic animals. It is further influenced by the availability of appropriate technology and access to

diagnostics, test characteristics, veterinary infrastructure and the experience and knowledge of the veterinary service providers in resource-limited settings. Regardless of the varying sensitivity and specificity of PPR diagnostic tests and prevalence of disease, it is clear that diagnostics play a valuable and critical role in early detection of PPR in infected animals with disease and those at risk of developing the disease.

Most of PPR field deployable IC-LFDs have lower sensitivity than IC-ELISA, LIPS and nucleic acid-based tests, but they are still useful tests on the PPR-battlefield in extremely remote settings, away from traditional veterinary laboratories. For instance, in the entire flock, if a less sensitive test has scored some animals' positive for PPR, the herd would be grouped as infected or at risk of developing a disease. Therefore field level diagnosis should be institutionalised for early screening and transportation of PPRV suspected samples from rural remote areas and ecosystem containing susceptible wildlife. In turn, early control measures will be put in place to prevent further spread of PPRV to neighboring flocks or a distant PPRV free zone (Fig. 3). A good example is a death toll of the saiga antelope; whereby a rapid diagnostic tool (PPR Rapid—BD SL Pirbright UK) was deployed with subsequent confirmation by PCR and sequencing [17].

The characteristics of an ideal diagnostic test include accuracy wherever used; heat-stable reagents with an extended shelf life; portability; minimal technical skills for operation; rapid, sensitive, and specific results; on-demand testing capability or minimal batch sizes; cost effective tests; and suitable for a broad range of clinical samples [131]. The current PPRV antigens and nucleic acids based tests meet some, but not all, of these standards. This has led to the development of automated diagnostic tools. The newer automated tests using nanotechnology that no longer require the addition of multiple reagents are being used for point-of-care diagnosis. Therefore, for recognition of PPRV during early phases of the disease and for clinical samples that gave equivocal results in other tests and require re-confirmation, highly sensitive nucleic-acid-based diagnostic tests could be used [6]. A good number of these tests are available commercially including IC-LFDs, RT-LAMP and Oxford nanopore MinION sequencers and their uses continue to increase logarithmically and the cost of instruments and their assays continue to decrease and are becoming of high value in resource-limited nations.

Moreover, in cases of co-infections, the multiplex assays utilizing real-time amplification methods may be of value for simultaneous detection of multiple viral infections in PPR infected animals as some viruses are preferentially replicating in PPR infected hosts [132]. These assays may also detect the nucleic acid of viruses that are previously unrecognised and/or not cultivatable in vitro. Inevitably, high mutation rates in RNA viruses including PPRV can be rapidly identified using nucleic acid amplification coupled with sequencing platforms such as Oxford nanopore MinION sequencers, to curb highly emerging or reemerging virulent strains. In resource-limited countries, conventional methods are more readily replaced in virology because the tissue

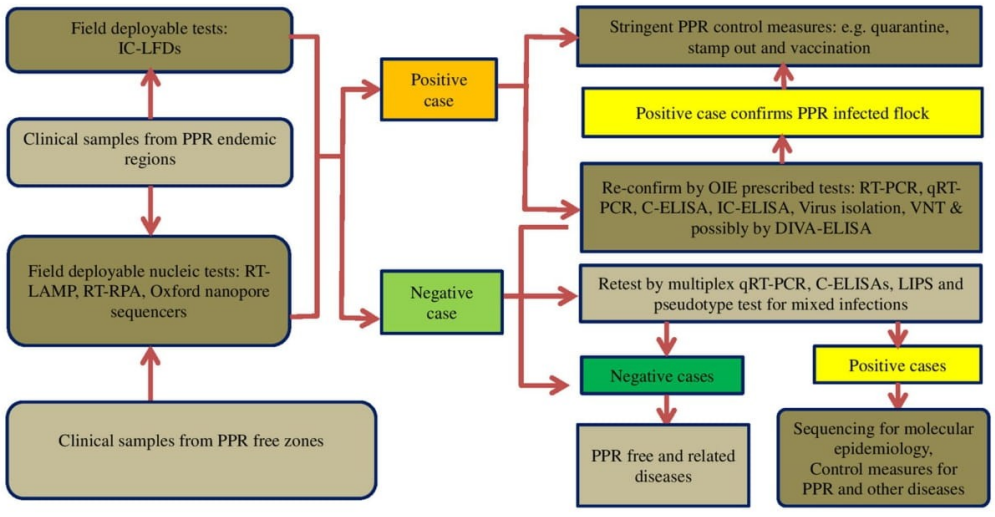

Fig. 3 Rational integration of diagnostic tests for rapid and correct identification of peste des petits ruminants

culture based virology methods are costly and generally less sensitive than newer molecular methods.

Civil unrest, global climate and environmental change from hurricanes, flooding, and earthquakes have a dramatic influence on the frequency of certain diseases in new locations. A minute or dramatic change in the environment can have a significant impact on the spread of diseases including PPR. These catastrophes including droughts or wars may lead to humans' movements from place to place with their belongings, including animals. In such situations, contagious diseases like PPR may spread very rapidly. Therefore to curb PPR spread and control animals' movements, highly sensitive nucleic acid-based field-deployable diagnostic tools are critical tools in keeping up with continuously changing disease dynamics. In addition, prompt outbreak identification is central to controlling the spread of highly contagious diseases like PPR, but recognizing that an outbreak has occurred can be difficult. Most molecular methods to detect different viral strains require that specimens are sent to distant reference laboratories, with confirmation of an outbreak possibly requiring days to weeks in resource-constrained countries. The development and use of both field deployable immunoassays and molecular diagnostic tools such as IC-LFDs, RT-LAMP, RT-RPA and Oxford nanopore MinION sequencers may play a significant role in controlling disease outbreaks in extremely remote areas in which transportation of clinical samples in optimum cold chain is unreliable (Fig. 3).

Prospects of nanobodies for use in immunoassays

In the early 90's, Hamers-Casterman and her colleagues made a remarkable discovery where they found a structurally different kind of antibodies which are part of the humoral immune response in the serum of camelids [133]. The heavy chain-only antibodies (HCAbs) in camelids or similar molecules in shark (Ig-NAR) are devoid of light chains [134]. The antigen binding domain of Ig-NARs or HCAbs involves a single domain only, referred to as V-NAR when derived from Ig-NARs and VHHs (nanobodies) when derived from HCAbs. The nanobodies have proven to be powerful tools in diagnostics due to their unique characteristics [135]. In particular, the recombinant expression of nanobodies in microbial systems and straightforward purification using His-tag by immobilised metal affinity chromatography makes their purification easy and very cheap. In contrast, traditional MAbs (used in virus detection) need more support costs and they are difficult for massive production compared to nanobody generation strategies [136, 137]. Furthermore, nanobody proteins are robust against thermal denaturation, which obviates a cold chain for transport and storage. Interestingly, despite that nanobodies recognise their cognate antigen via one single domain-only, they still achieve a high affinity and specificity. In addition, the convex paratope of the nanobodies comprising three antigen binding loops or complementary determining regions prefer to interact with a concave surface on the antigen, an architecture that is not antigenic for classical antibodies [134]. Therefore, the use of nanobodies may circumvent binding interference caused by the host's antibody response. Thus, nanobodies should detect both free antigens as well as those bound by host antibodies, which would make nanobodies based diagnostic tests rather attractive [138]. In contrast, the large size of MAbs prevents them from reaching cryptic epitopes andhost IgG molecules might as well conceal the epitopes from the MAbs employed in the diagnostic test [139]. The use of the nanobody coupled with lateral flow device may accelerate the development of cost effective, highly sensitive, specific and rapid immunoassays. In the light of global PPR eradication, a cost-effective, multiplex (multi-disease) diagnostic test would be very useful for concurrent bio-surveillance of PPR and similar infectious diseases such as contagious caprine pleuropneumonia, bluetongue, contagious ecthyma and foot-and-mouth disease. These multiplex assays may also be indispensable in all phases of PPR eradication operation to rule out mixed infections.

Conclusions

Peste des petits ruminants incidence is growing at an alarming rate worldwide and continues to undermine the economic activities of the poorest farmers and threatens biodiversity. However, in resource-limited setting, expensive sophisticated diagnostic tools are at risk of becoming redundant, due to insufficient funds for consumables, maintenance and expertise. Thus, the availability and distribution of cost effective field-deployable diagnostic tools in developing countries will improve diagnostic capacity and early containment of PPR. Field deployable and OIE prescribed laboratory based diagnostic tools have inherent strengths and weaknesses, thus optimal amalgamation is essential for rapid and accurate diagnosis of PPR.

Abbreviations

AGID: agar gel immunodiffusion; CIE: counter-immunoelectrophoresis; C-ELISA: competitive enzyme-linked immunosorbent assay; ELISA: enzyme-linked immunosorbent assay; FAO: Food and Agriculture Organisation; HCAbs: heavy chain-only antibodies; IC-ELISA: immunocapture enzyme-linked immunosorbent assay; IC-LFD: immunochromatographic lateral flow devices; IgG: immunoglobulin G; IgM: immunoglobulin M; LIPS: luciferase immunoprecipitation system; MAbs: monoclonal antibodies; OIE: World Organisation for Animal Health; PPR: peste des petits ruminants; PPRV: *peste des petits ruminants virus*; RT-LAMP: reverse transcription loop mediated isothermal amplification;

RT-PCR: reverse transcription polymerase chain reaction; RT-RPA: reverse transcription recombinase polymerase amplification assay; SACIDS: Southern African Centre for Infectious Diseases Surveillance; SACIDS-ACE: SACIDS-Africa Centre of Excellence for Infectious Diseases of Humans and Animals in East and Southern Africa; SLAM: signaling lymphocyte activation molecule; VHH: nanobody; VNT: virus neutralisation test.

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Authors' contributions
EK designed the study, collected the data, analyzed the data and drafted the manuscript. SO, SM, RK and GM participated in the study design, coordination and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The dataset analyzed during the current study are available from the corresponding author on reasonable request.

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PAPER TWO

2.1 DEVELOPMENT OF NANOBODIES TARGETING PESTE DES PETITS RUMINANTS VIRUS: THE PROSPECT IN DISEASE DIAGNOSIS AND THERAPY

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Development of Nanobodies Targeting Peste des Petits Ruminants Virus: The Prospect in Disease Diagnosis and Therapy

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Simple Summary: Peste des petits ruminants virus (PPRV) causes a highly devastating disease, peste des petits ruminants (PPR) of sheep and goats, that threatens food security, small ruminant production, and the conservation of wild small ruminants. Current efforts are directed towards the global control and eradication of PPRV, an initiative of the World Organisation for Animal Health and Food and the Agriculture Organisation of the United Nations. A plethora of diagnostic tools for PPR were primarily developed for livestock. New innovative diagnostic tools are needed to detect PPRV in atypical hosts (e.g., *Camelidae*, *Suidae*, and *Bovinae*), in wildlife ecosystems, and in complex field situations. Recent studies confirmed that single-domain antigen binding fragments (nanobodies) derived from heavy-chain-only camelid antibodies have proven to be a powerful tool in diagnostics and therapeutics due to their unique properties, such as small size and strong antigen-binding affinity. Therefore, the main objective of this study was to generate PPRV-reactive nanobodies in order to set a pace for the development of diagnostic and possibly therapeutic nanobodies in the future. Initially, a strategy was developed whereby an alpaca was immunized with PPRV in order to raise an affinity-matured immune response, from which an immune nanobody library was constructed. Following phage display, nine nanobodies that specifically recognise PPRV were identified on enzyme-linked immunosorbent assay. This study has generated PPRV-reactive nanobodies and have significant implications in the development of cost-effective diagnostic tools in context with the planned eradication of PPR in the world.

Abstract: Peste des petits ruminants virus (PPRV) causes a highly devastating disease, peste des petits ruminants (PPR) of sheep and goats, that threatens food security, small ruminant production, and the conservation of wild small ruminants in many developing countries, especially in Africa. Robust serological and molecular diagnostic tools are available to detect PPRV infection, but they were mainly developed for domestic sheep and goats. The presence of a wide host range for PPRV does present serological diagnostic challenges. New innovative diagnostic tools are needed to detect PPRV in atypical hosts (e.g., *Camelidae*, *Suidae*, and *Bovinae*), in wildlife ecosystems and in complex field situations. Interestingly, single-domain antigen binding fragments (nanobodies) derived from heavy-chain-only camelid antibodies have emerged as a new hope in the development of accurate, rapid, and cost-effective diagnostic tools in veterinary and biomedical fields that are suitable for low-income

countries. The main objective of this study was to construct an immune nanobody library to retrieve PPRV-reactive nanobodies that enable the development of diagnostic and therapeutic nanobodies in the future. Here, a strategy was developed whereby an alpaca (*Vicugna pacos*) was immunized with a live attenuated vaccine strain (PPRV/N/75/1) to raise an affinity-matured immune response in the heavy-chain-only antibody classes. The nanobody gene repertoire was engineered in pMECS-GG phagemid, whereby a *ccdB* gene (encoding a lethal protein) was substituted by the nanobody gene. An immune nanobody library with approximately sixty-four million independent transformants was constructed, of which 100% contained an insert with the proper size of nanobody gene. Following phage display and biopanning, nine nanobodies that specifically recognise completely inactivated PPRV were identified on enzyme-linked immunosorbent assay. They showed superb potency in rapidly identifying PPRV, which is likely to open a new perspective in the diagnosis and possible treatment of PPR infection.

Keywords: peste des petits ruminants virus; camelid; alpaca; nanobody; heavy-chain only antibody; diagnosis; therapeutics

1. Introduction

Peste des petits ruminants virus (PPRV) causes a highly contagious viral disease, peste des petits ruminants (PPR) of sheep and goats, that poses serious socio-economic losses in the small ruminant industry [1,2]. The PPRV infection has been confirmed in both *Ruminantia* and *Camelidae,* as well as in *Suidae* [3–7]. Considering the importance of sheep, goats, and wildlife in the livelihood of more than 300 million farmers, landless villagers, and pastoralists in Africa, the Middle East and Asia, PPR causes food insecurity and poverty, and threatens biodiversity [4,8]. On an annual basis, PPR causes economic losses of the equivalent to around US $1.2 to 1.7 billion due to animal deaths, reduced production, and the cost of fighting the disease [9]. Approximately one-third of the financial losses occur in Africa and a quarter in South Asia [10,11]. However, current efforts are now being directed towards the PPR Global Control and Eradication Program (PPR GEP), an initiative of the global animal health community coordinated through the World Organisation for Animal Health (OIE) and the Food and Agriculture Organisation (FAO) of the United Nations [12,13]. It was estimated that an investment of US $7.1 billion on PPR GEP could be recovered within five years of successful eradication [11]. This gives an overall benefit–cost ratio of 33.8 for the most likely situation, which makes PPR eradication economically feasible [12,14]. However, some economists and scientists believe that the actual cost of eradication could be much lower than US $7.1 billion [11,12]. Unfortunately, the slowness of the response to PPR spread in disease-free zones and atypical host species (e.g., *Camelidae*, *Suidae*, and *Bovinae*) increases the likely eradication cost [12]. For instance, economic losses associated with the saiga antelope (*Saiga tatarica mongolica*) death toll in Mongolia, a critically endangered species in central Asia, was estimated at US $7.27 million [2].

Multiple wildlife and atypical host species can be infected with PPRV, which poses diagnostic challenges in multi-host system testing [3,15,16]. Commercially available serological and molecular diagnostic tools to detect PPRV infection were mainly developed for domestic sheep and goats [17–19]. Thus, accurate diagnosis and standard protocols for interpretation of PPR diagnostic tests in atypical host species need to be established. For instance, previous studies showed that the haemagglutinin protein (H)-based competitive enzyme-linked immunosorbent assay (H cELISA) has a lower sensitivity in cattle compared to domestic sheep and goats [20–23]. The differences between PPRV H cELISA and neutralisation tests in buffalo sera have also been reported, indicating that differential antiviral immune responses among host species may affect the serology and interpretation of results [24]. The serological tool spectrum for PPR diagnosis (Virus Neutralization Test, immunochromatographic lateral flow devices, blocking ELISA, pseudotype-based neutralization assays, and PPR-Luciferase Immunoprecipitation System) have inherent strengths

and weaknesses that require parallel optimization and validation [17,25]. Diagnostic tools to detect active infection, such as antigen ELISA and reverse transcriptase polymerase chain reaction (RT-PCR), are critical to the prompt implementation of control measures [26]. The presence of in-depth genomic information has the potential to clarify the roles of wildlife and domestic animals in PPRV circulation, viral evolution, and direction of transmission at wildlife-livestock interfaces. This highlights the need to develop diagnostic tools and current protocols need to be standardized and adequately validated for atypical species affected and type of sample collected [15,27].

The access of required diagnostic tools, vaccines, and therapeutics are limited or available in short supply in distant centralized laboratories in low-income countries [18,28]. This is also compounded by the restrictions in transporting clinical samples across international borders for confirmation and further studies of PPRV in the OIE reference laboratories, due to the Convention on International Trade in Endangered Species of Wild Fauna and Flora, and Nagoya Protocol regulations [15,24]. Thus, deployment of cost-effective technology to break the limitations in the development of novel innovative diagnostic and therapeutic tools for PPR in the developing world can be of additional value to the Global PPR Control and Eradication Program [17]. Recent advances in technologies such as material sciences, genomics, biotechnology, nanotechnology, and microfluidics provide opportunities to develop cost-effective diagnostics and therapeutics reagents for PPR, suitable for resource-limited settings [17,29]. The development of vaccines, diagnostics, and therapeutics greatly rely on detailed insights into the PPRV genome [30,31]. Like any member of Morbillivirus, the genome of PPRV is organised into six genes in the order of 3′-*N*, *P*(*C*/*V*), *M*, *F*, *H*, and *L*-5′, and each of these genes code for a distinct protein [30,32]. The encoded proteins bear the acronym of the respective gene of origin: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H), and large polymerase protein (L) [33,34]. The *P* gene also codes for two additional non-structural proteins designated by C and V [35]. The N protein is abundant in PPRV-infected cells because the *N* gene is located near the genomic promoter and is hence the most transcribed gene [36]. Given its abundance and antigenic stability, the N protein has been a preferred candidate in PPR diagnostic development and the most appropriate gene for the molecular characterization of closely related isolates [37]. Most of the neutralizing antibodies are directed against the surface glycoprotein H [38,39]. For this reason, the N and H proteins are appealing targets in diagnostics and vaccine development, respectively [38].

A vital step towards eradication of PPR will be the cessation of vaccination and a switch to active surveillance in domestic and wild animals to identify the pock of endemicity responsible for PPRV persistence [40]. In this phase, active surveillance and disease reporting require robust and rapid diagnostic tests, which provide pen-side diagnosis [19,41]. Moreover, the lessons learnt from rinderpest eradication in 2011 meant that rapid and simple diagnostic tests based on monoclonal antibodies were available in the last phase of rinderpest eradication [41,42]. These tests were developed based on innovative diagnostic technologies that include Clearview chromatographic strip tests for rinderpest (Unipath, Bedford) and improved chromatographic strip tests for rinderpest and PPR detection (Svanova Biotech) [42]. The latter test recognised a wider range of rinderpest virus strains, including several strains of lineage 2 which had proved difficult to detect previously by the Clearview device [42]. In the final phase of PPR eradication, the development and use of serology that can differentiate vaccinated from naturally infected animals (DIVA) may play a significant role in controlling PPR outbreaks, enabling detection of cryptic foci, inadequate vaccine deployment, and other challenges in the midst of an eradication campaign. Thus, continued research funding is necessary to improve existing diagnostic tests, vaccines, and use of new innovative technologies, such as nanobodies, the Oxford nanopore MinION sequencer and the DIVA vaccine, to handle complex epidemiological situations that may arise during eradication [17,29].

Nanobody technology has emerged as a new hope in the development of accurate, rapid, and cost-effective diagnostic tools in veterinary and biomedical fields that are suit-

able for low-income countries [43–47]. A nanobody is the single-domain antigen binding fragment (12–15 kDa) of heavy-chain-only antibodies derived from *Camelidae* blood, devoid of light chains [48,49]. The nanobodies have proven to be powerful tools in diagnostics and therapeutics due to their unique biophysical, biochemical, and pharmacological signature advantages [50,51]. In particular, the recombinant expression of nanobodies in microbial systems and straightforward purification using His-tag by immobilised metal affinity chromatography makes their purification easy and at an affordable cost [49,52]. Nanobodies are generated at a large scale in bacterial systems or lower eukaryotes with superiority, which is crucial for their use in diagnostics and therapeutics [51,53]. Moreover, nanobodies are thermally stable, soluble, and ten times smaller than classical antibodies, and can be easily generated using the golden gate cloning strategy [54]. The golden gate cloning strategy generates a high-quality immune nanobody library by employing pMECS-GG phagemid in cloning whereby a *ccdB* gene (encoding a lethal protein) is substituted by the nanobody gene [54]. In this strategy, a considerably large immune library is produced where 100% of the clones possess a phagemid carrying an insert with a length of a nanobody gene [54]. The immunization, bleeding for peripheral blood, lymphocyte preparation, and cDNA synthesis are all performed in the golden gate cloning strategy [52,54]. Thus, the immune library can be constructed within a week and is more cost-effective than previous standard approaches using classical restriction enzymes and ligations [52,55].

The present study was carried out to generate PPRV-reactive nanobodies, so that nanobodies with diagnostic and therapeutic applications could be developed in the future. The availability of PPRV-specific nanobodies provides an opportunity for the development of rapid and accurate diagnostic tests and with perspective for therapeutic purposes.

2. Materials and Methods

2.1. Antigens and Antibodies

The live attenuated PPRV/N/75/1 vaccine strain was outsourced from Botswana Vaccine Institute, Botswana for immunisation of the alpaca. Whole killed PPRV antigen mixture generated from completely inactivated PPRV was obtained from The Pirbright Institute, United Kingdom for affinity selection of PPRV-reactive nanobodies. For enzyme-linked immunosorbent assay (ELISA) tests, rabbit anti-camel VHH antibody, goat anti-rabbit-horseradish peroxide (HRP, BioRad, Hercules, CA, USA), anti-M13-HRP, mouse anti-His tag antibody, and goat anti-mouse alkaline phosphatases (Sigma-Aldrich, St. Louis, MO, USA) were all provided by Vrije Universiteit Brussel, Brussels, Belgium and all were used according to the manufacturer's instructions.

2.2. Short Immunisation Scheme

An adult alpaca was subcutaneously injected in the shoulder with 1 mL of live attenuated PPRV/N/75/1 vaccine strain at $\geq \times 10^{2.5}$ $TCID_{50}$ per dose in two-week intervals (day 0, 14 and 28). Blood was collected from the jugular vein on day 40.

2.3. Nanobody Library Construction

On the 40th day from the start of the immunisation, 50 mL of blood was taken, and peripheral blood lymphocytes (PBLs) were purified on Leucosep® tubes (Greiner Bio-One, Monroe, NC, USA). An immune nanobody library was constructed as previously described [54,56]. In brief, total mRNA isolated from PBLs was used as a template to synthesise cDNA using oligo dT primers. The cDNA was subsequently amplified with a variable-domain heavy chain leader-specific primer CALL001 and a CH2-specific primer CALL002 to amplify the heavy chain gene fragments from the variable region to the CH2 region from conventional and heavy chain-only antibodies, as previously described by Romão et al. [54]. The resulting first PCR amplicons with lengths of approximately 700 bp (which contain the *VHH* of heavy chain only camelid antibodies (HCAbs) and 1000 bp (which contain the *VH* of the convention IgG) were separated by agarose gel electrophoresis. The 700 bp fragment was cut out of the gel with a scalpel blade and purified using QIAquick

gel extraction kit (Qiagen, Hilden, Germany). Second PCR was performed with VHH-BACK SAPI, which anneals at the *VHH* template strand and introduces the *Sap*I recognition sequence in VHH-genes family-3 whilst VHH-FORWARD SAPI hybridizes with the *VHH* coding strand and also has a *Sap*I recognition sequence, as previously described [54]. The resulting amplicons were purified, cut with the *Sap*I restriction enzyme and ligated in the phagemid vector pMECS-GG in frame with a hemagglutinin (HA)-tag and a His6-tag, and transformed in electro competent *Escherichia coli* TG1 cells, as previously described by Romão et al.

2.4. Biopanning and Screening of PPRV-Reactive Nanobodies

Biopanning is an affinity selection technique in which specific binders (i.e., peptides, antibodies, nanobodies) against a target of choice are enriched from a phage display library during consecutive cycles of incubation, stringent washing, amplification and re-selection of bound phages [57,58]. Several rounds of affinity selection (biopanning) and washing away unbound phages are necessary to enrich specifically binding phage particles [53,59]. In this study, four rounds of panning were performed to enrich PPRV-specific nanobodies, as previously described [53]. The nanobodies from the library were displayed on phage particles after M13K07 helper phage infection of the *E. coli* TG1 cells. An aliquot (1 mL) of the cloned library with a complete nanobody repertoire (at least 100× the library size) was grown to exponential phase before superinfection with M13 helper phage (20× excess of bacteria). Phage particles were then recovered through precipitation with sodium chloride polyethylene glycol solution and subjected to four rounds of panning on solid phase coated with whole killed PPRV antigen mixture (2 µg/well). The PPRV antigen-bound phage particles were eluted by adding 100 mM Triethylamine, pH 11.5, neutralised by 1.0 M Tris-HCl, pH7.4. The neutralised solution with eluted phage particles was used to infect *E. coli* TG1 cells. Parts of infected cells were used in subsequent rounds of selection on 2xTY/AMP-KAN (AMP is ampicillin and KAN is Kanamycin) and the rest were used for evaluation of enrichment on LB-AMP/GLU agar plates. Afterwards, independent colonies were grown on a master reference plate, cultured, and expressed. Expression of the nanobody protein was induced overnight in the presence of 1 mM isopropyl-ß-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA). After extraction by osmotic shock, the periplasmic extracts containing the nanobodies were added to wells of a microtiter plate coated with PPRV antigens (1 µg/well). The presence of PPRV-specific nanobodies was detected in an ELISA using a mouse anti-His antibody and anti-mouse alkaline phosphatase (BioRad, Hercules, CA, USA). The VHH gene inserts in pMECS-GG of colonies scoring positive in ELISA were sequenced and analysed. The pMECS-GG vector containing unique PPRV-specific nanobodies were then transformed and expressed in the non-amber codon suppressor *Escherichia coli* WK6 cells. Soluble nanobodies from the periplasmic extract were tested in ELISA for their capacity to recognize native PPRV antigens.

2.5. Enzyme-Linked Immunosorbent Assay

Native PPRV antigens were coated in test wells (1 µg per well) in a 96-well plate (Maxisorp Nunc) and were incubated overnight at 4 °C in cold room. The plate was washed five times with 300 µL of phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS/Tween-20) in each step and blocked for 2 h at room temperature with 200 µL of 2% skimmed milk powder in PBS in both test and control wells. Periplasmic extract nanobodies of 100 µL were added into each test and control well and incubated for 1 h at an ambient temperature. Then, the plate was emptied and washed five times to remove unbound and excess periplasmic extract. Anti-PPRV soluble nanobodies were detected by adding 100 µL of primary antibody (mouse anti-His tag antibody diluted in 1/2000 blocking solution) in each well. The reaction was incubated for 1 h at an ambient temperature. The wells were emptied and washed five times to remove the unbound primary antibody, followed by adding 100 µL of conjugate secondary antibody (anti-mouse alkaline phosphatase antibody diluted in 1/2000 blocking solution) into each well and incubating at an ambient

temperature for 1 h. The ELISA plates were developed by adding 100 µL of freshly prepared *p*-nitrophenyl phosphate disodium salt solution (0.06 g in 30 mL of distilled water). The results were read at a wavelength of 405 nm. Thus, a colony was considered "positive", i.e., expressing a nanobody that recognizes PPRV antigens in ELISA, when an absorbance in the antigen coated well was at least twice that of the well without the antigen for the same periplasmic extract, as previously described by Vincke et al. [53].

3. Results

3.1. Nanobody Library Size

A nanobody gene pool containing the original diversity of the antigen-binding domains of the HCAbs was generated from 2.26×10^8 lymphocytes. These nanobody genes were ligated in the pMECS-GG phage display vector. Thus, following electrotransformation in TG1 cells and selection on absence of cytotoxic ccdB protein and presence of ampicillin resistant colonies, we obtained a considerable large nanobody library of 6.4×10^7 independent transformants.

3.2. Enrichment of Nanobody Library

The PPRV nanobody binders were generated on four consecutive rounds of in vitro selections in ELISA plates coated with completely inactivated PPRV whole antigen. Using this strategy, a clear PPRV-specific enrichment was observed from the second round of panning onwards with an approximately hundred-fold enrichment, as previously described [60].

3.3. Selection of PPRV Reactive Nanobodies

Four rounds of panning enriched the phage particles with anti-PPRV antigen-specific nanobodies. Ninety-four individual colonies from round two to four of panning were screened for the presence of nanobodies that recognise native PPRV antigens. Nine nanobody clones that were positive in ELISA for PPRV recognition were obtained. The PPRV-reactive clones were sequenced and the resulting VHH inserts were classified into families based on different complementarity determining regions (CDRs). The obtained clones belonged to three families based on their complementarity determining region-3 (CDR3) (Figure 1). The nanobody clones represented by NbPPRV9 had an imprint in their framework-2 region (the conserved region between CDR1 and CDR2) that resembles a VH of a classical antibody. It contains V42, L50, and W52 hallmark amino acids (numbering according to IMGT). The two other clones clearly have a VHH framework-2 imprint with Y42 or F42, R50, and L52 or A52. One of these nanobodies (clone NbPPRV31) contains an interloop disulphide bond between C55 and a C in the middle of its CDR3 (Figure 1). After expressing the nanobody proteins in non-amber suppressor WK6 cells with a C-terminal HA-tag and His6-tag, three families of PPRV-specific nanobodies periplasmic extract were further tested on indirect ELISA to detect PPRV antigens. They demonstrated clear rapid detection signals on ELISA plate upon development (Figure 2).

```
IMGT numbering 1       10        20        30        40        50        60
NbPPRV9        QVQLQESGG-GLVQPGGSLRLSCAAS GFTF----SSYP MTWVRQAPGKGLEWVSD INSG--GGTT
NbPPRV28       QVQLQESGG-GLVQPGGSLRLSCEAS GSAF----SIHT MAWYRQAPEKNRELVAA ITTS---GGT
NbPPRV31       QVQLQESGG-GFVQPGGSLRLSCAAS GFNL----DRYA IGWFRQTPGKEREAIAC TKSH--DDQT
               <---FRAMEWORK REGION-1--->             <------FR-2----->

IMGT numbering    70        80        90       100                    120
NbPPRV9        HYADSV-KGRFTISRDNAKNTLYLHMNSLKPEDTAVYYC GKGGARYGSTWYGGN WGQGTQVTVSS
NbPPRV28       RYADSV-KGRFTISRGNSKNTLYLQMNSLNEEDTAVYYC NIRPR----ITHHGY WGQGTQVTVSS
NbPPRV31       YYAESV KGRFAISRDNFKNTVYLQMNSLKPEDAGVYYC ASYSGSY-CLGSVRH WGQGTQVTVSS
               <------- FRAMEWORK REGION-3----------->                  <-- FR-4-->
```

Figure 1. Alignment of single domain antibody sequences of three nanobody families that recognise peste des petits ruminants virus antigens in enzyme-linked immunosorbent assay based on complementarity determining regions (CDRs) of nanobody.

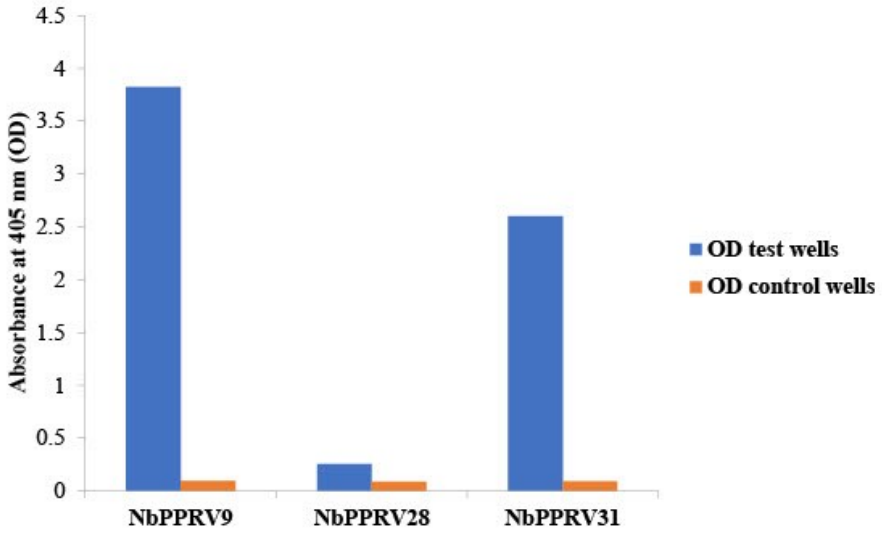

Figure 2. The bar chart represents an optical density of potent nanobodies directed against peste des petits ruminants virus (PPRV) in indirect enzyme-linked immunosorbent assay (ELISA). These nanobodies were rapidly detecting PPRV antigens with distinct signal strength in each nanobody family upon development of ELISA.

4. Discussion

Following the PPR Global Control and Eradication Strategy, an initiative of the OIE and the FAO of the United Nations, diagnosis remains the cornerstone towards the implementation of appropriate control measures including quarantine, vaccination, and possible stamping out [9]. Robust serological and molecular diagnostic tools are available to detect PPR infection, but they were mainly developed for domestic sheep and goats [17–19]. The presence of a wide host range does present diagnostic challenges [24]. Thus, deployment of cost-effective technology to break the limitations in the development of novel diagnostic and therapeutic tools for PPR is critical for effective surveillance of PPR in wildlife and atypical host species [17]. Interestingly, the rapid technological advances in areas such as material sciences, genomics, nanotechnologies, and microfluidics provide opportunities to develop cost-effective diagnostics and therapeutics reagents for PPR [17,29]. The applications of nanobodies in diagnostics and therapeutics are exponentially growing in biomedical and veterinary fields [46,61–63]. A very limited number of and extremely expensive biological antiviral treatments are available to control PPRV infections in sheep and goats [64–66]. The PPR antivirals cost high from the perspective of animal production [64–67]. For the same economic reasons, there is no OIE-prescribed veterinary antiviral curative treatment to fight against PPRV in infected animals [68,69]. The only treatments used are preventive vaccinations using live attenuated vaccines, including Nigeria/PPRV/75/1, Sungri 96, Arasur 87, and Coimbatore 97 [70,71]. The present study was carried out to generate nanobodies directed against PPRV, so that nanobody-based assays and therapy could be developed in the future.

In the present study, three nanobody families were identified from the immune nanobody library that are rapidly recognising PPRV antigens in ELISA (Figure 1). The nanobodies within the same family share similar CDR3 sequences, but might differ from each other by several point mutations, mainly spread between CDR1 and CDR2. The variation of the binding intensity between these nanobodies in ELISA may be due to different in expression of nanobodies in bacterial cells or varying affinities to the target

PPRV antigens. Similarly, previous research has demonstrated that if the concentration of the nanobody is not normalised, the intensity of the ELISA signal is a function of both the expression level of the nanobody and the affinity of the nanobody–antigen interaction [55]. These potent nanobodies directed against PPRV antigens may mark the beginning of the use of nanobodies as analytical tools for the diagnosis and possible therapy of PPRV infection in the future. Thus, this study confirms the practicality of isolating a panel of PPRV-specific nanobodies from an immunised alpaca without having prior knowledge of the antigens involved, as previously reported [72]. The rapid detection of PPRV antigens with nanobodies that exert strong binding signals in ELISA is likely to open new perspectives in the diagnosis or therapy of PPR, as demonstrated in recent studies, including the coronavirus disease 2019 (COVID-19) [43,46,50,73]. Thus, further characterization of PPRV-reactive nanobodies is required to determine their binding affinities, target PPRV proteins, and neutralisation potential. For diagnostic purposes, the reactivity against the N protein will be sufficient, but for a therapeutic application, the virus neutralisation based on the surface glycoproteins H or F reactivity must be available [19,74,75].

Previous studies demonstrated that PPRV-infected camels raise strong immune responses and develop an active clinical syndrome [6,7,76]. It was clear that an immunised alpaca provides direct access to the in vivo affinity-matured antibodies, an advantage to identify highly specific and affinity-matured nanobodies. Thus, the blood of this immunised alpaca can be a good source to clone the nanobodies for subsequent selection of a panel of PPRV-specific nanobodies with diagnostic and therapeutic potential. Following the recent advances in nanobody production through the golden gate cloning strategy, an immune library can be cost-effectively constructed within a week [54]. In fact, panning on PPRV antigens yielded a high and clear enrichment of phage nanobodies from the second round of panning onwards, as previously reported [60]. This is considered as a clear indication for the abundance of PPRV antigen-specific binders in our immune library. Furthermore, it has been repeatedly demonstrated that the phage nanobodies are readily amenable to produce soluble and highly expressed monomeric binders [52,60]. The PPRV nanobody library constitutes a source of nanobodies directed against PPRV antigens.

The diagnosis of PPR based on ELISA is available to assess seropositivity within sheep and goats, with high sensitivity that detects antibodies to either the N or the H proteins of the virus [77]. However, the presence of a wide host range does present diagnostic challenges; current protocols need to be validated for atypical species affected, and the need to improve diagnostic tests is highlighted [25]. New diagnostic tools are needed to detect PPR infection in atypical hosts in wildlife ecosystems and in complex field situations [26]. The availability of PPRV-specific nanobodies provides an opportunity for the development of rapid and accurate diagnostic tests and possibly therapeutic nanobodies. We envisage that these potent nanobodies with the capability for binding native PPRV antigens may play a significant role in controlling PPR outbreaks by enabling detection of cryptic foci and addressing inadequate vaccine deployment and other challenges in the midst of PPR GEP operations. Further studies are necessary to decipher the possible optimum detection combination of these nanobodies and their structural functional relationship for progression to developing a nanobody-based pen-side test for PPRV. A proof-of-concept experimental investigation yielded proficient nanobodies against native PPRV antigens that could enable the development of diagnostic and therapeutic nanobodies in the future. The use of novel innovative technologies such as PPRV-reactive nanobodies can be an additional diagnostic and therapeutic tool in context with the planned eradication of PPRV in the world.

In most cases, vaccines, prophylactics, therapeutics, and reliable diagnostic tools are largely inaccessible, absent, or available in short supply in distant centralized laboratories in developing countries [18,28]. Compared with other biologics, classical monoclonal antibodies produced with long-stablished hybridoma technology are acceptable as the gold standard in immunotherapy and diagnostics [78–80]. However, these classical monoclonal antibodies need more support costs and they are difficult for massive production compared

to their counterpart nanobodies [81–83]. The high production cost of classical monoclonal antibodies, limited tissue penetration, and less favourable pharmacokinetic stability have stimulated the use of smaller alternative antibody formats, such as the antigen binding fragments, single-chain variable fragments, and nanobodies [84,85]. Nanobodies have proven to be powerful tools in diagnostics and therapeutics due to their unique properties such as small size, strong antigen-binding affinity, high stability, water solubility, and preferential binding to cavities or grooves on the surface of the antigen, and resistance to extreme conditions (pH, pressure, chaotropic agents or proteases), often assisted by an extra interloop disulphide bond [62,86]. Furthermore, nanobody proteins are robust against thermal denaturation, which obviates a cold chain for transport and storage, suitable for the hot climate in sub-Saharan Africa, where cold-chain is unreliable. The low cost of high quality and the robustness of nanobodies will be an important feature for the development of cheap and sensitive diagnostic kits, either as lateral flow devices or as electrochemical detection assays suitable for low-income countries [44,45].

5. Conclusions

In conclusion, this study confirmed that PPRV-reactive nanobodies can be retrieved from an alpaca immune nanobody library. These proficient nanobodies against PPRV could open a new possibility in the diagnosis, vaccination, and treatment of PPR infection. Further studies need to be conducted to optimise the PPRV-potent nanobodies and determine their specificity and sensitivity to PPRV field isolates in comparison to other OIE prescribed diagnostic tests.

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Data Availability Statement: The genetic data sets of PPRV-reactive nanobodies were presented in this article. The following Springer Protocols used in this study; Construction of High-Quality Camel Immune Antibody Libraries by Ema Romão et al., 2018 and Generation of Single Domain Antibody Fragments Derived from Camelids and Generation of Manifold Constructs by Cécile Vincke et al., 2012.

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PAPER THREE

2.2 NUCLEOTIDE AMPLIFICATION AND SEQUENCING OF THE GC-RICH REGION BETWEEN MATRIX AND FUSION PROTEIN GENES OF PESTE DES PETITS RUMINANTS VIRUS

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Nucleotide amplification and sequencing of the GC-rich region between matrix and fusion protein genes of peste des petits ruminants virus

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ABSTRACT

Peste des petits ruminants virus (PPRV) causes a highly devastating disease of sheep and goats, that threatens the conservation of small wild ruminants. The development of PPRV vaccines, diagnostics and therapeutics, greatly depends on in-depth genomic data. Yet, high guanine-cytosine (GC) content between matrix (M) and fusion (F) genes of PPRV poses difficulty for both primer design and nucleotide amplification. In turn, this has led into absence or low nucleotide sequence coverage in this region. This poses a risk of missing important part of the genome that could help to infer viral evolution. Here, an overlapping long-read primer-based amplification strategy was developed to amplify the GC-rich fragments between M-F gene junction using nexus gradient polymerase chain reaction (PCR). The resulting amplicons were sequenced by dideoxynucleotide cycle sequencing and compared with other PPRV nucleotide sequences available at GenBank. Our findings indicate clear PCR amplification products with expected size of the GC-rich fragments on agarose gel electrophoresis. The sequencing results of these fragments indicate 99.5 % nucleotide identity with PPRV strain KY628761. An extremely difficult PCR target of 67.4 % GC contents was successfully amplified and sequenced using this long-read primer approach. The long-read primer set may be used in tiling multiplex PCR for complete genome sequencing of PPRV.

1. Introduction

Peste des petits ruminants virus (PPRV) causes a highly devastating viral disease, peste des petits ruminants (PPR) of sheep and goats, that threatens, small ruminants production and the conservation of small wild ruminants (Banyard et al., 2010; Pruvot et al., 2020). Sheep and goats are vital for day-to-day economic livelihoods of smallholder farmers especially in developing countries (Diallo, 2006; Jones et al., 2016). The fight against PPR should be considered as a program for the reduction of poverty and food insecurity in the world, an important agenda towards achievement of Sustainable Development Goals of the United Nations (OIE and FAO, 2015). Currently, efforts on PPR have been directed towards favourable technical attributes which facilitated rinderpest eradication in 2011 (Anderson et al., 2011). The Food and Agricultural Organisation (FAO) and the World Organisation for Animal Health (OIE) have jointly developed a strategy for the Global PPR Control and Eradication Program, which target the eradication of PPR by 2030 (Anderson et al., 2011; OIE and FAO, 2015). A plethora of diagnostic tools and effective vaccines exist, unfortunately the tardiness of the response to PPR spread in disease-free zones and atypical susceptible animal populations, increase the likely eradication cost and time (Jones et al., 2016; Kumar et al., 2004). Peste des petits ruminants progressively expanded its geographical distribution throughout Africa, Middle East, Asia and with few cases in European Union, in particular Bulgaria (Altan et al., 2019; Niedbalski, 2019). Incursion of PPRV in new locations is on rise with different PPRV lineages being reported in

Abbreviations: FAO, Food and Agricultural Organisation; OIE, World Organisation for Animal Health; PPR, peste des petits ruminants; PPRV, peste des petits ruminants virus; RP, rinderpest; GC, guanine-cytosine content; UTR, untranslated intergenic region; PCR, polymerase chain reaction; M, matrix protein; F, fusion protein.

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previously PPR-free zones (Bataille et al., 2019, 2016; Kock et al., 2015; Kwiatek et al., 2011). Several countries that had single lineage of PPRV are now simultaneously reporting the presence of two or more PPRV lineages (Luka et al., 2012; Mbuyi et al., 2019; Misinzo et al., 2015).

Historically, PPR started as a small nucleus in Côte d'Ivoire, West Africa in 1942 (Diallo et al., 2019; Gargadennec and Lalanne, 1942). However, it is believed that PPR emerged in the early part of the 20th Century whilst its presence was masked by ongoing rinderpest epidemics, which also affected sheep and goats and where immunity might have been cross protective (Libeau et al., 2014; Taylor, 1984). Over three decades, the causative agent of PPR was defined as a distinct viral entity (Gibbs et al., 1979). Based on PPRV morphological structure, cellular growth patterns, the composition of genetic materials, antigens, and its physical and chemical properties, PPRV was grouped as the fourth member of the genus Morbillivirus in the family *Paramyxoviridae* (Gibbs et al., 1979).

Structurally, PRRV virion is enveloped, pleomorphic with the diameter ranging from 400 to 500 nm (Gibbs et al., 1979). The genome of PPRV is linear, non-segmented negative sense, single stranded RNA, which is about 15,948 nucleotides long (Bailey et al., 2005; Bao et al., 2014). This genome constitutes six genes in order of 3′-*N, P/C/V, M, F, H, N,* L-5′ (Bailey et al., 2005). Each of these genes encode one structural protein with the exception of phosphoprotein (P) which codes for an additional two non-structural proteins C and V. The C and V non-structural proteins are generated from the P open reading frame through the utilisation of alternate start codons and RNA editing, respectively (Bailey et al., 2005; Mahapatra et al., 2003). Encoded proteins bear the acronym of the respective gene of origin that include; nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin (H) and large polymerase (L) proteins (Parida et al., 2015). In each of these structural protein genes there is untranslated intergenic region (UTR) of variable lengths that separate consecutive genes from each other (Meyer and Diallo, 1995). More importantly, there is unusual long UTR between M and F open reading frames in all four known PPRV lineages. This region constitutes about 1,080 nucleotides with 66–72 % guanine-cytosine contents (GC-rich) across the region (Haffar et al., 1999; Meyer and Diallo, 1995; Parida et al., 2015). The GC-rich region plays an important role in regulating translation by forming secondary structures in M and F mRNAs (Bailey et al., 2005; Meyer and Diallo, 1995). For instance, a recombinant measles virus lacking the long M/F UTR confirmed that the deleted region was not vital for viral replication, while its absence altered the viral phenotype (Takeda et al., 2005). Moreover, translational enhancement activity was observed in nine-nucleotide sequence of 5′UTR of PPRV F gene, which is complementary to the 18S ribosomal RNA (Chulakasian et al., 2013). The GC-rich region is not conserved between PPRV lineages and poses difficulty for both polymerase chain reaction (PCR) amplification and primer design (Eloiflin et al., 2019; Torsson et al., 2020). In turn, this has led into the absence or low nucleotide sequence coverage in this region (Dundon et al., 2014; Torsson et al., 2020). This poses risk of missing important changes within the genome that could help to infer viral evolution (Torsson et al., 2020).

Attempts have been made to address the nucleotides sequencing difficulty of the GC-rich region between M and F proteins using next generation sequencing (NGS) including Illumina and Oxford nanopore technologies(Hacıoğlu et al., 2020; Torsson et al., 2020). With NGS technologies, data of sufficient depth to characterize PPRV strains were generated, but with missing sequences at the GC-rich junction and genome extremities (Acevedo et al., 2014; Torsson et al., 2020). With Illumina short-read sequencing of PPRV, a short missing fragment of 359 bp was later amplified and confirmed by Sanger dideoxynucleotide cycle sequencing (Hacıoğlu et al., 2020; Rajko-Nenow et al., 2017). The missing sequences were at nucleotide position between 5,134 and 5,492 nucleotides when compared to the reference genome (Accession number: MN657232.1). It was demonstrated that Illumina was not able to span the repetitive structures that extend beyond the maximum read length generated, thus producing unresolvable loops during genome assembly and resulting in an assembly consisting of many unordered contigs (Alkan et al., 2011). Moreover, Illumina sequencing technology has difficulties in resolving GC-rich regions, making it problematic to reconstruct GC-rich sequences (Alkan et al., 2011). In order to circumvent these deficiencies, long-read Oxford nanopore sequencing technology was recommended and deployed for PPRV sequencing (Kinimi et al., 2020; 2021; Torsson et al., 2020). Nevertheless, low genome coverage was seen in the GC-rich region between 4,445 and 5,526 nucleotides but with extremities completely sequenced (Torsson et al., 2020).

The deployment of nanopore sequencing of PPRV, provides an opportunity for field-based molecular epidemiological surveillance of PPRV in resource-limited settings and challenging geographical landscapes, due to its rapidity, improved accuracy and relatively lower sequencing cost per sample (Torsson et al., 2020). Yet, the GC-rich region impedes PCR amplification and affects the downstream full genome sequencing of PPRV (Hacıoğlu et al., 2020; Torsson et al., 2020). The aim of the present study was to develop overlapping long-read primers to amplify and sequence the GC-rich region of PPRV, so that a complete genome of PPRV could be generated in single sequencing run using nanopore MinION sequencer. The development of long-read primer pairs with full potential to amplify an extremely difficult PCR target, the GC-rich region, creates an important opportunity for molecular epidemiological surveillance of PPRV.

2. Materials and methods

2.1. Long-read primer design

Long-read primer designing scheme was adapted from previous study on full genome sequencing of PPRV using Oxford nanopore MinION sequencing (Torsson et al., 2020). Three primer pairs targeting the GC-rich region were designed with overlaps of 195 and 310 bp using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and mapped to the reference genome (NC_006383) using Sequence Manipulation Suite version 2 (Oracle Corporation, Redwood, USA) (Table 1). The primer sequences were developed based on full genome sequences available at NCBI GenBank (Chard et al., 2008; Muniraju et al., 2014, 2013). These primer pairs were assigned names in respect to the target PCR amplicon such as fragment 7 (F7), fragment 8 (F8) and fragment 9 (F9), as previously described (Torsson et al., 2020). Primers targeting non-GC-rich fragments within the PPRV genome were used for PCR amplification as previously described (Torsson et al., 2020). Amplification of non-GC-rich fragment 10 (F10), previously described by Torsson et al. (Torsson et al., 2020), was used as a control during amplification of the GC-rich region of PPRV.

2.2. Sample selection

A PPRV-positive nasal swab collected from a goat in Momba district, Tanzania, in 2018 was used in the present study.

2.3. RNA extraction and PCR amplification

Viral RNA was recovered from an archived nasal swab using QIAamp Viral RNA Mini extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total viral RNA was reverse transcribed to cDNA by random hexamers using Invitrogen Superscript IV first strand cDNA synthesis kit (Invitrogen, Paisley, UK) following the manufacturer's instructions. The obtained cDNA was used as a template for subsequent PCR. Gradient PCR reactions were run in a final volume of 25 µL. The reaction mix consisted of 2 µL cDNA, 0.4 µM of each primer, and 12.5 µL of Q5 high fidelity 2x master mix (New England Biolabs, UK). Briefly, initial denaturation was performed at 94 °C for 30 s; followed by 35 cycles of denaturation at 94 °C for 15 s, gradient annealing

Table 1
Set of long-read primers 7, 8 and 9 targeting the GC-rich region of PPRV between M and F protein genes. The long-read primer 10, targets a non-GC rich region and it was used as positive control.

Primer name	Fragments	Sequence (5′ to 3′)	Tm*	GC%	Position on genome		Fragment size (bp)	Reference
					Start	End		
PPRV7LK	F7	CAACAACACTCCGCTGTCCT	60.30	55.00	3,778	3,796	770	This study
PPRV7RK		GAGTGGCTGTGTTGGTGCT	60.53	57.89	4,548	4,530		
PPRV8LK	F8	CAAGCCGTCCTACAGCCATC	60.81	60.00	4,353	4,372	895	This study
PPRV8RK		GTCCTCCCTCGGTCTGTCT	60.00	63.16	5,247	5,229		
PPRV9LK	F9	GAGGACACCCAACCACCGAAAC	59.00	59.09	4,972	4,993	815	This study
PPRV9RK		ACAGAGCATCCTCTACAGGCTT	55.00	50.00	5,787	5,766		
PPRV_10_LEFT	F10	CGAGCCAACAAACCCTGGTTAT	61.14	50.00	5,658	5,680	787	(Torsson et al., 2020)
PPRV_10_RIGHT		CATTCTTGTGCCCCGATGTTGT	61.72	50.00	6,445	6,423		

* Tm; melting temperature, GC%; GC content.

at either 64 °C, 64.1 °C, 65 °C or 65.5 °C for 1 min, and extension at 65 °C for 50 s; and with a final extension at 65 °C for 7 min. All PCR amplifications were carried out in an Eppendorf Mastercycler nexus gradient PCR System (Eppendorf AG Hamburg, Germany) followed by electrophoresis of PCR products on a 1.5 % agarose gel. Agarose was premixed with GelStar nucleic acid stain (Lonza Rockland, USA) and visualization of PCR products was done using a Gel DocTM EZ Imager agarose gel imaging system (Bio-Rad, Hercules, CA).

2.4. Sequencing of the GC-rich fragments between M and F protein genes of PPRV

The PCR products of PPRV GC-rich fragments were sequenced using long-read primers (Table 1). The sequencing reactions were performed in the DNA Master cycler pro-384 (Eppendorf) using BigDye® Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) following the protocols supplied by the manufacturer. The fluorescent-labeled fragments were purified from the unincorporated terminators with the BigDye XTerminator® Purification Kit (Applied Biosystems, Foster City, CA). The samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The quality of sequencing chromatogram was checked using Sequence scanner Version 2.0 software (Applied Biosystems, Foster City, CA). The reverse complement and forward nucleotide sequences delimited by reverse and forward primers sequence were aligned to obtain a consensus nucleotide sequence using Bioedit version 7.2.5 (Ibis Biosciences, Carlsbad, CA). The consensus nucleotide sequence was used in BLASTn to search for nucleotide identity in comparison with available nucleotide sequences at GenBank database.

3. Results

3.1. Amplification of the GC-rich region of PPRV between M and F protein genes

The long-read primers were tested for GC-rich PCR amplification of F7, F8 and F9 along with F10 as positive control, targeting non-GC-rich fragment. The resulting PCR amplification products had clear bands after conducting agarose gel electrophoresis with sizes corresponding to the expected sizes of targeted DNA fragments (Fig. 1). Unequivocal PCR amplicons were observed at 64 °C optimal annealing temperature for three GC-rich fragments (F7, F8 and F9).

3.2. Sequencing of the GC-rich fragments between M and F protein genes of PPRV

The GC-rich region consensus nucleotide sequences of F7, F8 and F9 indicate 99.5 % nucleotides identity compared to PPRV genome KY628761, after BLASTn. The highest sequence coverage was observed in F9 and with few nucleotides less in F7 and F8 (Accession numbers: MW580394; MW580395; MW580396). The position of these sequences in the genome were between 3,885 and 4,546 (662 bp) for F7; 4,479 and 5,100 (622 bp) for F8; and 4,991 to 5,787 (797 bp) for F9 when compared to PPRV genome available at GenBank (accession number KY628761). In combination, the generated sequences in F7, F8 and F9 covered an entire GC-rich UTR region at nucleotide position 4,445 and 5,524 (Fig. 2). The GC contents of F7, F8 and F9 at GenBank was 67.4 %, which is relatively similar to PPRV genomes presently available in the public database (Table 2).

4. Discussion

Peste des petits ruminants is economically important transboundary animal disease that is targeted for global control and eradication (OIE and FAO, 2015). The development of PPRV vaccines, diagnostics and therapeutics, greatly rely on detailed insights into the complete genomes (Parida et al., 2015). Yet, the GC-rich region between M and F protein genes of PPRV impedes PCR amplification and affects the downstream full genome sequencing using next generation sequencing technologies (Hacıoğlu et al., 2020; Torsson et al., 2020). In turn, it has led into the absence or low sequence coverage in this region (Torsson et al., 2020). In this study, long read primer amplification strategy was developed to

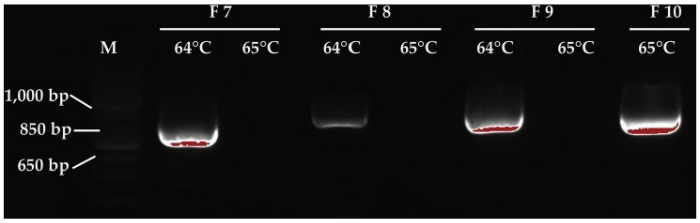

Fig. 1. Polymerase chain reaction (PCR) amplification of the GC-rich region between matrix (M) and fusion (F) protein genes of peste des petits ruminants virus (PPRV). Each PCR amplicon has an expected size of target DNA fragments (F7; 770 bp; F8; 895 bp and F9; 815 bp) and F10; 787 bp.

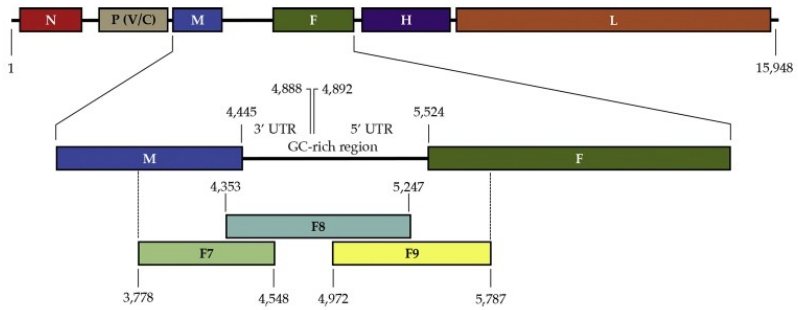

Fig. 2. Schematic illustration of peste des petits ruminants virus (PPRV) structural proteins (3'-N, P, M, F, H, L-5'). An overlapping long-read primer-based amplification strategy was developed to amplify and sequence the GC-rich fragments (F7, F8, and F9) between M-F gene junction. The numbers indicate genome position.

Table 2
Comparison of long GC-rich region of PPRV F7, F8 and F9 and other PPRV genome sequences available in public databases.

Genome	Accession number	Lineage	Nucleotide identity at GC-rich region (%)	GC % (M-F) (4,445–5,524)
Momba/Tanzania/2018	MW580394-6	II	100	67.4
Nigeria 75/1	KY628761	II	99.5	67.5
Benin/B1/1969	KR781450	II	95.2	68.3
Nigeria 76/1	EU267274	II	93.5	67.5
Ghana/NK1/2010	KJ466104	II	88.4	66.1
KN5/2011	KM463083	III	75.7	66.5
Uganda 2012	KJ867543	III	82.7	68.1
Sungri/96	KF727981	IV	83.5	68.0
SRMV/Ethiopia/2014	MK991799	IV	81.3	67.6
ICV89	EU267273	I	80.0	67.8
E32/1969	KP789375	I	82.0	67.5

amplify and sequence the GC-rich region of PPRV, belonging to lineage II.

An overlapping long-read primer-based amplification strategy was deployed to amplify the GC-rich fragments between M-F gene junction using nexus gradient PCR. Extremely difficult PCR target, the GC-rich region between M and F protein genes of PPRV was successfully amplified and sequenced in this study. Similarly, shorter notorious GC-rich region that posed difficulty to Illumina sequencing at nucleotide position 5,142 and 5,598 was also amplified and sequenced (nucleotide position between 4,991 and 5,787; accession number: MW580395) (Hacıoğlu et al., 2020). The GC-rich region of PPRV is a potential bottleneck for full genome sequencing with analytical methods that require PCR amplification of the PPRV (Chen et al., 2013; Hacıoğlu et al., 2020; Torsson et al., 2020). Polymerase chain reaction amplification of GC-rich templates is usually difficult compared to non-GC-rich targets. Poor nucleotide amplification in the GC-rich region might be due to the formation of stable hairpins and secondary structures that block DNA polymerase (Mamedov et al., 2008). These complex structures also impede primer annealing, resulting in incomplete or non-specific amplifications (Guido et al., 2016; Mamedov et al., 2008).

The annealing temperature of the primer was the most important parameter that a slight temperature change of 0.2 °C made a significant difference between specific and non-specific nucleotide amplifications. Gradient PCR reaction showed that the optimal annealing temperature of long-reads primers from this study was 64 °C, one degree of Celsius below that of long-read primers previously reported (Torsson et al., 2020). The optimal annealing temperature of primer usually depends on the nucleotide base composition and sequence length of the primer (Sahdev et al., 2007).

The GC-rich region at the junction of the M and F genes poses difficult for both primer design and amplification (Torsson et al., 2020). Attempts have been made to resolve the complex secondary structure formation at GC-rich regions by using PCR additives and adjusting thermal cycling conditions (Hubé et al., 2005; Ralser et al., 2006). The successful amplification in this study was achieved by re-designing three long-read primer pairs without use of PCR additives. Few less nucleotides sequences than expected in F7 and F8 may be due to slippage or secondary structure in GC-rich regions which produce shorter readable sequences (Choi et al., 1999). Normally, dimethyl sulfoxide is added to the sequencing reaction to ameliorate the effects of long GC-rich fragments (Choi et al., 1999).

Recent studies have shown that extremities and long GC-rich region of PPRV genome are usually very difficult to generate by NGS sequencing (Chen et al., 2020; Eloiflin et al., 2019). With Illumina sequencing technology, one more step is required to complete the extremities of PPRV genome using rapid amplification of cDNA-ends by polymerase chain reaction (RACE PCR) (Eloiflin et al., 2019). Whilst Oxford nanopore has recently shown capability for sequencing of PPRV genome extremities in a single sequencing run (Jain et al., 2018). With this technology, high quality long GC-rich amplification products can be effectively sequenced, (Bainomugisa et al., 2018; Torsson et al., 2020). In addition, the Oxford nanopore miniPCR and MinION sequencer can be powered by battery and solar panel for field use in resource limited setting, where electricity is unreliable or absent (Brunker et al., 2020; Quick et al., 2016; Torsson et al., 2020). This provides an opportunity for molecular epidemiological surveillance of PPRV in real time, necessary for PPR eradication strategy (Eloiflin et al., 2019; Torsson et al., 2020).

5. Conclusions

The GC-rich region residing at the end of the M open reading frame and before the start of the F gene coding sequences in PPRV genome, is an extremely difficult PCR target. Through overlapping long-read primer based-approach, the GC-rich region between PPRV genome position 4,445 and 5,524 was successfully amplified and sequenced without PCR additives. These three new long-read primer sets may be used in tiling multiplex PCR for full genome sequencing of PPRV.

Author contributions

EK designed the study, collected the data, analysed the data and

drafted the manuscript. JNH and GM participated in the study design, data analysis and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset generated during this research are publicly available at the NCBI GenBank (Accession numbers: MW580394; MW580395; MW580396).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PAPER FOUR

2.3 COMPLETE GENOME SEQUENCING OF FIELD ISOLATES OF PESTE DES PETITS RUMINANTS VIRUS FROM TANZANIA REVEALED A HIGH NUCLEOTIDE IDENTITY WITH LINEAGE III PPR VIRUSES

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Complete Genome Sequencing of Field Isolates of Peste des Petits Ruminants Virus from Tanzania Revealed a High Nucleotide Identity with Lineage III PPR Viruses

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Simple Summary: Peste des petits ruminants virus (PPRV) causes a highly devastating disease, peste des petits ruminants (PPR), in sheep and goats, which is targeted for global control and eradication. However, in many developing countries, access to expensive sequencing technologies is limited and is compounded by difficulties in transporting clinical samples across international borders. Oxford nanopore MinION is a relatively cheap sequencing technology using portable devices that require minimal supporting laboratory infrastructure or technical expertise for sample preparation and rapid sequencing. In this study, Oxford nanopore MinION sequencing was carried out to generate complete genomes of PPRV from archived PPRV-positive samples collected from PPR outbreaks in goats in Ngorongoro and Momba districts in Tanzania during 2016 and 2018, respectively. Complete genomes of PPRV of 15,948 nucleotides long were generated within four hours of sequencing. The phylogenetic analysis of the complete genomes revealed a high nucleotide identity (96.19–99.24%) with lineage III PPR viruses currently circulating in East Africa, indicating a common origin. The Oxford nanopore MinION sequencer can be deployed to overcome diagnostic and surveillance challenges in developing countries in the PPR Global Control and Eradication program. However, the coverage depth was uneven across the genome and amplicon dropout was observed between the matrix (M) and fusion (F) genes. Thus, larger field studies are needed to allow the collection of sufficient data to assess the robustness of nanopore sequencing technology.

Abstract: Peste des petits ruminants virus (PPRV) causes a highly devastating disease of sheep and goats that threatens food security, small ruminant production and susceptible endangered wild ruminants. With policy directed towards achieving global PPR eradication, the establishment of cost-effective genomic surveillance tools is critical where PPR is endemic. Genomic data can provide sufficient in-depth information to identify the pockets of endemicity responsible for PPRV persistence and viral evolution, and direct an appropriate vaccination response. Yet, access to

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the required sequencing technology is low in resource-limited settings and is compounded by the difficulty of transporting clinical samples from wildlife across international borders due to the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora, and Nagoya Protocol regulations. Oxford nanopore MinION sequencing technology has recently demonstrated an extraordinary performance in the sequencing of PPRV due to its rapidity, utility in endemic countries and comparatively low cost per sample when compared to other whole-genome (WGS) sequencing platforms. In the present study, Oxford nanopore MinION sequencing was utilised to generate complete genomes of PPRV isolates collected from infected goats in Ngorongoro and Momba districts in the northern and southern highlands of Tanzania during 2016 and 2018, respectively. The tiling multiplex polymerase chain reaction (PCR) was carried out with twenty-five pairs of long-read primers. The resulting PCR amplicons were used for nanopore library preparation and sequencing. The analysis of output data was complete genomes of PPRV, produced within four hours of sequencing (accession numbers: MW960272 and MZ322753). Phylogenetic analysis of the complete genomes revealed a high nucleotide identity, between 96.19 and 99.24% with lineage III PPRV currently circulating in East Africa, indicating a common origin. The Oxford nanopore MinION sequencer can be deployed to overcome diagnostic and surveillance challenges in the PPR Global Control and Eradication program. However, the coverage depth was uneven across the genome and amplicon dropout was observed mainly in the GC-rich region between the matrix (M) and fusion (F) genes of PPRV. Thus, larger field studies are needed to allow the collection of sufficient data to assess the robustness of nanopore sequencing technology.

Keywords: peste des petits ruminants virus; PPR; Oxford nanopore MinION; diagnosis; complete genome; sequencing; Tanzania

1. Introduction

Peste des petits ruminants (PPR) is a highly contagious viral disease of wild and domestic small ruminants caused by peste des petits ruminants virus (PPRV), a pathogen targeted for global control and eradication by 2030 [1]. PPR has been reported at an increased rate from its historical distribution across Africa and Asia into new areas where it has not been detected previously [2,3] since the global eradication of rinderpest. Owing to its spread and expansion beyond its known geographical boundaries, PPR causes significant economic losses between USD 1.2 and 1.7 billion globally per year, due to decreased production and animal death, as well as the cost required to overcome the disease [4]. Approximately one third of the economic losses occur in Africa and a quarter in South Asia, and the rest in East Asia, the Middle East and West Eurasia including Turkey, with costs being incurred predominantly by subsistence farmers [5,6]. However, an investment of USD 7.1 billion could be recovered within five years of a successful global eradication, with a 33.8 benefit:cost ratio that makes PPR eradication economically feasible [4]. Unfortunately, the delay of the response following rinderpest eradication and following the identification of PPR spread across disease-free zones and susceptible animal populations such as *camelidae*, *suidae*, and *bovinae*, has increased the likely eradication cost [1]. For instance, the financial losses associated with an outbreak of PPR in critically endangered species of saiga antelope (*Saiga tatarica mongolica*) in Mongolia were estimated at USD 7.27 million [1]. In order to avoid unprecedented financial losses in both wild and domestic small ruminants, an intensive, mass vaccination programme is required to reach and maintain high levels of herd immunity in sheep and goats [7]. It would be more efficient to target vaccination to identified pockets of endemicity responsible for PPRV persistence and create high levels of vaccination immunity in these defined populations, as was successfully carried out in the last phase of rinderpest eradication [7,8]. The identification of pockets of endemicity responsible for PPRV persistence requires an active surveillance programme in susceptible animal populations with rapid and cost-effective diagnostic tools.

The development of the necessary tools for PPR control, including vaccines, diagnostics and therapeutics, greatly depends on in-depth genomic information on the virus [9]. Unfortunately, few complete genomes of PPRV exist, and very few from East Africa, despite the existence of lineage II, III and IV PPRV [10]. The publicly available PPRV sequences from Tanzania were partial sequences, based on nucleoprotein sequencing, to study the phylogeny of PPRV isolates (Table 1). This restricts the ability to define important changes in the genome outside of pre-defined target genetic markers. Nevertheless, genetic changes could be important in viral evolution studies and in the development of novel diagnostic and therapeutic tools for PPR [9,11]. Interestingly, Oxford nanopore MinION sequencing is a rapid and relatively cheap technology that uses portable devices that require minimal supporting laboratory infrastructure or technical expertise for sample preparation [12]. The relatively low abundance of viral nucleic acids compared to that of host nucleic acids in clinical samples often necessitates the analysis of a substantial amount of sequence data, reflected in analysis times and associated costs [13]. In this study, the ARTIC method, based on tiling multiplex PCRs that were previously used to enrich PCR amplicons for Zika virus, Ebola virus and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was adopted to generate the complete genomes of field isolates of PPR [13,14]. Using this advanced approach, by assessing the long overlaps among multiplex amplicons, the accurately assembled and complete viral genome can be obtained, which can facilitate the rapid genomic surveillance of PPRV for better understanding its pathogenicity, evolution and transmission. Although a protocol for Oxford nanopore sequencing of PPRV has been established, adoption of the technology has been limited due to concerns around accuracy and high error rates associated with homopolymer lengths [15]. The nanopore device exhibits lower read-level sequencing accuracy than its short-read platform counterparts [16]. Moreover, the longest untranslated intergenic region between *M* and *F* genes, with about 66–72% GC content, impedes full-genome sequencing with next-generation sequencing technologies such as Illumina and Oxford nanopore MinION [9,17]. This region is an extremely difficult PCR target to sequence due to repetitive sequences and secondary DNA structure formation. In order to circumvent this deficiency, a method was successfully developed to amplify the GC-rich fragments (F7, F8 and F9) by redesigning long-read primers targeting the GC-rich region, prior to the full-genome sequencing in another study (manuscript in preparation).

The etiological agent PPRV belongs to the genus Morbillivirus of the family *Paramyxoviridae* [18]. The PPRV genome is linear, non-segmented, negative-sense, single-stranded RNA, which is 15,948 nucleotides long [19]. Its genome length complies with hexamer length and the "rule of six," in which the total number of nucleotides must be a multiple of six for the virus to replicate efficiently in infected cells [19]. However, a longer variant of PPRV of 15,954 nucleotides long has been reported in China [20]. The tolerance for variation is particularly constrained in the genomic termini, as they contain essential elements for replication, such as the signal for encapsidation and promoters for genome and antigenome replication [20,21]. Generally, this genome comprises six genes in order of 3′-N, P, M, F, H, L-5′, with each gene coding for a distinct structural protein, the exception being the phosphoprotein (P) gene [9,19] which also codes for additional non-structural proteins. These non-structural proteins, designated C and V, are generated through alternative start codons (leaky scanning) and RNA editing, respectively [22]. The encoded proteins bear the acronym of the respective gene of origin; the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin protein (H) and the RNA-dependent RNA polymerase also known as large protein (L). The N protein is abundant in PPRV-infected cells because the *N* gene is located near the 3′ proximal genomic promoter, and hence it is the most transcribed gene [23]. Given its abundance and antigenic stability, the N protein is frequently used in PPR diagnostic development and is the most appropriate gene for molecular characterization of closely related isolates [24]. Based on the partial molecular genetic characterization of N and fusion F protein genes, PPRV has been grouped into four distinct lineages (I, II, III and IV) that exist as a single serotype [25–27].

The current geographical distribution of PPRV lineages shows lineages I, II and III as restricted primarily to the African continent [10], whilst lineage IV is found throughout Asia and the Middle East, although early detection includes Central Africa as well as contemporary detections in North and sub-Saharan Africa [2,10]. In 2008, lineage IV PPRV was reported to have caused a devastating epidemic of PPR in Morocco [28]. Phylogeographic analysis suggests that lineage IV PPRV has spread from Eastern Africa, most likely from the Sudan 2000 outbreak, into Northern Africa, resulting in the 2008 Moroccan outbreak [29]. Later in 2009, lineage IV was also detected in several countries in sub-Saharan Africa [25,30–32], where there was no clear-cut association with trade of movement from either Morocco, the Middle East or South Asia. In the 1990s, Tanzania was free from PPR based on comprehensive serological investigations in goats and sheep [33]. In 2008, PPR was serologically confirmed in northern Tanzania for the first time [34]. However, a retrospective study that was conducted on archived samples collected from Ngorongoro district between 1998 and 2004 found antibodies against PPRV already present in samples collected in 2004, indicating that PPR might have been present in Tanzania before it was officially confirmed in 2008 [35]. Molecular confirmation of PPRV was carried out at a much later time (2010) than would have been expected for the correct identification of outbreaks (Table 1).

Since the 1980s, the diagnosis of PPR has constantly been improved through advances in material sciences, genomics, bioinformatics, biotechnology, nanotechnologies, microfluidics and the miniaturization of electronic devices [36]. The first partial nucleotide sequence of PPRV was generated from the cloned N gene of PPRV/N/75/1 vaccine strain in 1994, which was followed by the sequencing of the F protein gene [37,38]. The sequencing of the *N* and *F* genes was pivotal in the development of important molecular diagnostic tools for PPR detection and confirmation [26,39]. More importantly, in 2005, a complete genome of PPRV was publicly available for the first time using Sanger standard methods [19]. Sanger dideoxynucleotide cycle sequencing has been a standard method for the sequencing of PPRV and confirmation of sequences that are difficult to generate with other methods [10,19,40]. With recent advances in sequencing technologies, the complete genomes of PPRV isolates are now being generated using next-generation sequencing technologies, mainly Illumina, and recently Oxford nanopore MinION [17]. The Oxford nanopore MinION sequencing technology has been proven powerful in the genetic characterization of infectious disease agents, including PPRV [13]. The establishment and deployment of nanopore sequencing of PPRV provides an opportunity for the molecular epidemiological surveillance of PPR in resource-limited settings and challenging geographical landscapes, due to its rapidity, improved accuracy and relatively low sequencing cost per sample [12]. This sequencing tool may play a significant role in controlling PPR outbreaks, enabling the detection of cryptic foci, inadequate vaccine deployment and other challenges in the midst of an eradication campaign. Moreover, Oxford nanopore has recently shown a capability for sequencing a full or nearly full genome of PPRV in a single sequencing run within 4 h of sequencing. This provides an opportunity for genomic surveillance of PPRV in real time, necessary for the early implementation of control measures in low-income and in resource-constrained countries, where PPR is endemic.

The present study was undertaken to generate complete genomes of PPRV isolates collected from goat in the Ngorongoro and Momba districts of Tanzania in 2016 and 2018, respectively, using the Oxford nanopore MinION sequencer. The use of the Oxford nanopore MinION sequencer permits sequencing of PPRV in resource-limited settings and in addition makes the production of a complete genome possible within a day. The availability of the full genome of PPRV provides important insight in viral evolution, transmission routes and the implementation of appropriate control measures.

Table 1. Molecular epidemiological studies of peste des petits ruminants in different districts of Tanzania, based on partial N gene and complete genome sequence analysis from 2010 to 2020. The sequences from these studies clustered into three lineages (II, III and IV) and they were isolated from domestic sheep and goats, and a wild small ruminant (Grant's gazelle).

Region/District	Study Period	Host	Sequence	Lineage	References
Arusha, Kilimanjaro, Manyara and Tanga	2010	sheep and goats	partial	III	[40]
Tandahimba and Newala	2011	sheep and goats	-	-	[41]
Mvomero	2013	sheep and goats	-	-	[42]
Ngorongoro and Mvomero	2013	goats	partial	III	[43]
Ngorongoro	2014	sheep and Grant's gazelle	partial	II	[44]
Tandahimba	2015	sheep and goats	partial	II and IV	[45]
Ngorongoro	2015	sheep and goats	partial	III	[46]
Ngorongoro	2016	sheep and goats	-	-	[47]
Mbeya, Iringa, Dodoma Morogoro, Pwani Serengeti, Tanga and Arusha	2018	sheep and goats	-	-	[48]
Mvomero	2020	sheep and goats	complete	III	[17]

2. Materials and Methods

2.1. Sample Source Description and Storage

The nasal swab samples used in this study were collected from goats in Chilulumo ward in Momba district and the Loliondo area of Ngorongoro district in 2018 and 2016, respectively. Momba district is located in the north-western part of the Songwe region of the Southern highlands of Tanzania (Figure 1). The district borders the Rukwa region and Zambia to the west, with Mbozi district to the east, Chunya district to the north and Ileje district to the south, whilst the Ngorongoro district is located in the northern part of the country in the Arusha Region. The samples collected from the Loliondo area of Ngorongoro district in 2016 were previously described and tested by Kgotlele et al. [47]. However, Momba samples were collected during PPR sero-survey in September, 2018. The key clinical signs investigated in 13 goats suspected of PPRV infection include; fever, nasal and ocular discharges, diarrhoea and laboured breathing (Figure 2). The rectal temperature of clinically sick goats ranged between 40 and 41.5 °C, with an average temperature of 41 °C. It was noted that all sick goats (n = 51) recruited for study were an indigenous breed aged between 1 and 2 years. The study recorded 10 deaths with case fatality rates of 19.6% and 100% morbidity. The nasal swabs from live goats were collected in universal viral transport medium (BD Biosciences, Maryland, USA) followed by flicking to dislodge cells from the swabs, and were stored at −80 °C until RNA extraction was undertaken.

2.2. Sample Selection

Seventy-three samples that were previously screened for PPR from different outbreaks in Tanzania were used in this study. When the samples were re-tested by conventional reverse transcription polymerase chain reaction (RT-PCR) prior to full-genome sequencing, most were negative for PPRV, indicating RNA degradation due to temperature fluctuations in our ultralow temperature freezers (freeze and thaw) as a result of frequent power cuts [26]. For example, all 36 samples collected during a PPR outbreak in Tandahimba in 2011 were negative. A total of 7 out of 24 samples collected during a PPR outbreak in Ngorongoro were positive. Out of these 7 PPRV-positive samples, only one had a strong visible band on agarose gel electrophoresis after RT-PCR. Similarly, only 1 out of 13 samples collected during a PPR outbreak in Momba in 2018 produced a strong visible band on agarose gel electrophoresis after RT-PCR. Thus, in total we had 8 PPRV-positive samples with only 2 strongly positive samples that yielded full PPRV genomes after next-generation sequencing. The remaining samples had very low PPRV genome coverage.

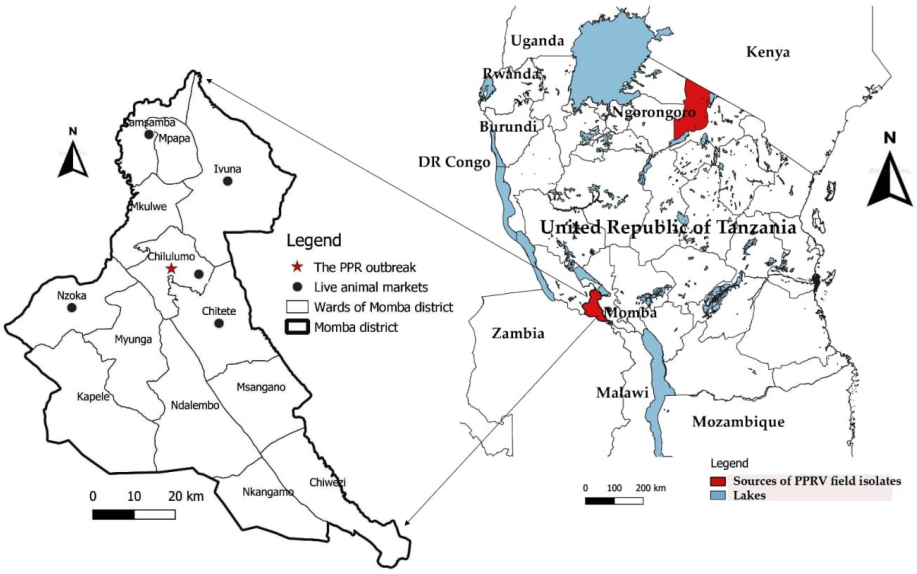

Figure 1. A map of Tanzania showing sources of PPRV field isolates used in this study. The archived PPRV-positive samples analysed in this study were collected from goats in Chilulumo ward in Momba district and in Loliondo area in Ngorongoro district, marked in red.

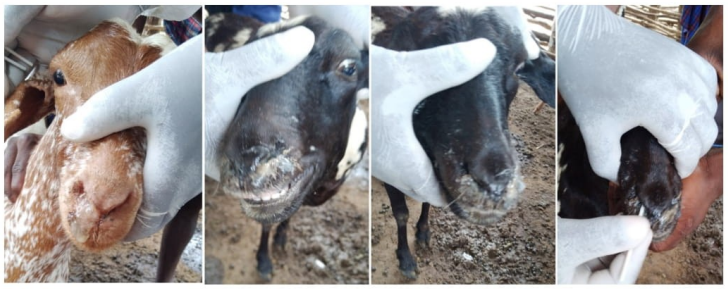

Figure 2. Nasally discharging goats suggestive of peste des petits ruminants virus (PPRV) infection, during a peste des petits ruminants (PPR) sero-survey at Chilulumo ward in the Momba district, in 2018. The nasal swab samples were collected and tested for the presence of PPRV infection. The PPR-positive samples were stored at −80 °C for further research.

2.3. RNA Extraction, cDNA Synthesis and PCR Amplification

Total RNA was extracted using the QIAamp Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA synthesis was carried out using the Superscript IV First-Strand Synthesis System (Invitrogen, Paisley, UK) using 11 µL of RNA, according to the full wet lab protocol [17]. Polymerase chain reaction (PCR) nucleotide amplification reactions were performed using the Q5 Hot Start High Fidelity Polymerase (New England BioLabs, UK). Twenty two pairs of multiplex primers of 800 bp with an overlap of 100 bp were used as previously described by Torsson et al. [17], plus three newly designed pairs of primers targeting the long guanine cytosine untranslated region (GC-rich) of PPRV (Table 2). Briefly, two separate PCR reactions were carried out

for each PPRV-positive sample. Two pools of primers were made; 'pool 1′ contained eleven primers that generated the odd-numbered tiled amplicons, while 'pool 2′ contained eleven primers that generated the even-numbered tiled amplicons for the 800 bp set. The three primers targeting the GC-rich fragments (F7, F8 and F9) were used separately in each PCR tube. The PCR reactions were performed using a nexus gradient master thermocycler (Eppendorf AG Hamburg, Germany). The resulting PCR amplicons were pooled and then purified using AMPure XP magnetic beads (Beckman Coulter, Redwood, USA) with a 1.8x bead ratio and quantified using Qubit 1.0 Fluorometer dsDNA HS assay (Thermo Fisher Scientific, Waltham, USA). The PCR amplicons were run on a 1% agarose gel and visualized using a Gel Doc™ EZ Imager agarose gel imaging system (Bio-Rad, Hercules, CA, USA). The detailed methods for reverse transcription, tiled multiplex PCR and library ligation were followed according to the full wet laboratory protocol available (doi.org/10.17504/protocols.io.pnxdmfn) since April 2021.

Table 2. List of long-read primers, 7, 8 and 9, that were developed to amplify an extremely difficult PCR target, the GC-rich region between matrix (M) and fusion (F) protein genes of PPRV.

Primer Name	Fragments	Sequence (5′ to 3′)	Tm *	GC%	Position on Genome		Fragment Size (bp)
					Start	End	
PPRV7LK	F7	CAACAACACTCCGCTGTCCT	60.30	55.00	3778	3796	770
PPRV7RK		GAGTGGCTGTGTTGGTGCT	60.53	57.89	4548	4530	
PPRV8LK	F8	CAAGCCGTCCTACAGCCATC	60.81	60.00	4353	4372	895
PPRV8RK		GTCCTCCCTCGGTCTGTCT	60.00	63.16	5248	5229	
PPRV9LK	F9	GAGGACACCCAACCACCGAAAC	59.00	59.09	4972	4993	815
PPRV9RK		ACAGAGCATCCTCTACAGGCTT	55.00	50.00	5787	5766	

* Tm; melting temperature, GC%; GC content.

2.4. Nanopore Library Preparation and Sequencing

Sequencing libraries were prepared using the SQK-LSK109 ligation sequencing kit and EXP-NBD104 native barcode expansion (Oxford Nanopore Technologies, UK) following the nanopore sequencing protocol [17]. The concentration of 0.12 pmol PCR products was diluted in 25 µL of nuclease-free water. This generated 60 ng of the PCR product in 25 µL water for our amplicons (800 bp) (https://nebiocalculator.neb.com/ accessed on 11 November 2020). The final concentration of the DNA library was 42 fmol. Briefly, the purified PCR amplicons were repaired and A-tailed using the NEB Next Ultra II End Repair/dA-Tailing module (New England BioLabs, Ipswich, MA, USA). Native barcodes and adaptors were ligated to end-repaired PCR amplicons using Blunt/TA Ligase Master Mix (New England BioLabs, Ipswich, MA, USA), to generate the nanopore library. The library was then sequenced on a MinION Flow cell for 4 h. The nanopore sequencing raw read datasets were generated in standard fast5 format. The nanopore raw reads were basecalled and demultiplexed to generate fastq files using GUPPY software built in MinIT.

2.5. Nanopore Dataset Analysis

The composition and quality of reads were assessed using nanoplot and qScore, and the reads below a qScore of 7 were removed by the EPI2ME software version 2019.7.9, (as per ONT pass/fail threshold) before downstream analysis (PycoQC (https://usegalaxy.org, accessed on 5 January 2021). Additional demultiplexing and adaptor removal were performed using porechop in NanoGalaxy platform [49,50]. The trimmed nanopore reads were checked for purity using Q-score in the nanoplot [49,51]. The reads were aligned to the PPRV reference genomes (RefSeq accession number: KM463083) using minimap2 version 2.17 [52]. The resulting binary alignment map (BAM) file was sorted and converted into an indexed BAM file for additional processing with samtools version 1.9 [53]. Following this, a consensus sequence was created by obtaining the majority vote for the bases from the pileup of the BAM file. The Katuali (https://github.com/nanoporetech/katuali, accessed on 7 January 2021) pipeline was used to assemble the genome. The

reads were assembled with Canu version 2.0 [54] and polished with Racon and Medaka (https://github.com/nanoporetech/medaka, accessed on 7 January 2021) [55]. For the Momba/Tanzania/2018 PPRV field isolate, the missing 5′ region (from 15890 bp onwards) in the nanopore assembly was completed using the matching region from the consensus. When the final assembly was compared with KM463083, 11 indels were found. We did not observe any clustering of the indels. We manually removed the indels where the frequency of the reads supporting an indel was below 40% and the frequency of the reads that did not support the indel exceeded 60%. The browser extensible data (BED) files were created, representing the coverage of the sequence reads against the reference genome, and the results were visualized using integrative genomics viewer (IGV). Finally, the consensus sequences were annotated using genome annotation transfer utility [56] and whole-genome comparison was performed using the basic local alignment search tool (NCBI BLAST version 2.12.0, Rockville Pike, MD, USA). With comparable sequences from GenBank, a phylogenetic tree was constructed using the Maximum likelihood method, and the Kimura 2-parameter model with a bootstrap frequency of 1000 replicates, as implemented in MEGA X software [57].

2.6. Temporal Phylogenetics

To identify the nearest common ancestor and hence likely dates of divergence, the Tanzania/Momba/2018 and Tanzania/Ngorongoro/2016 (Accession Numbers: (MZ322753 and MW960272) sequences were compared using the coalescent-based Bayesian Markov chain Monte Carlo (MCMC) approach to selected complete genomes of PPRV available in GenBank (n = 38) [58]. The gamma distribution and general time-reversible nucleotide substitution model for rate variation and the proportion of invariant sites were selected on the basis of Akaike information criterion scores. Bayesian time-scaled phylogenetic analysis molecular evolutionary rate and divergence times were estimated. A Bayesian maximum clade credibility (MCC) phylogenetic tree was constructed by using Bayesian Markov chain Monte Carlo (MCMC) analysis and Bayesian evolutionary analysis sampling trees (BEAST), and the Tree Annotator software package v1.10.4 (Auckland, New Zealand). For the sequence dataset, the best-fit nucleotide substitution model was determined on the basis of Akaike information criterion scores using JModel Test software v2.1.4 (Boston, MA, USA), as previously described [59]. An input file for BEAST analysis was obtained by using Bayesian evolutionary analysis utility software, BEAUti v1.10.4 (Auckland, New Zealand) in which sequences were tip dated according to the year of collection. The relaxed molecular clock with coalescent exponential growth was the most appropriate model for this analysis, as previously reported [29,59]. The Bayesian analyses were run for 50,000,000 iterations sampled every 5000 in duplicate; duplicate runs were combined for final analysis with effective sample size (ESS > 200) and were assessed for their proper mixing, convergence and consistency by Tracer v1.7.2 with 10% burn in. The two individual runs were combined by using LogCombiner v1.10.4 in the BEAST software package (Auckland, New Zealand). The nucleotide substitution rate (substitutions/site/year) and the time to the most recent common ancestor (TMRCA) (year) values were obtained from Tracer v1.7.2. The posterior tree distributions were summarized by using TreeAnnotator v1.10.4 and exclusion of the first 10% of the trees as burn in. Phylogenetic MCC tree with median node heights were visualized in FigTree software v1.4.2 (Auckland, New Zealand). The phylogenetic MCC tree in Newick file format together with metadata file with accession number and country of origin were uploaded to PastML for ancestral state reconstruction (https://pastml.pasteur.fr/, accessed on 2 May 2021).

3. Results

3.1. Nucleotide Amplification

Out of eight archived PPRV-positive samples screened for PPR, only two samples had strong bands on agarose gel electrophoresis using gene-specific primers (NP3/NP4) targeting the nucleocapsid protein gene. The two positive samples were collected from

goats in Ngorongoro and Momba districts in the northern and southern highland of Tanzania. The cDNA obtained from these positive samples were used subsequently in tiling multiplex PCR. Analysis of the PCR amplicons on agarose gel electrophoresis exhibited strong PCR bands of expected size (~800 bp), confirming successful amplification of the fragments (Figure 3). However, relatively faint bands were observed in the PCR fragments that targeted the GC-rich region of PPRV.

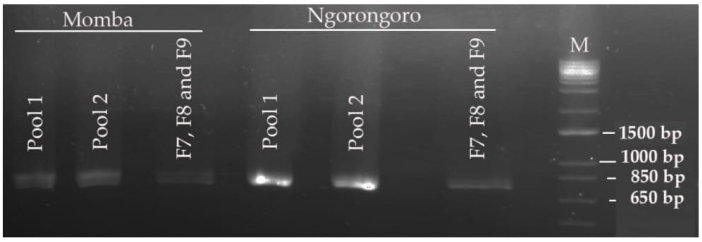

Figure 3. Gel electrophoresis of tiling multiplex polymerase chain reaction (PCR) amplification products of peste des petits ruminants virus isolates from Momba and Ngorongoro districts in Tanzania. The PCR amplicons indicate an expected band size of around 800 bp in accordance with primer targets.

3.2. Long-Read Nanopore Sequencing of PPRV Field Isolates

Sequencing of PCR amplicons produced over 1.5 million raw reads from each sample (Table 3). The read coverage from this isolate was much higher, up to 8000 reads per position, with low reads (34–163 per position) in the GC-rich region between the 4 and 6 kb genome positions (Figure 4). Similarly, low reads were observed in the leader and trailer regions, as low as 189 and 13 per position, respectively. In the region where a missing region was constructed using the consensus for the Momba sequence, the average read coverage was 84.4.

Table 3. Results from full-genome sequencing of PPRV field isolates from goats in Ngorongoro and Momba districts of the northern and southern highland of Tanzania, using Oxford Nanopore MinION sequencer.

Sample	Raw Reads	Total bp	N50 Length (bp)	Reads Mapped to PPRV	Average Coverage Reads	Genome Coverage >50× (%)	Genome Coverage >25× (%) Source	PPRV Lineage
Ngorongoro	1,881,426	2,203,973,564	793	1,784,633	4575	99.2	99.8	III
Momba	1,712,393	1,605,543,198	816	1,688,419	3906	98.7	99.5	III

3.3. Annotation of Peste des Petits Ruminants Virus Isolate

The nanopore sequencing of PPRV field isolates generated full-length genomes with 15,948 nucleotides. The graphical view of the PPRV open-reading frames (ORFs) in comparison with the reference sequence showed that the PPRV genomes encoded six structural proteins with transcription units for the N, P, M, F, H and L proteins, and two non-structural proteins C and V transcribed from the *P* gene, using the genome annotation transfer utility (Figure 5). The Tanzania/Ngorongoro/2016 PPRV isolate showed the highest protein identity with lineage III isolate, KN/2011(KM463083.1), N (99%), P (99.8%), V (99.33%), C (100%) M (100%), F (98%), H (100%) and L (99.7%). Equally, high protein identity was observed with the Tanzania/Momba/2018 PPRV isolate, N (99.6%), P (98.4%), V (95.97%), C (97.7%) M (100%), F (99.5%), H (99.3%) and L (99.4%). Moreover, the PPRV isolates, Tanzania/2016 and 2018, contained 1080 nucleotides of untranslated region (UTR) at the genome position between nucleotides 4446 and 5526, with 66.9 and 69.4% GC contents, respectively, between the M and F open-reading frames.

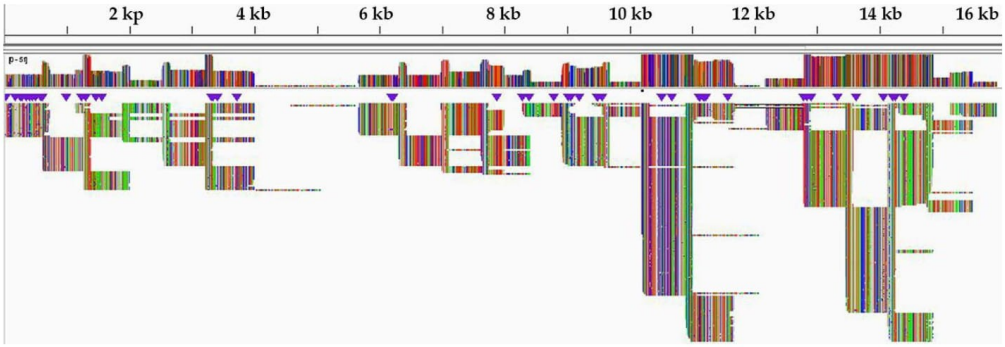

Figure 4. Integrative genomic viewer (IGV) of peste des petits ruminants virus (PPRV) sequence reads against reference genome (KM463083).

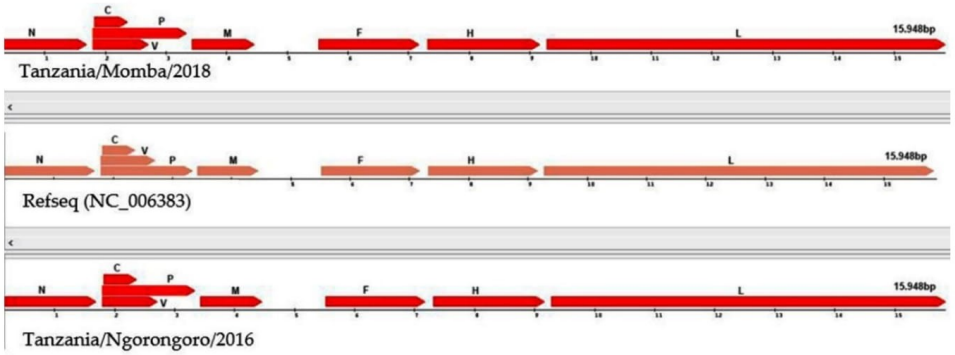

Figure 5. Graphical view of peste des petits ruminants virus (PPRV) open-reading frames (ORFs) in comparison with reference sequence (NC_006383), using genome annotation transfer utility.

3.4. Phylogenetic Analysis of Peste des Petits Ruminants Virus

Two complete genome sequences obtained from this study were submitted to GenBank and assigned accession numbers MW960272 and MZ322753. Another 19 full genomes representing all the four lineages were retrieved from GenBank for further analysis, making it a total of 21 sequences. The sequences showed good conformity with the PPRV complete genome sequences available on GenBank (Table 4). A phylogenetic analysis of the sequences showed that the PPRV sequences obtained from Loliondo in Ngorongoro and Chilulumo in Momba districts clustered into lineage III of PPRV (Figure 6). The comparison of their full genomes with those of other PPRV strains revealed the highest nucleotide identity (96.19 to 99.24%) with the PPRV isolate KN5/2011 (KM463083.1) from Kenya; B3 isolate from Burundi (MK686066.1) and Ugandan isolate (KJ867543.1).

3.5. Temporal–Spatial Spread of Peste des Petits Ruminants Virus

A Bayesian time-scaled MCC tree using complete PPRV genomes was constructed (n = 40), alongside Tanzania/Momba/2018 and Tanzania/Ngorongoro/2016 (Figure 7). In estimation of the route of entry of lineage PPRV III into East Africa, we visualized the summarized results of the Bayesian phylogeographic analysis of the complete genome (Figure 8). Analysis of the posterior probabilities suggests a strong historical and geo-

graphic connection between the Tanzanian isolates and PPR viruses isolated in East Africa (Figure 9). These analyses show a very strong likelihood >72% that the lineage III viruses currently circulating in East Africa are closely related to and, in the absence of further material, likely originated from the Ethiopia 1994 outbreaks (Figure 7).

Table 4. Publicly available complete genome sequences of peste des petits ruminants virus strains from East Africa and selected strains from other parts of Africa and Asia used for comparative genomic analysis in this study.

Isolate Name	GenBank	Country of Origin	Year of Collection	Lineage	Percentage Nucleotide Identity with Tanzania /2016 PPRV Isolate	Percentage Nucleotide Identity with Tanzania/ 2018 PPRV Isolate	Host Species	Reference
Tanzania/2016	MW960272	Tanzania	2016	III	100.00	97.39	goat	This study
Tanzania/2018	MZ322753	Tanzania	2018	III	97.39	100.00	goat	This study
KN5/2011	KM463083.1	Kenya	2011	III	99.24	97.92	goat	[60]
B3	MK686066.1	Burundi	2017	III	98.44	97.22	goat	[61]
Uganda 2012	KJ867543.1	Uganda	2012	III	97.38	96.19	goat	[62]
Ethiopia 1994	KJ867540.1	Ethiopia	1994	III	95.55	95.57	goat	[62]
UAE 1986	KJ867545.1	United Arab Emirates	1986	III	94.49	94.57	Dorcas gazelle	[62]
Oman 1983	KJ867544.1	Oman	1983	III	94.47	94.56	goat	[62]
Nigeria/75/1	HQ197753.1	Nigeria	1976	II	88.58	88.73	goat	[63]
Benin/B1/1969	KR781450.1	Benin	1969	II	88.85	89.00	goat	[64]
Ng76/1	EU267274.1	Nigeria	1976	II	88.50	88.66	goat	[65]
Ghana/2010	KJ466104.1	Ghana	2010	II	87.69	87.81	sheep	[66]
Benin/10/2011	KR781449.1	Benin	2011	II	87.59	87.72	sheep	[64]
ICV89	EU267273.1	Cote d'Ivoire	1989	I	87.92	88.02	goat	[65]
Ethiopia 2010	KJ867541.1	Ethiopia	2010	IV	87.20	87.35	goat	[67]
Georgia/2016	MF737202.1	Georgia	2016	IV	86.94	87.04	sheep	[68]
Morocco 2008	KC594074.1	Morocco	2008	IV	87.35	87.44	goat	[28]
Turkey/2018	MN657232.1	Turkey	2018	IV	86.85	87.01	sheep	[69]
Mongolia/2016	KY888168.1	Mongolia	2016	IV	86.86	86.88	sheep	[70]
China/33/2007	KX421388.1	China	2007	IV	87.30	87.36	goat	[71]
China/Tibet/07	FJ905304.1	China	2007	IV	87.28	87.34	goat	[72]

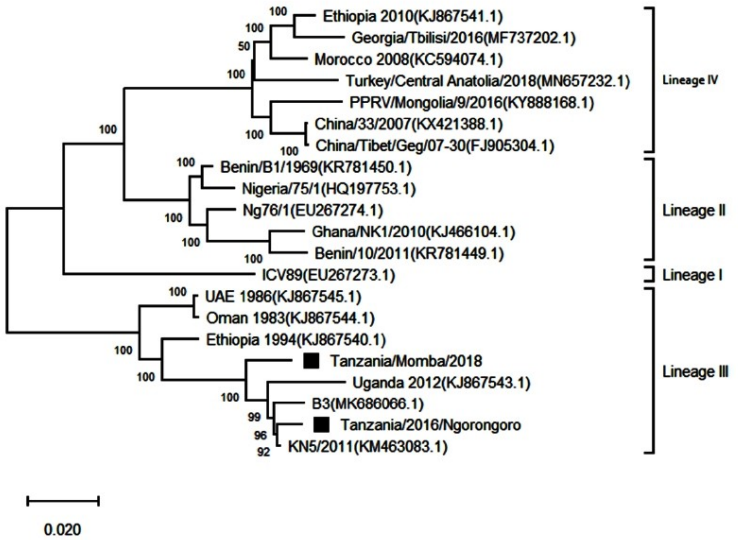

Figure 6. Maximum likelihood phylogenetic tree obtained after multiple sequence alignment of complete genomes of peste des petits ruminants virus strains from East Africa and selected strains from other parts of Africa and Asia. The viruses described in this study are indicated by black squares and the scale bar indicates nucleotide substitution per site, while the node values show percentage of bootstrap support. The analysis involved 21 nucleotide sequences with 15,962 positions in the final dataset.

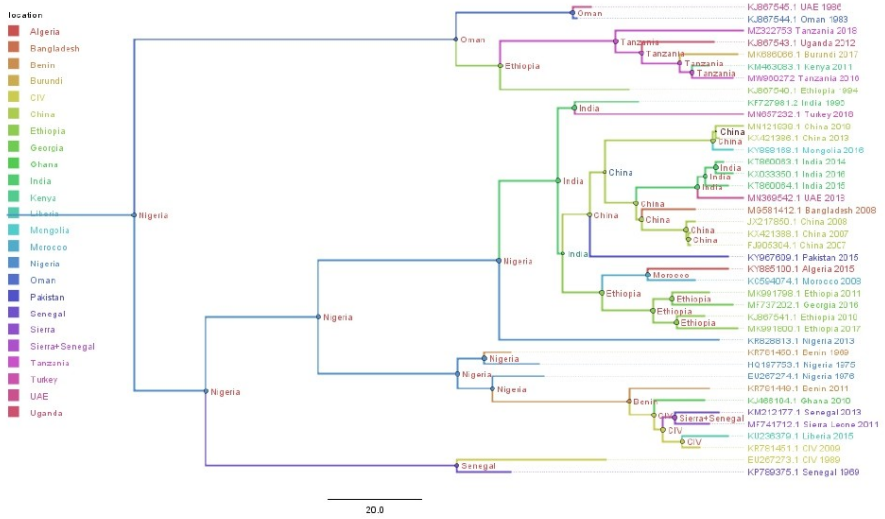

Figure 7. Maximum clade credibility (MCC) trees constructed for the phylogeographical reconstruction of peste des petits ruminants virus PPRV using complete genomes. Posterior probability values are indicated by the size of the node and posterior probability distribution, indicated as a location at the side of each node. Branches are coloured according to the most likely location at the preceding node in the tree. The year of the samples for which PPR viruses were sequenced and GenBank accession numbers are given against each sequence. A phylogeographic method estimated the probability of the root location of an ancestral PPRV and individual lineages as being Nigeria for PPRV, as previously reported by Muniraju et al., 2014.

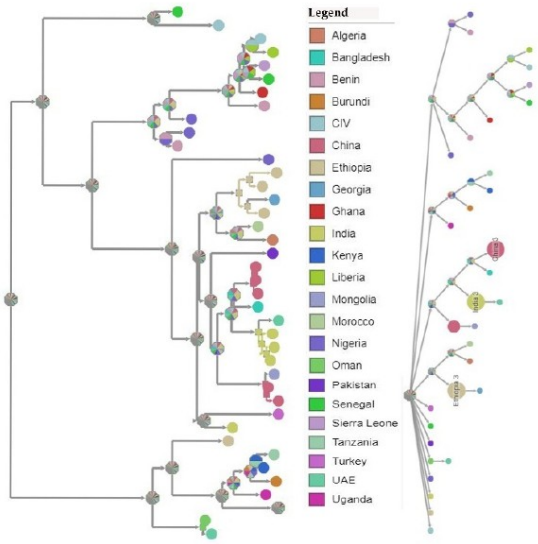

Figure 8. Ancestral rooted phylogenetic map of the selected complete genomes of peste des petits ruminants (PPR) viruses with annotated tips, using maximum likelihood (https://pastml.pasteur.fr/, accessed on 3 May 2021).

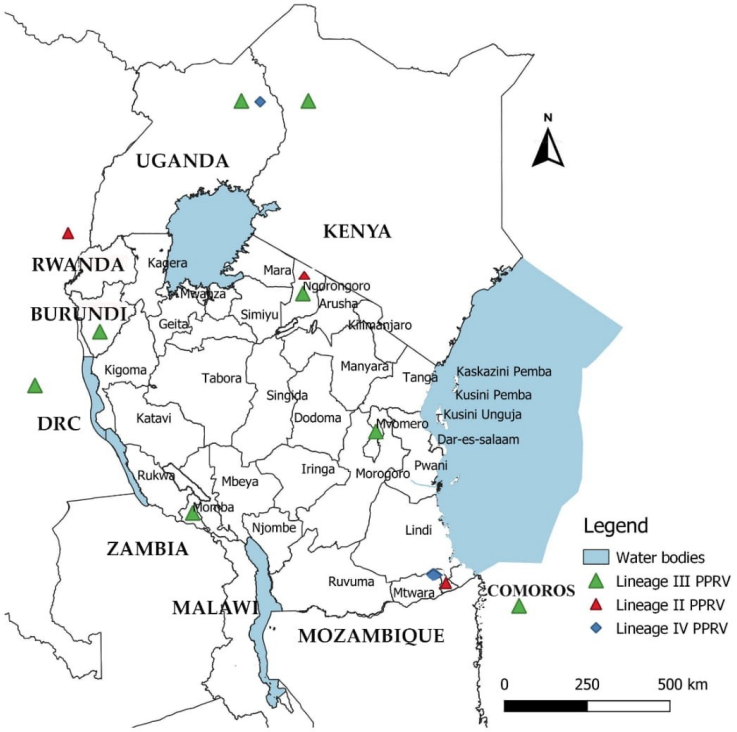

Figure 9. Distribution and molecular characterization of peste des petits ruminants virus in Tanzania and neighbouring countries (Kenya, Uganda, DR Congo, Burundi and Comoros). The colour of triangles and diamonds indicate the viral lineages reported in these countries: lineage III, light green; lineage II, red; and lineage IV, blue. This map was created using QGIS software version 3.16.8 downloaded from https://qgis.org/en/site/, accessed on 12 June 2021.

4. Discussion

Peste des petits ruminants is an economically important transboundary animal disease of sheep and goats, which is targeted for global control and eradication, as a joint initiative of the Food and Agriculture Organisation of the United Nations (FAO) and World Organisation for Animal Health (OIE). Molecular epidemiological surveillance using in-depth genomic data has the potential to clarify the roles of a wide host range in PPRV circulation, direction of transmission at domestic–wildlife interfaces and how viral evolution may alter host range and virulence [73]. However, in many developing countries, access to the required sequencing technology is limited, compounded by the difficulty in transporting wildlife clinical samples across international borders due to the Convention on International Trade in Endangered Species of Wild Fauna and Flora, and Nagoya Protocol regulations [3]. The Oxford nanopore sequencing devices are portable, cheap, require minimal supporting laboratory infrastructure or technical expertise for sample preparation and can be used to perform rapid sequencing analysis with flexible scalability. Thus, the present study was carried out to generate complete genomes of PPRV field isolates using the Oxford nanopore MinION sequencer. With nanopore sequencing, two complete genomes of PPRV were generated within four hours. The resulting sequences had the same length as all of the other PPRV genomes sequenced to date and were in agreement with the "rule of six" for

paramyxoviruses [19,20]. The organization of the genome was the same as those described previously, with transcription units for the N, P(C/V), M, F, H and L proteins [9]. The complete genomes generated with this technology comply with molecular epidemiology sequencing standards, as being "Coding complete", which means 90–99% of the genome is sequenced with no gaps, and that all ORFs are generated (Figure 5).

Following the use of the nanopore sequencing protocol, drop out of amplicons 7, 8 and 9 were observed in the GC-rich region, resulting in no PCR amplicons using this protocol [17]. This bottleneck was resolved by redesigning three pairs of long-read primers for regions 7, 8 and 9, which facilitated the generation of PCR amplicons in this problematic region (Figure 3). The GC-rich region is not conserved between PPRV isolates and poses difficulty for both PCR nucleotide amplification and primer design [74]. Equally, studies have shown that this region has led to the absence of or low nucleotide sequence coverage in the PPRV genome [66]. Next-generation sequencing technologies, such as Illumina and nanopore MinION, generate data of sufficient depth to characterize PPRV strains, but in most cases with no or very low sequence coverage at the GC-rich junction (between nucleotides 4444 and 5526 within the genome) and 3′ and 5′ genome extremities [17]. The missing short sequences are usually amplified using a new set of primers. The genome extremities are also amplified using rapid amplification of cDNA ends by polymerase chain reaction (RACE PCR) [74]. The amplified fragments are later confirmed by Sanger dideoxynucleotide cycle sequencing [69]. Similarly, a systematic drop out of problematic amplicons 18 and 76 was noticed during sequencing of SARS-CoV-2, which has led to the modification of the ARTIC nanopore sequencing protocol [75]. Within this study, the coverage depth was very uneven across the genome (Figure 4). Thus, the accumulation of genetic diversity in PPRV over time may necessitate further changes in the nanopore sequencing protocol. A larger field study is needed to allow the collection of sufficient data to assess the robustness of the protocol.

The sequence coverage was much higher, up to 8000 reads per position (Figure 4). The majority of the reads mapped against the reference genome with an average of 96.73% (Table 3). This indicated high-quality viral RNA, with no degradation of the viral RNA genomes in selected PPR-positive samples. However, very low reads were observed in the GC-rich region at the genome position between 4 and 6 kb (Figure 4). The GC-rich region was approximately 1080 nucleotides long, with an average of 68.15% GC content residing between the M and F genes of PPRV. Equally, previous studies have described that the M and F untranslated intergenic region is the longest intergenic region in the PPRV genome and is very rich in GC content (66–72%) [9]. Owing to secondary and hairpin structures in the GC-rich region, this region is an extremely difficult PCR target for both primer design and nucleotide amplification [76]. The development of overlapping long-read primers targeting this region was critically important to generate nucleotide sequences covering this region (Figure 4). Similarly, previous studies have confirmed that the GC-rich and genome extremities were regions with the lowest or absent sequence coverage in all four known PPRV lineages [17].

Genomic comparison of Tanzanian field isolates, together with other available complete genomes of PPRV available on GenBank, showed a high level of sequence conformity, and Tanzanian isolates clustered together with other isolates of lineage III PPRV (Figure 6). Phylogenetic analysis revealed a high nucleotide identity (96.19–99.24%) with lineage III PPR viruses currently circulating in East Africa, indicating a common origin. The M and H sequences were the most conserved of the genes with lineage III PPR viruses in East Africa (Table 4). Equally, previous studies showed that between isolates, the PPRV genome is relatively conserved, with a maximum divergence of 12% at the nucleotide level and 7% at the amino acid sequence levels [59]. Further analysis of posterior probabilities suggests a strong historical and geographic connection between the Tanzanian field isolates and PPR viruses isolated in East Africa (Figure 7). These analyses show a very strong likelihood >72% that the lineage III viruses currently circulating in East Africa spread from Ethiopia as the most likely origin, although genomic surveillance for PPRV is poor and

a lack of full-genome sequencing from other regions likely biases this result (Figure 8). A phylogeographic method estimated the probability of the root location of an ancestral PPRV and individual lineages as being Nigeria for PPRV, as previously reported [59]. The phylogeographic reconstruction with spatial and temporal information of PPRV isolates has enabled an understanding of the historic emergence and dispersal patterns involved in PPRV evolution [29,59]. As the available number of complete genome sequences is very small in East Africa, to explore the relationship between African virus isolates, further sequences and phylogeographic analyses are needed. As with many other areas, partial sequences are most commonly available but do not enable a thorough analysis of PPRV genetics (Table 1). Certainly, the paucity of full genome data significantly limits the opportunity to evaluate genetic changes outside of the target sequences. Certainly, a more thorough genetic analysis is critical in the study of PPRV pathogenesis and viral evolution studies, alongside the development of novel diagnostics and therapeutic tools for PPR control and eradication (10).

Peste des petits ruminants may have passed unrecognized for several years in some areas of Tanzania, because it is often confused with other diseases that cause respiratory problems and mortality in small ruminant populations [28,29]. A retrospective study that was conducted on archived samples collected from Ngorongoro district between 1998 and 2004 demonstrated anti-PPRV-specific antibodies in samples collected in 2004, indicating that PPR was likely present in Tanzania before it was officially confirmed following diagnostic evaluation in 2008 [35]. The presence of small ruminant diseases that can be considered in differential diagnosis, including bluetongue, contagious caprine pleuropneumonia, Orf disease, capripox and foot and mouth disease, often overlap in syndromic evaluation, leaving laboratory diagnosis as the only mechanism to diagnose PPR [28,30,31]. In addition, secondary infection caused by *Pasteurella multocida* and *Mannheimia haemolytica* can also complicate syndromic diagnosis [32]. Molecular confirmation of the occurrence of PPR in PPR-free zones and endemic settings is critically important for the accurate diagnosis and confirmation of outbreaks [33].

To enable the successful eradication of PPR, rapid diagnosis remains a cornerstone for the implementation of control measures, including vaccination, quarantine and possible stamping out [36,77]. The wide host susceptibility of species to PPR presents logistic challenges around sampling, diagnosis and diagnostic protocols need to be adequately validated for atypical species affected, and the type of sample being collected [78–80]. The application of Oxford nanopore MinION sequencing technology demonstrated it to be an effective and rapid option for the molecular sequencing of PPRV. Importantly, the sequencing protocol described has been developed and implemented for the sequencing of PPRV in the Molecular Virology Laboratory at Sokoine University of Agriculture in Tanzania as part of this study. This enhanced capability in an endemic setting has demonstrated that Oxford nanopore MinION sequencing is a viable option for minimally equipped diagnostic facilities in low-income and resource-constrained countries. Going forward, the Oxford nanopore MinION sequencer is likely to be an important tool in providing rapid and in-depth genomic information of circulating PPRV strains during the eradication programme. Critically, it will play a significant role in defining PPR outbreaks by enabling the detection of cryptic foci, as well as demonstrating virus circulation in areas where inadequate vaccine deployment may have occurred.

5. Conclusions

Two complete genomes of lineage III PPRV from Tanzania field isolates were generated for the first-time using Oxford nanopore MinION, in our Molecular Virology Laboratory at the Sokoine University of Agriculture in Tanzania. The development of this protocol in a resource-limited setting has demonstrated its utility for PPRV and other viral pathogens where full-genome sequence data acquisition is beneficial. Phylogenetic analysis of the complete genomes revealed a high nucleotide identity with lineage III PPR viruses currently circulating in neighbouring countries (Kenya, Burundi and Uganda), indicating a common

origin. There is a very strong likelihood, >72%, that the lineage III viruses currently circulating in East Africa spread from the Ethiopia 1994 outbreak as the most likely origin. However, the coverage depth was uneven across the genome, with amplicon dropout at the GC-rich region and genome termini. A larger field study is required to enable the collection of sufficient data to assess the robustness of the nanopore sequencing technology and to validate the protocol.

Author Contributions: Conceptualization, E.K. and G.M.; methodology, E.K., G.M., M.R.M., M.M., S.M., F.N., R.K. and S.P.; software, E.K., M.M. and S.P.; validation, R.K., S.P., G.M., S.O. and S.M.; formal analysis, E.K., C.T. and G.M.; investigation, E.K., T.K. and M.R.M.; resources, M.R., S.P., F.N. and G.M.; data curation, E.K., M.M., C.T. and G.M.; writing—original draft preparation, E.K.; writing—review and editing, M.M., T.K., M.R.M., C.T., F.N., S.O., S.M., R.K., S.P., M.R. and G.M.; supervision, G.M., S.M., R.K., S.O. and S.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the fact that we used laboratory achieved PPRV positive samples that were previously screened for PPR from different outbreaks in Tanzania.

Data Availability Statement: The nanopore datasets generated during this research are publicly available at the NCBI GenBank (accession numbers: MW960272 and MZ322753).

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CHAPTER THREE

3.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

3.1 General discussion

Peste des petits ruminats is spreading at an alarming rate across Africa, the Middle East and Asia, causing epidemics in both domestic and multiple wildlife species, despite of the presence of effective vaccines (Zhao *et al*., 2021). The presence of wide host range for PPR presents serological diagnostic and control challenges in multi-host environment (Fine *et al*., 2020). In resource-constrained settings especially in Africa where PPR is endemic, an access to an affordable sequencing technology, therapeutics and diagnostics are limited. This research reports an integration of diagnostic tools for PPR, establishment of nanobodies and complete genome nanopore sequencing of PPRV. It creates an important knowledge base as it registers cost-effective diagnostic and therapeutic material and methods for inclusion in PPR research in the context of planned GEP.

Integration of diagnostic tools is vital for prompt and accurate diagnosis of PPR. Disease diagnosis is one of the key pillars in any disease control and eradication programme. Since the Abidjan Conference in 2015 for PPR GEP, researchers have given the highest priority for diagnosis of PPR in domestic sheep and goats. The presence of a broad host range that is susceptible to PPRV infection, poses diagnostic challenge in testing both small ruminants and wildlife. Research on integration of PPR diagnostic tools in this study has elucidated the practicality, strength and weakness of existing PPR diagnostic spectrum. As a result, cost-effective and robust technologies such as nanobody Ablynx and nanopore

sequencing were proposed and later implemented in this study and elsewhere (Kinimi *et al*., 2020).

A proof-of-concept experimental investigation yielded proficient nanobodies against native PPRV antigens that could enable the development of diagnostics and possibly therapeutics in the future. For diagnostic use, the reactivity against the N or H protein will be sufficient, but for a therapeutic application, the virus neutralisation based on the surface glycoproteins H or F reactivity must be available as previously described (Baron *et al*., 2014). Thus, further studies need to be conducted to translate PPRV reactive nanobodies into diagnostic and possibly in therapy due to their relatively low cost of production.

Sheep and goats have a lower value per head compared with large ruminants, hence sheep and goat keepers may be less likely to invest in disease control given its higher relative cost. For instance, extremely expensive antivirals (small interfering RNAs) were successfully developed in 2005, at International Cooperation Centre for Agronomic Research and Development (CIRAD). These three synthetic siRNAs were able to inhibit over 80% of the *in vitro* replication of the PPRV, but they have not been approved for use due to high cost of their production (Liu *et al*., 2015). Consequently, a cost-effective therapeutic reagent is needed. However, the advances in mRNA vaccine technology during COVID-19 pandemic in humans may accelerate the use of siRNA (Anand and Stahel, 2021; Pardi *et al*., 2020). Alternatively, neutralizing nanobodies against PPRV may be an ideal candidates, for therapeutic development, as recent demonstrated for coronavirus disease of 2019 (COVID-19) (Huo *et al*., 2021). For example, trivalent nanobodies were able to neutralize both the original variant of the COVID-19 virus and the alpha variant that was first identified in Kent, UK. The tetravalent nanobodies chain was able to neutralize the beta variant first identified in South Africa. Once successful and approved,

COVID-19 nanobodies could provide an important treatment option around the world, as they are easier to produce than human classical antibodies and do not need to be stored in cold storage facility.

The development of PPR diagnostics and therapeutics tools rely on nucleotide sequencing of the virus. Such technology is often available in PPR OIE reference laboratories. The OIE reference laboratories for PPR include; CIRAD (Montpellier, France), the Pirbright Institute (Surrey, UK), and CAHEC (China Animal Health and Epidemiology Center, Qingdao, People's Republic of China) (Njeumi *et al*., 2020). For instance, currently available full genomes of PPRV from East African field isolates were sequenced outside the country of origin. The Kenyan and Ugandan PPRV isolates were fully sequenced at CIRAD and TPI, respectively (Table 1). The Tanzanian PPRV isolates (Tanzania/Dakawa 13a and 13b) were sequenced at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala (Torsson *et al*., 2020). However, the Burundian PPRV isolate (B3) was first confirmed by partial sequencing at the African Union-Pan African Veterinary Vaccine Centre (AU-PANVAC) laboratories following a joint investigative mission by the African Union-Interafrican Bureau for Animal Resources (AU-IBAR), AU-PANVAC and the East African Community (EAC) (Niyokwishimira *et al*., 2019). This was followed by complete genome sequencing at Animal Production and Health Laboratory (Seibersdorf, Austria). In this study, cost-effective and rapid nanopore sequencing technology was proposed and deployed for complete genome sequencing of PPRV.

Nanopore sequencing devices are portable, cost-effective and do not require equipped laboratory. However, the longest untranslated intergenic region that is rich in guanine-

cytosine (GC) content in PPRV genome impedes polymerase chain reaction (PCR) and affects the downstream complete genome nanopore sequencing (Torsson *et al*., 2020). In this study, a PCR method for unlocking the GC-rich region between matrix and fusion genes of PPRV was developed. As result, two complete genomes of PPRV were generated within four hours of sequencing in a single run.

The complete genomes generated by this sequencing technology comply to molecular epidemiology sequencing standards, that “Coding complete”, which means 90–99% of the genome is sequenced with no gaps (all open reading frames are generated). The sequences have the same length as all of the other PPRV genomes sequenced to date and are in agreement with the ‘‘rule of six’’ for paramyxoviruses. The organization of the genome is the same as those described previously with transcription units for the N, P(C/V), M, F. H and L proteins. The use of this genomic data becomes vital in understanding virus circulation, the distribution of different virus clades, and the differing roles these might play in the epidemiology of the disease in the field and direct appropriate vaccination response.

Phylogenetic analysis of the complete genomes revealed a high nucleotide identity with lineage III PPR viruses currently circulating in neighbouring countries (Kenya, Burundi and Uganda) indicating a common origin (Muniraju *et al*., 2014; Niyokwishimira *et al*., 2019; Dundon *et al*., 2020). A very strong likelihood >72% that the lineage III viruses currently circulating in the East Africa originated from Ethiopia 1994 outbreak in the absence of further materials. However, the coverage depth was uneven across the genome, with amplicon dropout at the GC-rich region and genome termini. A larger field study is

needed to allow the collection of sufficient data to assess the robustness of the nanopore sequencing technology.

3.2 Conclusions and recommendations

3.2.1 Conclusions

(i) Experimental investigation generated proficient nanobodies against native PPRV antigens as new material for inclusion in PPR research. This could enable the development of cost-effective diagnostic and therapeutic nanobodies in the future.

(ii) On other hand, a PCR method was established, that led into complete genome sequencing of PPRV for the first time in our laboratory and elsewhere in African research laboratories.

3.2.2 Recommendations

(i) The study of structure-function relationships of PPRV reactive nanobodies is necessary for diagnostic and therapeutic applications.

(ii) A larger study is required to enable the collection of sufficient data to assess the robustness of nanopore sequencing protocol. Thus, the development and use of cost-effective and robust diagnostic and therapeutic tools could be of additional value in the Global PPR Control and Eradication Strategy.

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