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Identification of African swine fever virus proteins that activate T-cell immune responses

By

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Submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Veterinary Tropical Diseases in the Faculty of Veterinary Science, University of Pretoria

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DEDICATION

I dedicate this work to the most important people in my life, which are; Angelinah Tshelane Mabetlela, Thato Fortunate Seabi, King Tlou Lethabo Mabetlela and Samuel Thapelo Mabetlela, who give me the energy, the reason and the encouragement to never give up, even in stormy weathers.

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DECLARATION

I, Freddy Mokadi Mabetlela, hereby declare that this dissertation, which is submitted for the degree Master of Science (Veterinary Science), in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, at the University of Pretoria (UP), is my work and has not been submitted by me for a degree at this or any other tertiary institution.

……………………………………………………… 28 September 2018

Signature Date

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------In time, He always provides------

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LIST OF ABBREVIATIONS

Abp1	Auxin binding protein 1
ADF-H	Actin depolymerizing factor homology
AEC	Animal Ethics Committee
APC	Antigen presenting cells
ARC	Agricultural Research Council
ASF	African swine fever
ASFV	African swine fever virus
BCA	Bicinchoninic acid
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary Deoxy-ribonucleic acid
CO_2	Carbon dioxide
Con A	Concanavalin A
CSF	Classical swine fever
Ct	Threshold cycle
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine C-X motif ligand
ΔΔCT	Delta delta threshold cycle
DAFF	Department of agriculture, forestry and fisheries
DC	Dendritic cells
dH_2O	Distilled water
DNA	Deoxy-ribonucleic acid
DMSO	Dimethyl sulphoxide
Dpi	Days post infection
DTTP	Deoxythymidine triphosphate
DUTP	Deoxyuridine triphosphate
dUTPase	Deoxyuridine triphosphatase nucleotidohydrolase
EDTA	Ethylene diamine tetra acetic acid
EEC	European Economic Community
EK	Enterokinase
ELISA	Enzyme linked immunosorbent assay

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ELISpot	Enzyme linked immunospot
ER	Endoplasmic reticulum
F	Fragment
FAO	Food and agriculture organization of the United Nations
FAT	Fluorescent antibody test
FBS	Foetal bovine serum
FCS	Foetal calf serum
GAPDH	Glyceraldehyde -3- phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBsAg	Hepatitis B surface antigen
HBS	Hanks balanced salt
HIP55	HPK1-interacting protein of 55 kDa
HP	His-patch
HPK1	Hematopoietic progenitor kinase 1
HRP	Horse-radish peroxide
HRPO	horseradish peroxidase substrate
IDT	Intergraded DNA Technologies
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAC	Lactose
LACZ	Lactose section Z coding for β-galactosidase on the lactose operon
LAK	Lymphokine-activated killer cells
LB	Lysogeny broth/ Luria broth
LEW	Lysis equilibration wash buffer
MAB-BIOTIN	Biotinylated monoclonal antibody
mAbp1	Mouse/ mammalian actin binding protein one
MAB-PO	Monoclonal antibody horseradish peroxidase
MAL	Malawi

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MARA	Ministry of Agriculture and Rural Affairs
MGF	Multigene family
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NEG	Negative
NF κβ	Nuclear factor kappa beta
NH	Non haemadsorbing
NI-IDA	Nickel ion- iminodiacetic acid
NK	Natural killer
Nm	Nanometres
NSP	Non-structural protein
OAZ1	Ornithine decarboxylase antizyme 1
OD	Optical density
OIE	Office International des Épizooties
ORF	Open reading frame
OVR	Onderstepoort Veterinary Research
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDNS	Porcine dermatitis and nephropathy syndrome
pi	Post infection
POS	Positive
pp	Polyprotein
qPCR	Quantitative real time polymerase chain reaction
RBC	Red blood cells
RBS	Ribosomal binding site
RNA	Ribonucleic acid
RPL-27	Ribosomal protein L27
RPMI	Roswell park memorial institute medium
RSA	Republic of South Africa
RT	Reverse transcription
SA	South Africa
SDS	Sodium dodecyl sulphate

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SGE	Salivary gland extracts
SH3P7	Mouse/ Mammalian actin binding protein (mAbp1)
SHA	*Salmonella* H-antigen
SOC	Super optimal broth with catabolite repression
Spmc	Spots per million cells
S-LA I	Surface Leukocyte antigen
SUMO	Small ubiquitin modifier
TAD	Transboundary animal diseases
TAP	Transporter associated with antigen processing
TBE	Tris-borate-ethylenediaminetetraacetic acid buffer
TCR	T cell receptors
TEMED	Tetramethylethylenediamine
Temp	Temperature
Th0	Naïve T helper cells
Th1	T helper 1
TNFα/β	Tumor necrosis factor alpha or beta
TOPO	Topo isomerase
UGA	Uganda
UP	University of Pretoria
USA	United States of America
UV	Ultra violet
V5	Variable epitope 5
VNT	Viral neutralization tests
WAHID	World animal health information database

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SUMMARY

Identification of African swine fever virus proteins that activate T-cell immune responses

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Co-Supervisors:
Dr Alri Pretorius
Dr Juanita van Heerden

African swine fever (ASF) is a highly contagious haemorrhagic disease of domestic pigs, which affects wild boar as well and for which there is currently no commercial vaccine. African swine fever virus (ASFV), the causal agent of ASF infects warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus)*, giant forest hogs and members of the soft tick genus (*Ornithodoros moubata* complex) without causing disease. Virulent isolates of this virus causes mortality in domestic pigs within seven to ten days post infection, leading to negative economic consequences. Vaccination attempts using different strategies only protect against homologous strains with inadequate protection against heterologous strains of ASFV. The immunological characterisation of ASFV proteins could assist in the development of an effective vaccine against ASF. The aim of this study was therefore to immunologically characterise a selected range of ASFV proteins (p22, CD2V, pp220 fragments (F)1-F4, pS273R, pA104R, pE165R, pF334L, pK205R and pL11L) by identifing the cytokines they induce in peripheral blood mononuclear cells (PBMC) from a pig that was infected with ASFV via tick feeding (pig 1) and an incontact ASFV infected pig (pig 2). The benefits of identifying immunilogical characteristics of the cytokines can lead to possible vaccine development and decrease of animal losses due to ASF.

Several proteins (p22, pp220 F1-4, pS273R, pA104R, pE165R and pK205R) from the genotype II ASFV isolate MAL/11/02 were successfully expressed using a pET102/D-Topo isomerase enzyme (TOPO)® bacterial expression system. Plasmids with gene inserts were amplified using the Invitrogen *E. coli* TOP10 cloning cells and proteins were expressed in BL21 DE3 cells using isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The protein product of genes EP402R (CD2V), pF334L and pL11L did not express and were excluded from futher experiments. The expressed proteins

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were purified by affinity chromatography using Nickel columns with affinity to the His-tag on the recombinant proteins. The immunological assays, interferon gamma (IFN)-γ enzyme-linked immunospot (ELISpot) and cytokine [Interleukin (IL)-2, IL-4, IL-8, IL 10, IL-12, IFN-γ and IFN-alpha (α)] reverse transcription (RT) quantitative real time polymerase chain reaction (RT-qPCR), were conducted using PBMCs. PBMC was collected from pig 1 after ten days post infection (pi) with the genotype XIX ASFV isolate RSA/12/15 strain of ASFV and pig 2 ten days after co-habitation with pig 1.

Only four of the nine recombinant ASFV proteins produced significant cytokine levels. The recombinant p22 protein up-regulated cytokines IL-2, IL-4, IL-8, IL-10 and IFN-γ in pig 1 and IFN-α in pig 2. Fragment four (F4) of polyprotein (pp) 220 up-regulated IL-4, IL-8, IL-12 and IFN-α in pig 1 and IFN-α in pig 2. Recombinant proteins pA104R up-regulated IFN-α in pig 1 and pS273R significantly down-regulated IFN-α in pig 1. In conclusion, the study revealed that ASFV recombinant proteins p22 and pp220-F4 induced all the study cytokines which are important in antiviral immunity. This data can be applied in future ASFV vaccinology studies.

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Chapter 1

1 LITERATURE REVIEW

1.1 Introduction

African swine fever (ASF) is endemic in sub-Saharan Africa and has spread to several European countries as well as the Caribbean and Brazil (Wilkinson, 1984; Costard *et al*., 2013). ASF is a notifiable disease as described by the Office International des Épizooties (OIE), 2015). Disease outbreaks are controlled through the slaughtering and insinuration of infected animals as well as decontamination processes (Manzano-Román *et al*., 2012). Prevention methods include tick control and strict animal movement restriction policies (Manzano-Román *et al*., 2012).

There is currently no commercialised vaccine against ASF. Vaccination attempts by using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, detergent-treated infected alveolar macrophages, Deoxy-ribonucleic acid (DNA) vaccines, naturally occurring virions, genetically modified virions or cell culture-adapted African swine fever virus (ASFV) did not induce protective immunity (Coggins, 1974; Forman *et al*., 1982; Kihm *et al*., 1987; Mebus, 1988; Krug *et al*., 2015). African swine fever virus infection in wild pigs is asymptomatic with low viral loads (Plowright *et al*., 1969). Domestic pigs surviving infections from low virulent ASFV isolates have been shown to gain immunity to subsequent challenges with related virulent isolates (Oura *et al.,* 2005). Neutralizing antibodies against structural proteins of ASFV p30, p54 and p72 have been described but offers no cross protection (Zsak *et al*., 1993; Borca *et al*., 1994; Gómez-Puertas *et al*., 1996). Furthermore, it has been shown that domestic pigs pre-exposed to an ASFV strain of low virulence like OURT 88/3 are protected from the parental virulent ASFV strain OURT 88/1 (Oura *et al.,* 2005). In the same study, domestic pigs without cluster of differentiation (CD) 8^+ T lymphocytes were no longer fully protected from parental virulent ASFV strain OURT 88/1 challenge. This highlights the importance of $CD8^+$ T cell immunity in ASF (Oura *et al.,* 2005).

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1.2 Virology

African swine fever virus (ASFV) has an icosahedral structure and is currently the sole member of the *Asfarviridae* family (Dixon *et al*., 2005). There are other similar double stranded DNA virus families with an icosahedral structure for example the *Herpesviridae* and *Iridoviridae* families (Sogo *et al*., 1984; González *et al*., 1986). *Poxviridae* is another family of large DNA viruses with a single linear segment of DNA. African swine fever virus does not fit with any of these families mainly because its genome structure is similar to that of Poxviruses but its structure is similar to that of *Herpesviridae* and *Irridoviridae* (Sogo *et al*., 1984; González *et al*., 1986). The icosahedral structure of ASFV is an assembly product of more than 50 proteins, including those with structural functions and contains packaged enzymes in the viral core (Simón-Mateo *et al*., 1997). The mature particle of ASFV contains all enzymes required for early Ribonucleic acid (RNA) synthesis and processing (Simón-Mateo *et al*., 1997). These viral particles can be found in all body tissues including fluids, but are more numerous in blood (Abworo *et al.,* 2017). When in blood, ASFV infects the pig's monocytes (Franzoni *et al*., 2017). Once cells are infected, replication of the virus DNA is initiated in the host cell nucleus after which the virus particles are assembled in the cytoplasm (Schmid *et al*., 2014). Four classes of Messenger ribonucleic acid (mRNA) have been identified as those expressed immediately, early, intermediately and late (Almazan *et al*., 1992, Broyles and Knutson, 2010). The required enzymes for ASFV DNA replication are expressed in the intermediate stage from partially uncoated core particles using pre-packaged resources such as enzymes (Salas *et al*., 1981; Almazan *et al*., 1993). Viral morphogenesis occurs in the perinuclear areas called viral factories where a late replication occurs (Galindo and Alonso, 2017). Virus maturation occurs when DNA is encapsulated (Stefanovic *et al*., 2005) after which the virus acquires an additional envelope as it buds out of the cell (Breese and De Boer, 1966).

1.3 Structure of African swine fever virus

ASFV particles are around 200 nm in diameter (Viñuela, 1985) and consists of more than 30 different structural proteins (Carrascosa *et al*., 1984). Starting from the centre of the virus to the surface (Figure 1.1), each virion consists of a linear double stranded DNA genome contained at the core of the virus known as the nucleoid (Galindo and

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Alonso, 2017). The nucleoid is enclosed by the matrix (Andrés *et al.*, 1998), a thick protein layer, which contains the mature protein products derived from polyprotein pp220 and pp62 (Andrés *et al.*, 2002a). The polyproteins pp220 and pp62 are processed into major structural proteins p150, p37, p35, p15 and p14 (Simón-Mateo *et al.*, 1993 and 1997). A membrane termed the matrix shell in turn covers the matrix protein (Galindo and Alonso, 2017). The matrix shell is surrounded by two membranes, the immediate membrane is called inner membrane two and is followed by inner membrane one as shown in Figure 1.1 (Andrés *et al.*, 1998). This is followed by the inner lipid envelope and thereafter, the outer icosahedral capsid (Carrascosa *et al.*, 1984). The inner lipid envelope is acquired during budding (Breese and De Boer, 1966; Carrascosa *et al.*, 1984; Andrés *et al.*, 1997). The cellular membranes are asymmetric in structure, with one face exposed to the cytosol and the other exposed to either the luminal or the extracellular space (Stephens and Compans, 1988; Pettersson, 1991). The viral membranes are formed from the host cell and adopt its topology (Griffiths and Rottier, 1992).

The inner envelope is derived from the endoplasmic reticulum (ER) cisternae (Andrés *et al.*, 1997). The viral particle at this stage therefore consists of three lipid membranes and is surrounded by capsid proteins (Andrés *et al.*, 1997). The capsid is formed by protein sub-units arranged in a hexagonal lattice (Andrés *et al.*, 1997). Amongst others, the p72 is a major capsid protein (López-Otín *et al.*, 1990). The capsid of ASFV does not have transmembrane domains (García-Escudero *et al.*, 1998). Lastly, the outer icosahedral capsid is covered by the outer lipid envelope as shown in Figure 1.1 (Andrés *et al.*, 1998). Viral membranes engulf the core materials while acquiring a polyhedral shape through the assembly of the outer capsid layer (Galindo and Alonso, 2017).

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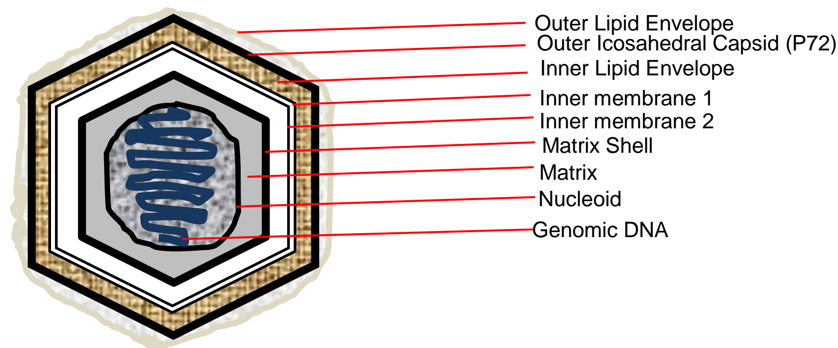

Figure 1.1: The structure of ASFV, Adopted from: Andrés *et al*, 1998.

1.4 African swine fever transmission

African swine fever can be transmitted through direct contact with infected animals, indirect contact through aerosols and through infected tick vectors (*Ornithodoros Spp*) (Vial *et al*., 2007). Soft ticks (*Ornithodoros moubata* complex) can remain infected with this virus for many years and transmit the virus to other ticks through sexual, trans-ovarial and transstadial transmission (Costard *et al*., 2009). Stable flies (*Stomoxys calcitrans*) have been experimentally proven to transmit the virus within 24 h after feeding from a reservoir (Showler *et al*., 2015). The natural reservoirs of ASFV are warthogs (*Phacochoerus africanus*) and bush pigs (*Potamochoerus porcus*) (Oura *et al*., 1998). Infections are generally asymptomatic in warthogs, bush pigs, giant forest hogs, collared peccary and white lipped peccary (Wilkinson, 1984). African swine fever virus is further circulated amongst hosts through an intermediate enzootic cycle in domestic pigs and *O. moubata* ticks, which serve as both vector and reservoir of ASFV (Wilkinson, 1984). Wild boars are also susceptible to ASFV (Wilkinson, 1984). Research with salivary gland extracts (SGE) of *Ornithodoros porcinus domesticus* has shown that pigs pre-exposed to *O. porcinus* ticks SGE develop increased hyperthermia and macrophage recruitment as well as delaying macrophage draining to the lymph nodes, thus enhancing local ASFV spread in pigs (Bernard *et al*., 2016). Although several transmission mechanisms have previously been outlined for ASFV, the main routes of transmission were found to be direct contact with ASFV infective materials and consumption of contaminated pig feeds (Guinat *et al*., 2016).

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1.5 Clinical symptoms of African swine fever

The clinical signs of ASF can be seen from five to 19 days after contact with infected pigs and within five to seven days of exposure to infected ticks (Sánchez-Vizcaíno *et al.*, 2015). Depending on the viral strain and the host immune system, ASF can be sub-acute, per-acute, acute, or chronic (Gallardo *et al.*, 2017). In sub-acute ASF, fever may persist or fluctuate for up to 20 days during which the host may develop symptoms or remain asymptomatic (Sánchez-Vizcaíno *et al.*, 2015).

The mortality rate in this disease form may vary between 30-70% (Sánchez-Vizcaíno *et al.*, 2015). The lesions in this form of ASF are milder in comparison to acute ASF (Sánchez-Vizcaíno *et al.*, 2015). Peracute cases of ASF occurs with few lesions and sudden death. Acute ASF is characterised by a high fever of 42°C, anorexia, sub-cutaneous haemorrhage, inactivity, breathing difficulty, loss of appetite, often massive mortality, lethargy, weakness and recumbence (Gallardo *et al.*, 2017). In white pigs, erythema is the most common symptom (Spickler *et al.*, 2010). Other noticeable symptoms are abdominal pain, constipation, shortness of breath, vomiting, nasal and conjunctival discharges, skin or mucoid to bloody diarrhoea, neurologic signs as well as cyanotic skin blotching on the ears, lower legs, tail or hams (Spickler *et al.*, 2010). Death mostly occurs from 7 to 10 days post infection in acute ASF (Spickler *et al.*, 2010) and lesions are usually found in this phase of the disease (Carrasco *et al.*, 1996). The morbidity rate approaches 100% in naïve herds and the mortality rate ranges from zero to 100% depending on the virulence of the viral strain and the susceptibility of the host (Wilkson, 1984). Most animals infected with ASFV die from pulmonary oedema and shock which is characterised by disseminated intravascular coagulation with multiple haemorrhages in all tissues (Villeda *et al.*, 1993). Programmed cell death of T and B lymphocytes occurs in lymphoid organs (Carrasco *et al.*, 1996) and endothelial cells in arterioles and capillaries of the swine host (Gòmez-Villamandos *et al.*, 1995) because of ASFV infection.

Pigs with the chronic ASF can have sporadic low fever, appetite loss and depression (Sánchez-Vizcaíno *et al.*, 2015). The signs may be limited to weight loss and stunting in some animals. The development of respiratory problems, swollen joints as well as

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coughing is common, and diarrhoea and occasional vomiting have been reported for other pigs.

1.6 Pathogenesis

Haemorrhagic fever is characterized by pathologic death of lymphocyte subsets and haemostatic impairment with consequent immunological function impairment (Blome *et al*., 2013). Tumor necrosis factor alpha (TNF-α), produced by virulent ASFV infected macrophages has been found to be elevated in pig serum and other pig tissues (Del Moral *et al*., 1999; Salguero *et al*., 2008). African swine fever virus pA238L inhibits activation of several immune-modulatory genes, including pro-inflammatory cytokines and chemokines at transcription level by inhibiting the activation of Nuclear factor kappa beta (NF kβ) and calcineurin phosphatase (Powell *et al*., 1996; Miskin *et al*., 1998) and the host transcriptional co-activator p300 (Granja *et al*., 2009). Inflammatory chemokines promote leucocyte migration to injured and infected cells (Nourshargh and Alon, 2014). These chemokines activate immune cells to initiate a response and begin wound healing (Baggiolini, 1998; Moser and Loetscher, 2001; Johnston and Butcher, 2002; Turner *et al*., 2014).

1.7 Epidemiology

The first recorded outbreaks of ASF were reported in pigs belonging to European settlers in Kenya in the year 1914 (Montgomery, 1921) and the first report of ASF from West Africa was from Senegal in 1978 (Penrith *et al*., 2013). In South Africa the first record of ASFV was in 1926 in the former Transvaal province (now Gauteng), followed by another outbreak that occurred in 1933 in the Cape Province (Boshoff *et al*., 2007). The outbreak of African swine fever (ASF) that occurred in Gauteng in 2012 was the first in more than 50 years to occur outside of the ASF control area. Investigations conducted by provincial veterinary services suggested that the sale and movement of infected animals caused the dissemination of disease across provincial borders. In 2016/2017, outbreaks of ASF were reported in North West, the Free State and Northern Cape (another outbreak occurred in 2018). Although investigations into the source of these outbreaks are still ongoing, preliminary indications are that cross-

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border movement of animals from neighbouring countries may have contributed to the introduction of the diseases into the affected areas.

In the years 2013-2015, severe outbreaks of ASF were reported to have occuered in 6 provinces of Zambia (Simulundu *et al*., 2018). Genetic characterization of ASF viruses (ASFVs) using standardized genotyping procedures revealed that genotypes I, II and XIV were associated with these outbreaks (Simulundu *et al*., 2018). Zimbabwe, had its first outbreak after 22 years, a genotype II ASF virus, in the year 2015 (Van Heerden *et al*., 2017).

The ASFV spread outside Africa for the first time to Lisbon, Portugal in 1957 through infected pork products (Wilkinson, 1984). There after, the disease was reported in Lisbon for the second time in 1960 and remained endemic in Spain and Portugal until the mid-1990's (Wilkinson, 1984). By 1978, ASF had spread to Sardinia, Malta and Brazil (Botija, 1982; De Paula Lyra *et al*., 1986). In 1980, ASF had managed to reach the Caribbean (Wilkinson, 1984; Giammarioli *et al*., 2011). The spread of ASFV reached Madagascar in 1998 (Rowlands *et al*., 2008; Rahimi *et al*., 2010). The disease was reported in Armenia, Georgia and Russia in 2007 (Costard *et al*., 2009). African swine fever virus was introduced to Iran in 2008 (Rahimi *et al*., 2010). The spread of ASF has extended to the Caucasus and the Russian federation (Figure 1.2) Food and agriculture organisation of the United Nations Food and agriculture organization of the United Nations (FAO), 2012; Jori *et al*., 2013). Except in cases where wild host are involved, ASFV eradication in a country can take around 30 years with a considerable amount of financial expenditure and herd loss (Geering *et al*., 2011). A few outbreaks of ASF have been recorded in Poland, Eastern Europe in June 2016 (Popiołek, 2016). This is following 76 cases of ASF recorded in wild boar and three outbreaks in domestic pigs in Poland since August 2015. However, only one case of ASF has been reported in August 2016 (Popiołek, 2016). In the year 2017, ASFV outbreaks have occurred in Czech Republic and near Warsaw (Stokstad, 2017). In the European Unions, ASFV outbreaks occurred in Latvia and Lithuania (European Commission, 2017).

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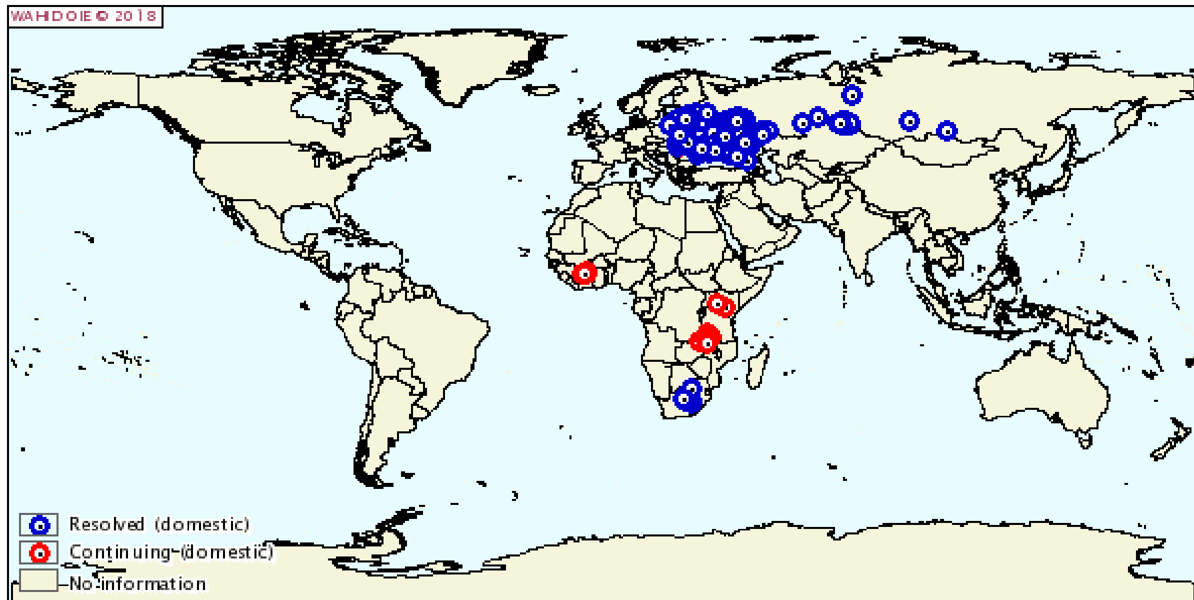

Figure 1.2: Distribution of ASF (Source: Office International des Épizooties (OIE) World animal health information database (WAHID), 2018).

The ASF disease in Belgium is currently seen in wild boar but remains well under control (Te Beek, 2018). In December 2018, carcasses of dead wild boar have been found outside the control zone near Gérouville, a village that is roughly 3 km from the French border (Te Beek, 2018). Another recent outbreak occurred in Shanxi province in Jincheng City in China, a farm with over 8 000 pigs infected with ASFV in mid-November 2018 (Te Beek, 2019b). In the second week of January 2019, a large herd of ~69 000 pigs were reported to have been infected with ASFV in Jiangsu province of China (Te Beek, 2019a).

1.8 Infection by African swine fever virus

The virus replicates in cells of the mononuclear phagocytic system, macrophages, reticular-endothelial cells in lymphoid tissue and organs of domestic swine (Gòmez-Villamandos *et al*., 1995). The virus enters the cell by receptor-mediated endocytosis (Alcamí *et al*., 1989) or micropinocytosis (Sánchez *et al*., 2012). This was confirmed by electron microscopy analysis that showed that ASFV enters susceptible Vero cells and swine macrophages by endocytosis (Geraldes and Valdeira, 1985; Alcamí *et al*., 1989, 1990). The virus is released into the cytoplasm of the cell by a mechanism that involves fusion of the virus envelope with that of an endocytic vesicle (Valdeira *et al*., 1998). The virus will then replicate within the cell and progeny virions are released to infect new cells (Bernard *et al*., 2016). The virus circulating in blood can be transmitted

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to ticks when they feed on infectious pigs and naïve pigs can be infected when an infectious tick feeds on them (Bernard *et al.*, 2016). Cell death caused by apoptosis occur in endothelial cells in arterioles and capillaries of swine hosts (Gòmez-Villamandos *et al.*, 1995).

1.9 Diagnosis

The diagnosis of ASF is an extensively researched subject and multiple diagnosis methods using multiple sample types with different principles have been developed. However, sensitive, reliable and specific diagnosis methods are required when it comes to the iagnisis of ASFV because it is often confused with classical swine fever (CSF) (Kleiboeker, 2002) and porcine dermatitis and nephropathy syndrome (PDNS) (Chae, 2005). The samples used in the diagnosis of ASFV includes whole blood in ethylene diamine tetra acetic acid (EDTA), serum, tissue homogenates, cell culture supernatants, lymph nodes, kidney, tonsils, spleen and tick materials (Gallardo *et al.,* 2013). African swine fever virus can be detected in the laboratory using various methods including: the diagnosis of genomic DNA by quantitative real time polymerase chain reaction (qPCR), conventional polymerase chain reaction (PCR), enzyme linked immunosorbent assay (ELISA), haemadsorption test, virus isolation in pig monocytes, indirect fluorescent antibody (IFA) testing as well as immunoblotting and fluorescent antibody test (FAT) (Bastos *et al.*, 2003; McKillen *et al.*, 2010). Genomic-based methods such as qPCR, conventional PCR and isothermal assays are used for ASFV detection (McKillen *et al.*, 2010) since serologic methods like ELISA's can be time consuming and may require a waiting period until the number of samples are enough to use a full plate (Chang *et al.*, 2012; Musso and La Scola, 2013). Quantitative real time polymerase chain reaction, conventional PCR and isothermal assays are the preferred methods used for ASFV diagnosis (Bastos *et al.*, 2003; Heather *et al.*, 2010; McKillen *et al.*, 2010).

Methods such as haemadsorption techniques have difficulties when it comes to non-haemadsorbing and non-cytopathic strains of ASFV (Gonzague *et al.*, 2001). Contemporary approaches for both diagnosis and genotyping of the 24 major genotypes known are based on the analysis of three independent conserved regions located in the middle of ASFV genome (Thoromo *et al.,* 2016). The three regions

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include the C-terminal end of genes *B646L*, *E183L* and *B602L* (Thoromo *et al.,* 2016). The central variable region of the *B602L* gene is mostly used for epidemiological purposes.

The ASFV is genotyped by amplifying and sequencing the *B646L* gene that codes for the p72 protein (Thoromo *et al.,* 2016) and constructing a phylogenic tree using reference *B646L* gene sequences from different known genotypes, however, genotypes are not distictly linked to origin of the samples as described section 1.7. Virus genotyping and phylogenetic analysis are not limited to a single gene such as *B646L* in the case of ASFV (Thoromo *et al.,* 2016).

1.10 Prevention and control

Different countries have respective although similar strategies that they employ in controlling ASFV. The fact that there is no commercialized or effective vaccine against ASFV and that the natural reservoirs of ASFV cannot be eliminated, causes the disease to become more difficult to control (Costard *et al*., 2009).

ASFV transmission to susceptible hosts can be avoided by preventing contact between the source of the virus and susceptible animals (Bellini *et al*., 2016). This includes movement restrictions, isolation, and aseptic disposals of infected carcasses by burning or deep burial of infected tissue materials, dis-infection of the premises and dis-infection of the materials used in handling the exposed materials (Bellini *et al*., 2016). In farms where ASFV has been detected, all pigs in that holding are culled under competent official supervision (Brown and Bevin, 2018). Animal tissues that can transmit the virus including muscles, semen, ova and embryo are prevented to move from the holding without written permission from competent authorities such as European Commission (EC), 2015 in the European union and the Department of agriculture, forestry and fisheries (DAFF) in SA (Section 20 of the SA animal diseases Act 35 of 1984). In addition to these control measures in South Africa, ASF is being controlled by restriction of animal movement within well-defined boundary lines (Magadla *et al*., 2016), prevention of contact between warthogs and domestic pigs and ensuring that infected pigs as well as material do not leave the area (Penrith and Vosloo, 2016).

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Acaricides are often used in tick population control thus indirectly controlling the virus spread (Manzano-Román *et al*., 2012). Educating the famers about ASFV and tick control is one of the ways in which ASF can be prevented (EC, 2015). Tracing surveillance helps in determining the aetiology and epidemiology of the disease (EC, 2015). The transmission from country to country can be prevented by import control and disposal of waste food in ships and aircraft from infected countries (European commission, 2015). Other measures involve hygienic piggeries and heating contaminated materials at 60°C for 30 minutes (EC, 2015).

1.11 General immunology

The immune response is composed of a network of organs, cells and proteins in association to defend the host against pathogens such as yeasts, bacteria, fungi, viruses, proteins, prions and chemicals (Craft, 2016; Abbas *et al*., 2017). Pathogens will first encounter the first line immune response that mainly refers to the animals physical barriers that seeks to prevent pathogenic agents to gain entry into the host. This includes the skin, mucus, mucous membranes, enzymes in tears, saliva, stomach acid and urine (Crow, 2016). Pathogens that penetrate the physical barriers face the second line host defence (Craft, 2016). Here, phagocytes (macrophages, neutrophils and immature dendritic cells (DC)) internalise a pathogenic agent through phagocytosis (Abbas *et al*., 2017). Monocytes and macrophages in blood, skin and other tissues can kill pathogens (Firestein, 2016).

Lymphocytes are all the white blood cells including B cells, T cells, Natural killer (NK) cells and monocytes (Tina and John, 2016). The adaptive immune system consists of mature B and T cells (Macleod *et al.,* 2014). B cells are antibody-producing cells (Bedoui and Greyer, 2014). Soluble antigens bind to B cells through their receptors and this activates these cells to produce antibodies against specific pathogens (Bedoui and Greyer, 2014; Sim *et al.,* 2016). Pathogens internalised through phagocytosis and proteins formed from pathogen degradation are presented on the surface of the antigen presenting cells (APC) via Major histocompatibility complex (MHC) class I and II to T lymphocytes (Craft, 2016; Crow, 2016; Firestein, 2016; Abbas *et al*., 2017). Protein antigen recognition by T cells require pathogen peptide fragments to be presented at the cell surface of APC by major histo-compatibility complex (MHC) class

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I or MHC class II molecules (Mantegazza *et al.*, 2013) for intracellular (endogenous) and extracellular (exogenous) peptides, respectively (Waldmann and Lefkovits, 1984).

In the endogenous pathway, proteins originating from pathogen replicating within cells are cleaved by proteasomes within proteolytic organelles into peptide fragments of approximately 20 amino acids (Blum *et al.,* 2013). These fragments are transported into the lumen of the ER through the transporter associated with antigen processing (TAP) complex, where they combine with MHC class I molecules and are transported to the cell surface (Ritz and Seliger, 2001). Antigens that are presented by MHC class I molecules are recognised by $CD8^+$ T cell receptors (Salter *et al.*, 1990).

In the exogenous pathway, pathogens or soluble protein components of the pathogen are phagocytosed into the cytoplasm of professional APCs (Mantegazza *et al.*, 2013). The antigens are processed within a series of endosomes that later combines with vesicles containing MHC class II molecules (Hohl, 2015). The $CD4^+$ T cells recognise antigens that are presented by MHC class II molecules on APCs surface membrane (Mantegazza *et al.*, 2013).

Dendritic cells (DC) infected with virus can use the endogenous pathway to degrade viral proteins to be presented by MHC class I (Gallo and Galluci, 2013). Alternatively, viral antigens phagocytosed by DC can be presented on MHC class I via cross-presentation (Mantegazza *et al.*, 2013). Cross-presentation allows for the delivery of exogenous antigens that are processed in DC by the exogenous pathway after phagocytosis to be presented by MHC I without requiring that the pathogen be of intracellular origin (Mantegazza *et al.*, 2013).

Virus killing mechanisms can be achieved by antibodies through a process called neutralization, where antibodies bind to viral particles thus preventing the particle from infecting a host cell (Braciale and Hahn, 2013). Agglutination, a process whereby virus-antibody complexes are grouped together, will activate phagocytosis (Braciale and Hahn, 2013). This happens when the FC portions of the antibody-virus complexes binds to the FC receptors on the surface of phagocytes and trigger phagocytosis (Laing, 2017). Alternatively, antibodies can activate the complement system (Laing, 2017). The complement system opsonises and promotes phagocytosis. The

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complement system leads to the destruction of the virus cell by making perforations within the phospholipid bilayer envelope (Bernet *et al.,* 2003). Binding of complement to foreign substances is influenced by opsonins and chemoattractants (Braciale and Hahn, 2013). Opsonins are factors that enhance phagocytosis of foreign immune invasive particles while chemoattractants are factors that recruits immune cells to areas of inflammation (Laing, 2017).

Circulating mature and immature T lymphocytes binds to antigens presented by APC (Macleod *et al.,* 2014; Bedoui and Greyer, 2014; Tina and John, 2016; Sim *et al.,* 2016). As part of the innate immune response macrophages and neutrophils recognise pathogen associated molecular patterns via their toll like receptors and subsequently activate other pathways of the immune response (Mogensen, 2009). Cytotoxic T cells and NK cells attack infected cells (Cook *et al.,* 2014). The main difference between cytotoxic T lymphocytes (CTL) and NK cells are that CTL falls under adaptive immune response thus recognise specific antigens complexed with MHC and respond to infection while NK cells falls under the innate immune response (Paust *et al.,* 2010). The latter do not recognise antigen, and thus non-specifically kills pathogens or infected cells (Iwasaki and Medzhitov, 2015). Natural killer cells bridge the innate immune response with adaptive immune response by producing cytokines and binding to non-specific or protein bound antigens (Sompayra, 2012). These specialised effectors of the innate immune system have cytotoxic properties in addition to producing cytokines that induce adaptive immune responses and destroys target cells by cell mediated cytotoxicity (Macleod *et al.*, 2014; Bedoui and Greyer, 2014; Tina and John, 2016; Sim *et al.*, 2016).

Many types of T cells such as helper T cells, cytotoxic T cells, γδ T cells and regulatory T cells play important roles in the immune system (Pennock *et al.*, 2013). These cells have proteins on their surfaces called T cell receptors (TCR) that recognise pathogen specific antigens that are complexed to MHC I or II on the surface of APC (Delves, 2006). Helper T cells secrete cytokines that regulate or assist in activating other systems of the immune response. Memory T cells live long term after infection has been cleared and result in an enhanced effector T cells response upon re-exposure to the same antigen (Sompayra, 2012). Regulatory T cells maintain the immune

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response by suppressing over active immune responses (Bedoui and Greyer, 2014; Macleod *et al.*, 2014; Da Silva *et al.*, 2014; Sim *et al.*, 2016; Tina and John, 2016).

Viruses are intracellular parasites, this implies that viral particles are not exposed to some of the immune surveillance such as antibodies that are in the extracellular fluid (Braciale and Hahn, 2013). The cell has therefore developed a system that allows it to signal cytotoxic T cells about its infection through MHC class I viral antigen presentation (Laing, 2017). Cytotoxic cells will release cytokines and cytotoxic granzymes after making contact with infected cells (Braciale and Hahn, 2013).

Interferons directly prevent viral replication within the host cell and signal nearby immune cells of viral invasion (Laing, 2017). Interferons will activate cells, resulting in increased expression of MHC class I molecules on their surface so that cytotoxic T cells can identify and eliminate viral infected cells (Braciale and Hahn, 2013). Cytotoxic cells have granules stored within them that are released only when the cytotoxic cell is exposed to infected cell (Mantegazza *et al.*, 2013). The granules contain perforins and granzymes (Mantegazza *et al.*, 2013). Perforins make pores on the cell membrane of the infected cell and allows granzymes to enter the cell through the pores to induce programmed cell death (apoptosis) (Mantegazza *et al.*, 2013; Braciale and Hahn, 2013; Laing, 2017).

The combination of antigen phagocytosis by DC and danger signal encounter is required for DC maturation and migration to secondary lymphoid organs (Gallo and Galluci, 2013). In the lymphoid organs, matured DC can activate naïve T cells (Th0) (Gallo and Galluci, 2013). The activation of $CD8^+$ T cells as well as polarisation of $CD8^+$ T cells towards CTL requires presentation of MHC class I peptide complexes, co-stimulation and the presence of cytokines like IFN-α as well as IL-12 (Kalinski *et al.*, 1999; Schiavoni *et al.*, 2013). Antigen-specific TCR on the T cell surface, specifically the α/β chains of the $CD3^+$ complex interacts with the appropriate peptide presenting MHC complex (Mantegazza *et al.*, 2013). Post activation differentiation of the $CD4^+$ Th0 results into functional subsets called T helper (Th) 1 and Th2 cells based on the produced cytokines (Trinchieri *et al.*, 2003). The IL-12 and IFN-γ are important cytokines that results in Th0 differentiation to Th1 T cells while IL-4 results in Th0 differentiation to Th2 T cells (Kaplan *et al.*, 1996; Trinchieri *et al.*, 2003). Although the

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divisions are not absolute as there is considerable overlap or redundancy in function between different T cell subsets, Th1 $CD4^+$ T cells produce IL-2, IFN-γ and TNF-α/β while Th2 $CD4^+$ T cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Mantegazza *et al*., 2013; O'Garra, 1998; Seder and Ahmed, 2003).

The Th1 subset has been found to be responsible for cell-mediated effector mechanisms and offer protection against numerous intracellular infections while the Th2 subset has been found to partake in the regulation of antibody production as well as protective immunity against specific extracellular infections (Biedermann *et al*., 2004). The $CD8^+$ T cell effector mechanism has been found to operate through IFN-γ and TNF-α production as well as cytolytic mechanisms (Mantegazza *et al*., 2013; Seder and Ahmed, 2003). The Th1 cells are crucial for intracellular pathogen elimination and plays a role in autoimmunity. Activation of Th1 cells can lead to cell division, lymphokine secretion by the T cell and expression of T cell antigens in the activated state (Biedermann *et al*., 2004). Alternatively, in the case of CTL, interaction with antigen through the specific TCR leads to destruction of target cells (Anikeeva and Sykulev, 2011).

1.11.1 Cytokines

Cytokines were originally discovered in the 1950's (Bernheim and Kluger, 1976). They are small glycoproteins synthesised by a broad range of cells like leukocytes (Khan, 2008). These proteins modulate and regulate the immune and pathological responses as well as the formation, physiological functions, development and differentiation of immune cells (Duque and Descoteaux, 2014). The secretion of cytokines is mostly brief and self-limited (Khan, 2008). Cytokines affect the physiology of cells and have effects on other cytokines (Khan, 2008). Cytokines bind to specific receptors on the target cell that respond by either mRNA or protein synthesis resulting in a specific physiological response (Khan, 2008). Cytokines of specific interest to the current project are discussed in the following paragraphs.

Interleukin (IL)-2 is produced by T cells and modulates the growth, activation, proliferation, maturation and differentiation of naïve lymphocytes into effector cells (Waldmann and Tagaya, 1999) and induces cellular immunological memory

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(Sakaguchi *et al*., 1995; Thornton and Shevach, 1998). It facilitates the production of immunoglobulin through B cell activation (Waldmann and Tagaya, 1999; Waldmann, 2006). Furthermore, IL-2 plays a role in removing self-reactive T cells (Waldmann, 2006) and stimulates cytotoxic cells and macrophage phagocytosis (Khan, 2008).

The IL-4 is produced by Th2 cells, NK cells, mast cells, basophils and eosinophils (Khan, 2008). The IL-4 stimulates Th2 cell proliferation and stimulates naïve B cell differentiation into mature plasma cells (Khan, 2008). The IL-4 up-regulates MHC class II production on resting B cells. In mice, IL-4 decreases the production of Th1 cell through the inhibition of IFN-γ production by macrophages and DC (Khan, 2008). The IL-4 also induces the production of IL-10 (Khan, 2008).

The IL-8 or Chemokine C-X motif ligand (CXCL)8 is an innate immune response cytokine and plays a major role in the pro-inflammatory response (Fishbourne *et al*., 2013). The CXCL8 attracts neutrophils, NK and T cells to the site of infection (Fishbourne *et al*., 2013).

The IL-10 is an anti-inflammatory cytokine (Scheller *et al*., 2011). It is well known for its inhibitory activity on IFN-γ production by Th1 cells (Kabeya *et al*., 2007). The IL-10 is secreted by macrophages, Th2 cells and mast cells (Mollazadeh *et al*.,2017). The IL-10 inhibits IL-2, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (Hashimoto *et al*., 2001). The IL-10 further limits IL-12 production by monocytes and increases the ability of IL-18 to stimulate NK cells (Khan, 2008). The IL-10 stimulates B cell activity and promotes antibody production (Vilanova *et al*., 1999).

The IL-12 is produced by macrophages and B cells and targets cytotoxic T cells, NK and lymphokine-activated killer cells (LAK) and functions in differentiation of Th0 into Th1 cells (Hsieh *et al*., 1993; Khan, 2008). The IL-12 stimulates T cell maturation so that it can perform its function, one of which is to produce IFN-γ (Liu *et al*., 2005). The IL-12 reduces IL-4 mediated suppression of IFN-γ (Temblay *et al*., 2007). Administration of IL-12 increases cytolytic activity and secretion of TNF-α (Cho *et al*., 1996).

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The IFN-γ, the only type II interferon is produced by activated T lymphocytes of CD8$^+$ phenotype, CD4$^+$ Th1, NK, mast, dendritic, professional APC and NKT cells (Kambayashi *et al*., 2003). This cytokine is known to induce pathogen clearance (Tau and Rothman, 1999). The IFN -γ promotes cytolytic T lymphocyte, NK, and macrophage activity (Martin, 2016). The production of this cytokine is regulated by IL-12 and IL-18 (Khan, 2008). This cytokine induces apoptosis as well as Th1 immune responses (Nakagome *et al*., 2009). Some of the important roles of IFN-γ include elevation of antigen presentation and lysozyme activity while inhibiting viral replication, and IL-4 induced isotype switching (Khan, 2008; Fishbourne *et al*., 2013).

The IFN-α is produced by leukocytes and serves as an inhibitor of viral replication in infected cells and up-regulates MHC I (Gil *et al*., 2008).The IFN-α possesses anti-tumour activity and it has been shown to stimulate antiviral immunity by cytotoxic lymphocytes and NK cells (Gil *et al*., 2008).

1.12 Immune response to ASFV

Immune responses to ASFV encompass cellular as well as humoral components (Lokhandwala *et al.,* 2016). The ASFV infect and replicate in soft ticks of the *Ornithodoros sp*, bush pigs of the *Potamochoerus sp.* and warthogs of the *Phacochoerus sp* (Björnheden, 2011). In these species, few clinical signs are observed if present and chronic infections do establish (Wilkinson, 1984). Hence, the development of a protective immune response is suspected to occur in ASFV's natural hosts (warthog and bush pig) (Costard *et al.,* 2009). The development of a protective immune response has been shown to occur in domestic pigs that had recovered from infection when challenged with a homologous isolate (Malmquist, 1963). Research has further shown that ASFV induce neutralizing antibodies in domestic pigs (Gómez-Puertas *et al*., 1996).

Passive immune transfer experiments using anti-ASFV immunoglobulins demonstrated an important role of protective antibodies in immunity in that ASFV antibodies alone might protect pigs against lethal infection (Wardley *et al*., 1985; Onisk *et al*., 1994). These studies have shown that viral proteins *E183L*/p54, *K205R*, *A104R*/histone-like and *B602L*/CVR can elicit strong total immunoglobulin G (IgG)

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antibody responses while protein *K205R* stimulate immunoglobulin M (IgM) production (Reis *et al.,* 2007). Proteins *NP419L*/DNA ligase, *CP312R*, *B646L*/p73, *K196R*/thymidine kinase and *K205R* were found to elicit high antibody titres in animals developing lesions (Reis *et al.,* 2007). Antibody response to *A104R*/histone-like protein was found to be higher in asymptomatic pigs than in chronically infected pigs, implicating an effective or protective immune response (Wardley *et al*., 1985; Onisk *et al*., 1994). Fourteen serological epitopes of ASFV have been characterised by exhaustive screening of a representative lambda phage Complementary Deoxy-ribonucleic acid (cDNA) expression library of the tissue culture-adapted Ba71V strain of ASFV (Kollnberger *et al*., 2002). These included six proteins encoded by open reading frames (ORF's) *B602L*, *C44L*, CP312R, *E184L*, *K145R* and *K205R*, structural proteins *A104R*, p10, p32, p54, p73 and non-structural proteins RNA reductase, DNA ligase and thymidine kinase (Kollnberger *et al*., 2002). Furthermore, Zsak *et al*. (1993) and Ruiz-Gonzalvo *et al.* (1986) have demonstrated an *in vitro* antibody-mediated reduction of infectivity of the virulent ASFV isolates in both Vero cell cultures and swine macrophages; however, the complete neutralization of the virus was not achieved (Ruiz-Gonzalvo *et al*., 1986; Zsak *et al.,* 1993). In 2016, Lokhandwala's group demonstrated ASFV antigen specific antibody and cellular immune responses when adenovirus-vectored ASFV multi-antigen chimera of p32, p54, pp62 and p72 was used as a trial vaccine (Lokhandwala *et al.,* 2016). Lokhandwala's group observed an antibody response that undergoes isotype switching within a week and increases over a period of two months with rapid recall upon a subsequent boost (Lokhandwala *et al.,* 2016). In 2017, Lokhandwala's group studied seven more ASFV antigens (Lokhandwala *et al.,* 2017). The antigens included *A151R, B119L, B602L, EP402R∆PRR, B438L, K205R* and *A104R* (Lokhandwala *et al.,* 2017). In this study, Lokhandwala's group demonstrated antigen-specific IFN-γ responses with all the antigens under their study when using adenovirus vectors (Lokhandwala *et al*., 2017).

The presence of ASFV-specific CTLs has been demonstrated in 1993 (Martins *et al*., 1993). Research shows that ASFV-specific CTLs are able to lyse infected macrophages with a non-haemadsorbing isolate (Martins *et al*., 1993). Oura *et al.* (2005) has shown experimentally that in pigs pre-exposed to the non-virulent ASFV OURT 88/3, those with $CD8^+$ T cells were fully protected against the virulent ASFV OURT 88/1 challenge and those without $CD8^+$ T cells were no longer fully protected

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from OURT 88/1 challenge. This highlights the importance of $CD8^+$ lymphocytes in protective immune response against ASFV and highlights that anti-ASFV antibodies alone from OURT 88/3 immunisation is not sufficient to protect pigs from OURT 88/1 challenge (Oura *et al.*, 2005). It was also observed that inbred pigs of the CC haplotype pre-exposed to the OURT 88/3 ASFV were not always protected against the virulent ASFV OURT 88/1 challenge (Oura *et al.*, 2005). In this case, viremia as well as fever have been witnessed (Oura *et al.*, 2005). Oura *et al.* (2005) also observed that elevated viremia was always correlated with elevated numbers of circulating $CD8\beta^+$ T cells resulting in decreased viremia and hence protection. This emphasizes the importance of $CD8^+$ T cells in ASF immune response.

Other studies have described high levels of IFN-γ production by ASFV-immune lymphocytes stimulated *in vitro* with ASFV (Revilla *et al.*, 1992) and elevated NK cell activity during infection (Leitáo *et al.*, 2001). Golding *et al.* (2016) shown that the sensitivity of type I IFNs are linked to the multi-gene family (MGF) 360 and MGF 505 as virulent ASFV with MGF 360 and MGF 505 induced biologically active IFN *in vivo* when viruses such as OURT 88/3 not having these MGF's did not induce biologically active IFN *in vivo*. Oura *et al.* (2005) has experimentally found the NK cells levels to be very low compared to the combined number of $CD8\beta^+$, $CD4^+$ and $CD8^+$ lymphocytes. However, the immune response to ASFV is not yet fully characterised and remains to be explored.

1.13 ASF vaccine development

The development of a successful ASF vaccine is significantly hindered by insufficient knowledge on the virus and the complexity of virus-host interactions in infected pigs and the immune response to ASFV (Neilan *et al.*, 2004). Furthermore, knowledge on protection mechanisms of ASF is not sufficient and most of the experimental evidence is out dated (Schlafer *et al.*, 1984; Onisk *et al.*, 1994). Vaccination attempts against ASF by using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, detergent-treated infected alveolar macrophages, DNA vaccines, naturally occurring virions, genetically modified virions or cell culture-adapted ASFV did not induce protection (Coggins, 1974; Forman *et al.*, 1982; Kihm *et al.*, 1987;

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Mebus, 1988; Krug *et al*., 2015). However, a live attenuated ASFV that contains engineered deletions of specific virulent ASFV or pathogen range genes were reported to only protect against homologous virus (Lewis *et al*., 2000). Domestic pigs pre-exposed to ASFV strain of low virulence like OURT 88/3 are protected from the parental virulent ASFV strain OURT 88/1 with lack of cross protection as well (Oura *et al*., 2005).

Viral neutralizing epitopes have already been identified on structural proteins of ASFV p30, p54 as well as p72 (Zsak *et al*., 1993; Borca *et al*., 1994; Gómez-Puertas *et al*., 1996). Neutralizing antibodies against p30, p54 and p72 have been described to confer protection but offers no cross protection against heterologous strains (Zsak *et al*., 1993; Borca *et al*., 1994; Gómez-Puertas *et al*., 1996). Furthermore, a neutralizing antibody response to p54 and p30 together has been shown to provide partial protection against ASF and significantly modify the disease course, resulting in a delayed onset of the disease to complete protection against virus infection (Gómez-Puertas *et al*., 1998; Barderas *et al*., 2001). Immunization of pigs with p22, p30, p54, and p72 briefly delayed disease onset (Neilan *et al*., 2004). Regardless of the delayed disease onset, no effect on disease development, progression or outcome was observed (Neilan *et al*., 2004). Other proteins that have been studied include attachment protein p12 (Gómez-Puertas *et al*., 1998). Immunisation with p12 has been shown to block specific binding of viral particles to susceptible cells (Angulo *et al.,* 1993).

Research has demonstrated that DNA vaccines were unable to induce neutralizing antibodies against ASFV p30 and p54 proteins and, they might even exacerbate ASFV infection (Argilaguet *et al*., 2011). Two surface leukocyte antigen (SLA) I-restricted $CD8^+$ T cell epitopes have been identified with a putative protection potential (Argilaguet *et al*., 2012). The potential adjuvant effect of *Salmonella* H-antigen (sHA) is currently being explored to improve the immune responses induced against other antigens in pigs (Argilaguet *et al*., 2012). Scientists are also trying to identify as many CTL epitopes as possible within the ASFV polypeptides by using expression library immunization (Lacasta *et al*., 2014) developed in the laboratory from the entire ASFV genome. Research into broader and optimal cellular and humoral responses is being done by testing several strategies of proven efficacy in veterinary medicine, including

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electroporation to improve DNA vaccine delivery (Van Drunen Littel-van den Hurk *et al.*, 2010).

In order to design a good protein, DNA or epitope vaccine, viral proteins that elicit a protective immune response as well as long lasting immunological memory should be used. Unfortunately, the immunological profile of the ASFV proteome has not been generated (Li *et al.*, 2014).

1.14 ASFV proteins important for this study

On average, 170 proteins are coded for by the genome of ASFV (Dixon *et al.*, 2013). These include structural proteins with several known and unknown physical and immunological characteristics as well as non-structural proteins that were reported to induce immune responses in pigs (Rascón-Castelo *et al.*, 2015). Structural proteins are likely to be immunogenic proteins, which activate an antibody response, as they are the first molecules that physically interact with the host's immune system (Goldman and Prabhakar, 1996). Other proteins such as non-structural protein (nsp)1, nsp2, nsp4 and nsp7 may play a pivotal immunological role even if they are not structural proteins (Rascón-Castelo *et al.*, 2015). A selected group of genes, chosen for immune characterisation in this project, are discussed in the section below.

1.14.1 P22

The protein coded for by the gene *KP177R* has been identified as a structural protein of 22 kDa known as p22 (Dixon *et al.*, 2013). This member of the p22 MGF proteins forms part of the ASFV envelope (Dixon *et al.*, 2013). The surface and structural proteins of the virus are most likely to interact with the host immune system during the phase before the onset of the disease (Dixon *et al.*, 2013). The p22 is one of the early structural proteins in the life cycle of ASFV (Camacho and Viñuela, 1991). Induction of an early immune response is ideal for ASF vaccine because virulent ASFV kills domestic pigs within ten days post infection (pi) (King *et al.,* 2011).

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1.14.2 CD2V

The *EP402R* gene codes for the CD2V protein of ASFV (Monteagudo *et al.,* 2017). Expression of CD2V is required for the haemadsorption of erythrocytes around ASFV-infected cells leading to the dissemination of the virus within the host (Ruiz-Gonzalvo and McColl, 1993; Borca *et al*., 1994). The CD2V protein contains a signal peptide, a 147 amino acid cytoplasmic tail and a predicted trans-membrane domain (Craney *et al*., 2011). The extra-cellular section of this protein has been found to contain significant similarities to the CD2 protein of T cells but it contains a unique cytoplasmic section of unknown function (Borca *et al*., 1994). Studies reported that CD2V inhibits mitogen-dependent proliferation of lymphocytes and the deletion of the gene encoding CD2V abrogated the inhibition of peripheral blood mononuclear cell (PBMC) culture (Borca *et al*., 1994).

The cytoplasmic tail of the CD2V protein has been reported to bind to mouse/mammalian actin binding protein (SH3P7), also known as mouse/mammalian actin binding protein one (mAbp1) or hematopoietic progenitor kinase one (HPK1)-interacting protein of 55 kDa (HIP55) which is involved in several cellular functions that includes vesicle transport and signal transduction (Kay-Jackson *et al*., 2004). The SH3P7 protein is a mammalian homologue of the yeast actin binding protein Auxin binding protein 1 (Abp1) (Kay-Jackson *et al*., 2004). It contains a SH3 domain, an actin depolymerizing factor homology (ADF-H) domain (Lappalainen *et al*., 1998) and two tyrosine's, which is phosphorylated after T and B cell receptor (BCR) stimulation (Larbolette *et al.,* 1999; Kessels *et al*., 2000, 2001). The SH3P7 protein is linked with endocytosis, vesicle trafficking through the Golgi and signal transduction (Fucini *et al*., 2002; Warren *et al.,* 2002; Mise-Omata *et al.,* 2003). This CD2V–SH3P7 interaction was identified using the yeast two-hybrid system and was shown by confocal microscopy to co-localize in ASFV infected cells in the cytoplasm around the Golgi network known as the virus factory (Kay-Jackson *et al*., 2004).

The viral CD2 proteins have been demonstrated to be partially resistant to endo-H digestion indicating that they can be transported beyond the endoplasmic reticulum (ER) to the Golgi network (Kay-Jackson *et al*., 2004). This may be important for the activation of cellular immunity since proteins trafficking through the Golgi and

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endocytosis are presented on MHC class I (Kay-Jackson *et al.*, 2004) thus making this protein a good candidate for detection by CD8⁺ T cells. Most importantly, CD2V expression is required for the haemadsorption of erythrocytes around ASFV-infected cells (Ruiz-Gonzalvo and McColl, 1993). The *CD2V* protein has been documented to convey immunomodulatory characteristics as well as reduce the mitogen-dependent proliferation of uninfected lymphocytes when ASFV infection of mononuclear cells occur (Borca *et al.*, 1994).

1.14.3 Pp220

The gene *CP2475L* codes for the polyprotein (pp) 220 (Andrés *et al.*, 2002b). The pp220 undergoes proteolytic processing by enzyme pS273R to form mature viral major structural proteins p150, p37, p14 and p34 (Jia *et al.*, 2017). This polyprotein is an N-myristoylated precursor polypeptide (Jia *et al.*, 2017). The pp220 is localized at the core shell (matrix-like virus domain) between the DNA-containing nucleoid and the inner envelope (Andrés *et al.*, 2002a). Electron microscopy showed that repression of pp220 results in the assembly of an icosahedral particle with no apparent core structure (Andrés *et al.*, 2002a).

1.14.4 PS273R

The *S273R* gene protease contains a “core domain” with the conserved catalytic residues characteristic of small ubiquitin modifier (SUMO)-1-specific proteases and the adenovirus protease (Dixon *et al.*, 2013). This gene codes for a SUMO-1-like protease of around 31 kDa, with a pivotal role of cleaving structural polyproteins that forms the major components of the core shell such as the pp220 and pp60 of ASFV (Dixon *et al.*, 2013). It cleaves the poly-protein pp60, the product of the *CP530R* gene, into mature viral proteins p35 and p15. The SUMO-1 like protease is thought to be a cysteine protease because its activity was shown to be shattered by the introduction of a mutation in the predicted catalytic histidine and cysteine residues (Simón-Mateo *et al.*, 1993, 1997; Andrés *et al.*, 1997). The SUMO-1 like protease is expressed late pi where it localises in the cytoplasmic viral factories associated with virus precursors and mature virions as well as the products of the viral polyprotein (Andrés *et al.*, 2001).

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1.14.5 PA104R

The *A104R* gene of ASFV codes for the p11.6 protein (Dixon *et al*., 2012). This is a histone-like structural and DNA binding protein of around 14.9 kDa in size (Borca *et al*., 1996). The *A104R* recombinant protein induced a substantial antibody response in asymptomatic pigs compared to chronically infected pigs (Borca *et al*., 1996). A chimera of *K205R-A104R* induced IFN-γ post boost when domestic pigs were vaccinated with adenovirus vectored ASFV antigen *K205R-A104R* (Lokhandwala *et al*., 2017).

1.14.6 PE165R

The *E165R* gene is homologous to the deoxyuridine triphosphatase nucleotidohydrolase (dUTPase) (Baldo and McClure, 1999). This protein plays a role in viral replication and it is essential for the organism's viability (Dixon *et al*., 2013). This enzyme catalyses the pyrophosphate cleavage of the substrate deoxyuridine triphosphate (dUTP) into the product deoxythymidine triphosphate (dTTP) affecting the dUTP/ dTTP ratio (Oliveros *et al*., 1999). The protein product of the *E165R* gene shows that the virus dUTPase is trimeric, highly specific enzyme that requires a divalent cation for its activity (Oliveros *et al*., 1999). This enzyme is expressed at both early and late stages of infection and localises within the cytoplasm of host cells (Rodríguez *et al*., 2009). Deoxyuridine triphosphatase nucleotidohydrolase activity is unessential for virus replication in dividing cells but is required for productive infection in non-dividing swine macrophages (Oliveros *et al*., 1999). It is a 21.7 kDa protein important in replication, because it prevents the use of DNA polymerases during replication and repair (Oliveros *et al*., 1999).

1.14.7 PF334L

The *F334L* gene codes for a protein of about 40.4 kDa in size, an RNA reductase protein important in DNA replication (Kollnberger *et al*., 2002). This oxidoreductase enzyme is useful in deoxyribonucleotide diphosphate metabolic processes (Dixon *et al*., 2013).

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1.14.8 PK205R

The protein coded for by the *K205R* gene has not been assigned a function yet, but it has been shown to stimulate IgM production (Gutiérrez-Castañeda *et al.*, 2008). The *K205R* coded protein is transcribed early and translated around four hours pi. The translated protein is around 33 kDa in size (Gutiérrez-Castañeda *et al.*, 2008). This protein has been shown to sustain antibody titres and inhibit IFN-β expression and as a result, it is speculated that the protein may serve as a serological immuno-determinant of ASFV (Correia *et al.,* 2012). The protein diffuses and metastasis within the cytoplasm and subsequently in viral factories late post infection (Gutiérrez-Castañeda *et al.*, 2008). The *K205R* ORF appears to be conserved between viruses isolates (Gutiérrez-Castañeda *et al.*, 2008).

1.14.9 PL11L

The protein coded for by the *L11L* gene may be an integral membrane protein as membrane-spanning segments have been predicted within the protein (Kleiboeker *et al.*, 1998). This protein is expressed late in the virus replication cycle (Kleiboeker *et al.*, 1998). In 1998, the *L11L* gene coding for this protein pL11L was reported to be highly conserved in pathogenic ASFV isolates (ASFV pathogenic tick isolates Chiredzi/83/1, Crocodile/96/1, Crocodile/96/3, Malawi Lil-20/1, Nooitverwacht/96/6, Noord Biabant/96/1, Pretoriuskop/96/4 and Wildebeeslaagte/96/1; pathogenic pig isolates Camaroon, European-70, Kerita, Spencer, Tengani, Uganda'61, Victoria Falls and Zimbabwe; and the pathogenic tissue culture-adapted isolate Dominican Republic 2. Chiredzi/83/1 and Malawi Lil-20/1 isolated from *Ornithodoros spp*. ticks in 1983; Crocodile/96/1, Crocodile/96/3, Nooitverwacht/96/6, NoordBiabant/96/1, Pretoriuskop/96/6 and Wildebeeslaagte/96/1 isolated from *Ornithodoros porcinus* ticks from Republic of South Africa in 1996) from various sources such as African, European and Caribbean ASFV isolates (Kleiboeker *et al.*, 1998). The *L11L* exists either in a short form, encoding a protein of 77-78 amino acids (9±1 kDa) or in a longer form of 93-94 amino acids (11±1 kDa) (Kleiboeker *et al.*, 1998). Furthermore, the deletion of *L11L* gene did not show to affect virus replication in swine macrophage cell cultures or virulence in domestic pigs, indicating that *L11L* gene may not be essential for growth *in vitro* and for virus virulence in domestic pigs (Reis *et al.*, 2017).

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1.15 Justification

African swine fever (ASF) outbreaks mainly affect the resource poor farmers who practise free-range pig farming. Their pigs are mainly kept as a protein source and money reserve as they can be easily converted to cash during emergencies or as a source of household income to pay school fees, hospital bills and traded for draught power. An ASF outbreak thus threatens the communities' food security and livelihood.

Despite all the data available and several attempts at vaccine development, there is currently no effective vaccine against ASF. Attempts to protect animals with inactivated vaccines failed (Stone, 1967; Forman *et al*., 1982; Mebus, 1988). Attenuated vaccines proved protection against homologous virulent virus with rare cross protection obtained (Wardley *et al*., 1985; Boinas *et al*., 2004; King *et al*., 2011; Monteagudo *et al*., 2017). Attenuated viral strains have resulted in biosafety issues because they retain some virulence and produced sub-clinical infections in pigs, and occasionally result in chronic infection (Arias *et al.,* 2017).

The pathogenesis and mode of infection of ASFV are not yet fully understood and there is a huge gap in the immunological characterisation of the ASF viral proteins. However, research has shown that both a cellular and humoral immune response is involved in protection against ASF. Identifying proteins that induce similar immune responses will aid recombinant vaccine development. Cytokines have been described to play a very important role in mediating and regulating the protective immune response to a pathogen and they will therefore be the focus of this project.

The ASFV genome codes for 150 to 167 proteins of which few are characterised immunologically (Jia *et al.,* 2017). The development of an effective vaccine against ASFV may be hindered by the limited knowledge on the immunological nature of these ASFV proteins. Therefore, a broader range of ASFV proteins should be studied in order to add to the current knowledge on the immunological characteristics of ASFV proteins.

Once the proteins with suitable cytokine profiles are discovered, a more investigative study with a larger sample size is desired followed by vaccine candidate epitope

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identification and vaccine trials. The human hepatitis B vaccine is evidence that a successful vaccine can be derived from a pathogen's genes as hepatitis B surface antigen (HBsAg) was successfully used to produce hepatitis B vaccine (Mc Aleer *et al.*, 1984).

The need for a safe and efficacious vaccine to prevent further spread of ASF is imperative. Overall, a vaccine will enhance the control and eradication of the disease in affected countries and therefore contribute to the reduction of the sanitary barriers to trade and the negative impact this disease causes in affected countries.

1.16 Objectives of the current study

1.16.1 Aim

The aim of this study was to identify ASFV recombinant proteins that induce cellular immunity by investigating cytokines (IL-2, IL-4, IL-8, IL 10, IL-12, IFN-γ and IFN-α) induced by PBMC isolated from ASFV infected pigs.

1.16.2 Specific objectives

I. To clone and express ASFV genes *KP177R, EP402R, CP2475L, S273R, A104R, E165R, F334L, K205R* and *L11L*.
II. To investigate the cytokines induced by recombinant ASFV proteins using cytokine qPCR and ELISpot assays.

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Chapter 2

2 MATERIALS AND METHODS

2.1 Selection of virus isolates

A genotype II African swine fever virus (ASFV) isolate from Malawi (MAL) MAL/11/02 and haemadsorbing genotype XIX ASFV isolate from the republic of South Africa (RSA) RSA/12/15 were selected for the purpose of the current study. The ASFV isolate MAL/11/02 led to an outbreak in Malawi (MAL), South-eastern Africa in the year 2011. The MAL/11/02 strain was isolated from infected pig's spleen and passaged once in primary pig bone marrow culture (Boshoff, 2017). The ASFV isolate RSA/12/15 was isolated from ASFV positive ticks. The RSA/12/15 virus has been successfully passaged three times on pig bone marrow culture. The two isolates are both highly virulent in nature. The ASFV MAL/11/02 isolate was selected for gene expression for the purpose of cross-reaction investigations with peripheral blood mononuclear cells (PBMC) isolated from ASFV isolate RSA/12/15 infected pigs (Boshoff, 2017; Raselabe, 2017).

2.2 ASFV DNA isolation

The ASFV DNA template was extracted from ASFV MAL/11/02 using the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) according to manufactures instructions. The isolation of ASFV MAL/11/02 DNA was confirmed by amplifying the partial sequences of the 478 bp ASFV gene B646L with P72 U (5' GGCACAAGTTCGGACATGT 3') and P72 D (5' GTACTGTAACGCAGCACAG 3') primers in a conventional polymerase chain reaction (PCR) (Bastos *et al.*, 2003). Two micro litres of DNA extract was aliquoted into Q5 Hot start 2X master mix (New England BioLabs® Inc, USA) that contained 0.5 µM of the forward primer and 0.5 µM of the reverse primer. The reaction was subjected to 1 cycle of enzyme activation at 98°C for 30 s, followed by 30 cycles of 98°C denaturation for 10 s, 53°C annealing for 30 s, 72°C extension for 18 s and 1 cycle of 72°C final extension for 2 min in a thermal-cycler (GeneAmp® PCR system 9700, Applied Biosystems, USA). The PCR products and a 100 bp GeneScript DNA size marker (Promega, USA) were ran on a 1% agarose gel (Sigma-Aldrich, USA) that contained 2 µg/mℓ ethidium bromide (Promega, USA) in

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Tris-borate-EDTA buffer (TBE) (Sigma-Aldrich, USA) for 30 min at 100 V using an electrophoresis system (Bio-Rad, USA, USA). The gel was viewed under ultra violet (UV) light and captured as a picture using an intergrated camera-UV light viewer system (AlphaImager®HP Alpha, Innotech, USA). The ASFV positive culture of ASFV RSA/12/15 was used as positive DNA extraction control while ASFV Uganda (UGA) UGA/3/95 ASFV isolates DNA was used as positive PCR control and 2 negative controls were used. The two negative controls were Negative DNA extraction control from ASFV negative tissue and Negative PCR control consisting of nuclease free dH_2O (ThermoFisher Scienific, USA).

2.3 ASFV genes selection, primer design and gene amplifications

The ASFV genes *KP177R, EP402R, CP2475L, S273R, A104R, E165R, F334L, K205R* and *L11L* were selected for the current study. Some of the proteins investigated in this study were randomly selected. Others were selected based on factors such as structural, physical and immunological characteristics. These include structural proteins with incomplete immunological profiles such as p22, pp220, pA104R and pL11L (Camacho and Viñuela, 1991; Borca *et al.*, 1998; Larbolette *et al.*, 1999; Kay-Jackson *et al.*, 2004; Dixon *et al.*, 2013), non-structural proteins that were reported to induce immune responses in pigs and those which did not, such as CD2V, pS273R, pE165R, and pF334L (Gutiérrez-Castañeda *et al.*, 2008), proteins of known character but unknown immunological profile such as CD2V, pS273R, pp220, pA104R, pL11L, pK205R, pE165R and pF334L (Dixon *et al.*, 2013). Some of these proteins may play a pivotal immunological role even if they are not structural proteins and were therefore included in this study.

The ASFV MAL/11/02 sequence was not published in GenBank or sequenced therefore a closely related ASFV Georgia 2007/1 (Accession number: FR682468) isolate sequence was used for primer design. Primers were designed for directional cloning into the pET102 Directional cloning vector (Champion™ pET Directional TOPO® expression kit, Invitrogen, United States of America (USA)) in such a way that the forward primer had the nucleotide sequence 5'-CACC-3' before the start of the primed gene. The 5'-CACC-3' nucleotide sequence is complementary to the 5'-GTGG-3' nucleotide sequence on the ligation site of the plasmid. To prevent termination of

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translation of the cloned gene, the reverse primer was designed using the end of the gene omitting the stop codon so that translation extends as far as the six-histidine tags (included in the plasmid) that are later used for protein purifications. Both the bio-edit version 7.2.3 (Hall, 1999) and OligoAnaliser 3.1 software (Intergrated DNA technologies, USA) was used for designing the primers (Table 2.1). These primers were synthesised by Inqaba Biotechnologies (Inqaba Biotec™ Genomic Company, SA). The primers were re-suspended to 100 µM, stored at -80°C, and diluted to 10 µM working stock for gene amplification.

Table 2.1: ASFV genes, amplicon sizes and newly designed primer sequences used for gene amplifications with Intergraded DNA Technologies (IDT) melting temperatures (TM) provided.

GENE	amplicon SIZE (bp)	PRIMER SEQUENCE	TM (˚C)
KP177R	569	**KP177R F:** CACCATG TTT AAT ATT AAA ATG ACA ATT TCT RYA TTG CTT ATT G **KP177R R:** TGC ATG TTT ATG ATT TCT AGG TAA GGC	(55.4) (55.4)
EP402R	1082	**EP402R F:** CACC ATG ATA ATA CTT ATT TTT TTA ATA TTT TCT AAC ATA GTT TTA AG **EP402R R:** AAT AAT TCT ATC TAC GTG AAT AAG CG	(53.4) (51.1)
CP2475L-F1	1920	**CP2475L F1 F:** CACC ATG GGT AAC CGT GGA TCT **CP2475L F1 R:** GAA TCG GCG TTT TAC ATC ATA CTC	(52.9) (54.0)
CP2475L-F2	1920	**CP2475L F2 F:** CACC GTA GAC TGG AAG GCA ACC **CP2475L F2 R:** TGC TGC AGC CGC TTC	(52.6) (56.6)
CP2475L-F3	1920	**CP2475L F3 F:** CACC GAA ATC AAT ATG CGG CTT TCT ATG **CP2475L F3 R:** TTG TTC GCG AGC AAC TAC	(52.6) (53.0)
CP2475L-F4	1850	**CP2475L F4 F:** CACC ATG CCC GGA GTA CAG **CP2475L F4 R:** TAA AAT CCG AAT ATC ACT ATC ATA CTG TTT ATC	(50.7) (53.7)
S273R	821	**S273R F:** CACC ATG TCT ATA TTA GAA AAA ATT ACG TCA AGT CCC TCT GA **S273R R:** TGC GAT GCG AAA CAG ATG GG	(58.7) (58.3)
A104R	314	**A104R F:** CACC ATG TCG ACA AAA AAA AAG CCC ACA ATT ACC **A104R R:** GTT TAA CAT ATC ATG GAC AGG TTT CAA TGC TC	(58.7) (58.0)
E165R	497	**E165R F:** CACC ATG GCA ACA AAT TTT TTT ATT CAA CCT ATC ACC **E165R R:** AGT TCT CAT AAT CCC GGC CTC GCC	(57.3) (62.8)
F334L	1004	**F334L F:** CACC ATR TTA ATA TTT ATT TCA AAT ATG GAG G **F334L R:** AAA ATC ATC GTC GTT TAA AAA G	(47.8) (47.9)
K205R	617	**K205R F:** CACC ATG GTT GAG CCA CGC GAA C **K205R R:** CTT CTT CAT CAT CTC TTT GAC CAT TTT CTT CTG T	(58.7) (58.6)
L11L	361	**L11L F:** CACC ATG TTG GAG CCA ATA TTA GTA ATG GCC **L11L R:** TTT ATT ACA CTT AAC TTG AAA AGA ATT ATT GCC T	(57.2) (54.8)

The genes of MAL/11/02 ASFV isolate were amplified in a total volume of 50 µℓ consisting of 25 µℓ of Q5 hot start high fidelity 2x master mix (New England, Bio Labs®Inc, USA), 2.5 µℓ each of the 10 µM forward and reverse primers (Table 2.1), 2 µℓ of genomic DNA template isolated in section 2.2 and 18 µℓ of nuclease free dH_2O (ThermoFisher Scienific, USA) in a 0.2 mℓ PCR tube (ThermoFisher Scienific, USA). The enzyme was activated at 98°C for 30 sec before 30 cycles of 98°C for 10 sec denaturation, gene specific annealing temperature for 30 sec and 72°C for gene specific extension time period indicated in Table 2.2 in a thermal-cycler (GeneAmp® PCR system 9700, Applied Biosystems, USA). The final extension was performed at 72°C for 2 min.

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Table 2.2: The annealing temperatures and extension times used for ASFV gene amplification.

Gene	Annealing temperature (°C)	Extension times (s)
KP177R	54	30
EP402R	52	32
CP2475L-F1	53	60
CP2475L-F2	58	40
CP2475L-F3	53	60
CP2475L-F4	52	60
S273R	58	60
A104R	58	60
E165R	60	30
F334L	48	120
K205R	58	60
L11L	56	30

Amplified gene PCR products and a 1 kbp plus DNA Ladder (ThermoFisher Scientific, USA) were each loaded into a separate well and ran on a 1% agarose gel (Sigma-Aldrich, USA) that contained 2 µg/mℓ ethidium bromide (Promega, USA) for the purpose of DNA extraction from the gel. Each gel was run in TBE buffer (Sigma-Aldrich, USA) for 40 min at 100 V using an electrophoresis system (BIO-RAD, USA). The gel was viewed under UV light and captured as a picture using a camera (AlphaImager®HP Alpha, Innotech, USA). The negative PCR control of dH_2O (ThermoFisher Scientific, USA) was concurrently ran on agarose gel electrophoresis (BIO-RAD, USA). There was no PCR positive control as the genes were amplified for the first time. Expected amplicon size per gene as indicated in Table 2.1 was derived from the un-amplified gene size and primer-histidine tags size.

2.4 The PCR product cleaning

The gene amplicon was manually excised using a razer blade based on band size from the agarose gel under UV light immediately after electrophoresis and inserted into a 1.2 mℓ microcentrifuge tube (Eppendorf, USA). The Zymoclean™ Gel DNA Recovery kit (Zymo research) was used to clean the gene amplicon according to manufacturer's instructions. A micro litre of the cleaned product was run on 1% agarose gel and vizualised under a UV light to confirm quality of the DNA for

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sequencing. The concentration of the DNA was measured using a Spectrophotometer (NanoDrop™ 1000, ThermoFisher Scientific, USA) and stored at -20°C for a maximum period of 1 month.

2.5 Cloning of ASFV MAL/11/02 genes into pET102 directional cloning vector

Amplified complete genes were ligated into the pET102 directional cloning vector (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA). The materials used included 1 µℓ PCR product, 1 µℓ salt solution (1:4 v/v ratio of salt to dH_2O, (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) diluted in nuclease free dH_2O (ThermoFisher Scienific, USA) at 1:4 volume/volume ratio of salt to dH_2O (ThermoFisher Scienific, USA) dilution, 3 µℓ nuclease free dH_2O (ThermoFisher Scienific, USA) and 1 µℓ of TOPO pET102 directional cloning vector (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) to achieve a final volume of 6 µℓ. The ligation reaction was mixed very gently and incubated at ± 20°C for 30 min, placed on ice or at -20°C for long-term storage. Gene inserts of ≤1 Kbp were adjusted to 5 ng/mℓ while gene inserts of ≥2 Kbp were adjusted to 10 ng/mℓ in the final ligation reaction. Gene inserts between 1 and 2 Kbp were adjusted to 7.5 ng/mℓ in the final ligation reaction. The incubation period of the ligation reaction was extended for up to ±15 h at 4 ℃ for effective cloning. Chemical competent One Shot TOP10 cells (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) genotype [F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG] were used to make TOP10 electro-competent cells using the protocol by Magliery (2001). Two microliters of the transformation reaction was added into a 0.5 mℓ microcentrifuge tube (Eppendorf, USA) with 40 µℓ of TOP10 electro-competent cells and gently mixed before transferring into a 0.1 cm electroporation Pulser cuvette (Bio-Rad, USA). Cells were electroporated at 1.5 kV, resistance at 200 Ω and capacitance at 25 µF using the GenePulser X cell electroporator (Bio-Rad, USA). It was confirmed that the time constant was greater than three using the GenePulser X cell electroporator (Bio-Rad, USA). The super optimal broth with catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose (Sigma-Aldrich, USA)) was added immediately into the cuvette and mixed before transferring it into a 1.5 mℓ microcentrifuge tube (Eppendorf, USA) and

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incubation at 37°C (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA) for 60 min.

One hundred microliters of the transformation reaction were plated onto Luria Broth (LB) agar media (Sigma-Aldrich, USA) plates with the antibiotic ampicillin (Sigma-Aldrich, USA) added at 50 mg/mℓ concentration and incubated at 37°C for 24 h (LABEX and LABCON Shaking Incubators, Air and Vacuum Technologies, SA). To determine which of the colonies contained the plasmid with the correct gene insert, isolated colonies were screened using colony PCR. Before the colonies were transferred into the PCR master mix, they were first transferred onto a reference LB agar media plate (Sigma-Aldrich, USA) with 50 mg/mℓ ampicillin (Sigma-Aldrich, USA) for sub-culturing. Colonies were screened using plasmid primers TrxFus Forward and T7 Reverse (Champion^{TM} pET Directional TOPO$^{®}$ expression kit, Invitrogen, USA) in a conventional PCR setup using GoTaq$^{®}$ Hot Start Green Master Mix (Promega, USA). To screen the colonies for successful cloning, 25 µℓ of PCR mixture consisting of 10 µℓ of nuclease free dH_2O (ThermoFisher Scienific, USA), 12.5 µℓ of GoTaq$^{®}$ Hot Start Green 2x master mix (Promega, USA), 1.25 µℓ per each of the 10 µM forward and reverse primers were subjected to a thermal-cycler (GeneAmp$^{®}$ PCR system 9700, Applied Biosystems, USA) for conventional PCR using the kit cycling conditions (Champion^{TM} pET Directional TOPO$^{®}$ expression kit manual, Invitrogen, USA). The enzyme was activated at 95°C for 2 min before 30 cycles of 95°C for 30 sec denaturation, 53°C annealing temperature for 30 sec and 72°C for 60 sec extension. The final extension was performed at 72°C for 5 min. The PCR products were ran and viewed as previously described in section 2.3.

Clones of known sizes such as *CP2475L F1* RSA/12/15 was used as a positive control. The LB agar plates (Sigma-Aldrich, USA) with 50 µg/mℓ ampicillin (Sigma-Aldrich, USA) from which the colonies were screened were stored at 4°C in a fridge (Samsung, DEFY DAC518 C210 bottom freezer fridge, SA) to prevent over growth during the detection of positive colonies. Positive colonies were cultured in 5 mℓ LB broth (Sigma-Aldrich, USA) with ampicillin (Sigma-Aldrich, USA) and incubated in a shaking incubator at 200 rpm overnight (±15 h) at 37°C (LABEX and LABCON Shaking Incubators, Air and Vacuum Technologies, SA) with the lid loosely closed to allow for air exchange. Positive colonies were transferred from the LB agar plate (Sigma-

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Aldrich, USA) to the LB broth bottle (Sigma-Aldrich, USA) using a pipette tip. Positive colonies were regarded as colonies of band size equivalent to the insert gene size and the amplified plasmid size of 235 bp (Table 2.1). A millilitre of the overnight culture was archived in a 2 mℓ microcentrifuge tube (Eppendorf, USA) containing 1 mℓ 50% autoclave sterilized glycerol (Sigma-Aldrich, USA), mixed and immediately stored at -80°C for future reference. The RSA/12/15 *KP177R* of 804 bp clone was used as a positive control.

2.6 Plasmid isolation and correct clone confirmation

Plasmids were isolated from the overnight TOP10 *E. coli* cell culture using the Invisorb® Spin Plasmid Mini two Kit (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) according to the manufacturer's instructions. The concentration of the DNA plasmid isolated was measured using a Spectrophotometer (NanoDrop™ 1000, ThermoFisher Scientific, USA) and 10 µℓ of the isolated plasmid of ≥ 50 ng/mℓ was sent to Inqaba Biotechnologies (Inqaba Biotec™ Genomic Company, SA) for sequencing and the remaining volume was stored at -20°C for BL21 DE3 *E. coli* cells transformation during protein expression experiments. Sequences were screened for mutations on the T7 promoter of the plasmid, the ribosomal binding site (RBS), HP-thioidoxine, enterokinase, the portion of the Lactose section Z coding for β-galactosidase on the lactose (LacZ) operon before the gene insert, correct reading frame from the start codon of the plasmid ATG until the stop codon across the variable epitope 5 (V5) and the six histidine tags to the stop codon of the plasmid using Sequencher software version 5.2.3 (www.genecodes.com). Only the clones with all the above-mentioned regions still intact and correctly inserted sequence in the correct reading frame were used to transform BL21 DE3 *E. coli* cells.

2.7 Transformation of electro-competent BL21 DE3 *E. coli* cells and gene expression

The proteins coded for by the cloned genes were expressed in BL21 DE3 *E. coli* cells using the Champion™ pET 102 Directional TOPO® Expression Kit (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) according to manufacturer's instructions. Twenty millilitres of the overnight culture was poured into a 1 ℓ flask (Erlenmeyer, USA) with 100 mℓ of pre-warmed LB broth (Sigma-Aldrich, USA)

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containing 1.2 mℓ of 50 µg/mℓ ampicillin (Sigma-Aldrich, USA) and incubated in a shaking incubator (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA) at 200 rpm for 2 h at 37°C. After 2 h incubation, 1.2 mℓ of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Marbach and Bettenbrock, 2012) (Sigma-Aldrich, USA) of 50 µg/mℓ concentration was added into the 120 mℓ culture to induce protein expression and incubated in a shaking incubator (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA) at 200 rpm for an additional 4 h at 37°C. Protein expression was initially attempted with the same method but with the omission of the IPTG induction step.

2.8 Recombinant protein isolation

Expressed ASFV soluble recombinant proteins were isolated by harvesting BL21 DE3 *E. coli cells* (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) from 100 mℓ of LB broth (Sigma-Aldrich, USA), which was divided into two in a 50 mℓ centrifuge tube (Eppendorf, USA), by centrifugation (Eppendorf 5424/5810R, USA) at 16 000 *g* for 35 min at 4°C. Supernatants were thoroughly decanted and pellets resuspended with a plastic streaking loop in 2 mℓ Bug buster reagent (Merk Millipore, SA) until the colour changed from yellow to white or cloudy (Merk Millipore-Bug buster reagent manual, SA). Additional 3 mℓ of Bug buster reagent (Merk Millipore, SA) that contained 25 U/mℓ Benzonase nuclease (Sigma-Aldrich, USA) and 1 kU/mℓ R-lysozyme (Sigma-Aldrich, USA) was added to the 2 mℓ Bug buster reagent (Merk Millipore, SA) and gently mixed (Merk Millipore-Bug buster reagent manual, SA). Equal volumes of the mixture was aliquoted into three microcentrifuge tubes (Eppendorf, USA) of 2 mℓ capacity, which were incubated at room temperature ±20°C on a shaker (LABEX and LABCON Shaking Incubators, Air and Vacuum Technologies, SA) at 270 rpm for 20 min. The three microcentrifuge tubes (Eppendorf, USA) were centrifuged (Eppendorf 5604, USA) at 16 000 *g* for 30 minutes at 4˚C. The supernatants were collected and divided into another two microcentrifuge tubes (Eppendorf, USA) of 2 mℓ capacity and stored at -20ºC.

For recombinant insoluble protein isolation, the pellet was thoroughly re-suspended in 2 mℓ Lysis equilibration wash (LEW) buffer (50 mM NaH_2PO_4 and 300 mM NaCL at pH 8.0) (Protino® Ni-IDA, Macherey-Nagel, Germany) and centrifuged (Eppendorf

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5604, USA) at 10 000 *g* for 30 min at 4°C to wash Bug buster reagent (Merk Millipore, SA) off and other impurities. The supernatants were discarded and the pellet thoroughly re-suspended in 2 mℓ of LEW buffer (Protino® Ni-IDA, Macherey-Nagel, Germany) containing 8 M urea (Sigma-Aldrich, USA). The homogenates were incubated on a shaker (LABEX and LABCON Shaking Incubators, Air and Vacuum Technologies, SA) operating at 270 rpm on ice for 60 min and centrifuged (Eppendorf 5604, USA) at 7 000 *g* for 45 min at 20°C. The supernatants were carefully poured into sterile microcentrifuge tubes (Eppendorf, USA) and stored at -20°C as the insoluble protein isolates.

2.9 Screening recombinant protein isolation with PAGE

The crude, soluble and insoluble portions of each isolated recombinant protein obtained in section 2.8 were run on a 12% Sodium dodecyl sulphate (SDS)-PAGE gel (Bio-Rad, USA) for screening together with the Kaleidoscope™ Precision Plus Protein™ standard (Bio-Rad, USA). The method was used to determine if the protein is in the soluble or insoluble portion of the isolates and if the protein size is correct. The upper portion of the gel called the stacking gel was made up of 5% Acrylamide mix (Sigma-Aldrich, USA), 0.1 M Tris buffer (Bio-Rad, USA) at pH 6.8, 0.1% SDS (Bio-Rad, USA), 0.1% APS (Sigma-Aldrich, USA) and 0.1% Tetramethylethylenediamine (TEMED) (Bio-Rad, USA). The lower portion of the gel called the resolving gel was made up of 12% Acrylamide mix (Bio-Rad, USA), 0.4 M Tris buffer (Bio-Rad, USA) of pH 8.8, 0.1% SDS (Bio-Rad, USA), 0.1% APS (Sigma-Aldrich, USA) and 0.1% TEMED (Bio-Rad, USA). The resolving gel was poured into two glass plates (Bio-Rad, USA) sealed at the bottom with plastic (Bio-Rad, USA) to prevent leaking. Distilled water was slowly added on top of the resolving gel to straighten the resolving gel and allowed to set. Distilled water was poured out of the glasses and was replaced with stacking gel on top of the resolving gel. Combs (Bio-Rad, USA) were inserted into the stacking gel before it set to create wells. Ten microliters of the protein sample was mixed with 10 µℓ of 2X blue protein loading dye (Bio-Rad, USA) and heated in a water bath (Memmert, Lasec, SA) at 95°C for 5 min in order to denature the recombinant protein. The 20 µℓ denatured protein sample was loaded into the stacking gel wells of a 12% SDS-PAGE gel (Bio-Rad, USA) and placed in electrophoresis tank (Bio-Rad, USA) containing SDS running buffer (Tris/ glycine /SDS) containing 25 mM Tris

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(Sigma-Aldrich, USA), 192 mM glycine (Sigma-Aldrich, USA) and 0.1% SDS (Bio-Rad, USA) adjusted to pH 8.3. The gel was ran at constant voltage of 120 V using a power pack (Clever Science, United Kingdom (UK)) until the blue buffer front reached the bottom of the SDS-PAGE gel. The gel was carefully removed from the glass plates into a plastic container (ADDIS, Makro, SA) containing dH_2O of amounts that covered the gel. The gel was subsequently placed into a plastic container (ADDIS, Makro, SA) containing a ready to use Coomassie blue stain (Sigma-Aldrich, USA) of volume that covered the gel and incubated for ±15 h at ±20°C on a shaker operating at 200 rpm (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA). The following morning the gel was de-stainined by replacing Coomassie blue stain with dH_2O on a shaker operating at 200 rpm (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA). The dH_2O was replaced at 10 min intervals in order to obtain optimal results. Positive results were determined by the band size of the protein in relation to the expected band size in reference to the Kaleidoscope™ Precision Plus Protein™ standard (Bio-Rad, USA). The expected protein sizes were calculated as the actual ASFV ligated gene size in kDa and the fusion tags size in kDa of 18.3 kDa consisting of His-patch (HP) thioredoxin, enterokinase (EK) recognition site, V5 epitope and polyhistidine (6xHis) regions (Table 2.1). The gel was viewed and photographed using the Alpha imager instrument (AlphaImager®HP Alpha, Innotech, USA).

2.10 Confirmation of protein expression using western blotting

To confirm if the expressed recombinant proteins contained the His tag, western blotting. Briefly, proteins were ran on 12% SDS-PAGE gel (Bio-Rad, USA) as described in section 2.9 without staining the gel with Coomassie blue stain. Instead, the gel was used to transfer the protein band from the gel to a nitrocellulose membrane. One nitrocellulose membrane and two filter papers were cut to the size of the 12% SDS-PAGE gel (Bio-Rad, USA) using scissors (CNA, SA). The two filter papers and one nitrocellulose membrane were separately incubated for 5 min in a plastic container (ADDIS, Makro, SA) with Tris/Glycine (25 mM Tris and 192 mM glycine) transfer buffer of pH 8.3 in amounts that covered the membrane and the filter papers in order to activate the nitrocellulose membrane and wet the filter papers with the transfer buffer. A cassette was assembled using the negative electrode on plastic

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cover, sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge and positive electrode on plastic cover in that order. The cassette (Bio-Rad, USA) was placed into electrophoresis tank (Bio-Rad, USA) containing 25 mM Tris (Sigma-Aldrich, USA) and 192 mM Glycine (Sigma-Aldrich, USA) at pH 8.3. The gel was ran at constant voltage of 100 V using a power pack (Clever Science, UK) for 1 h. the membrane was carefully removed from the cassette and placed into a plastic container. One percent milk powder (Elite skimmed milk, Clover, SA) in phosphate buffered saline (PBS) (137 mM NaCl, 10 mM PO_4 and 2.7 mM KCl (Sigma-Aldrich, USA)) pH 7.4 was poured into the plastic container (ADDIS, Makro, SA) with the nitrocellulose membrane in amounts that covered the nitrocellulose membrane (Bio-Rad, USA). The plastic container (ADDIS, Makro, SA) with the nitrocellulose membrane and milk was incubated on a shaker with of amounts that covered the nitrocellulose membrane using SDS-PAGE gel (Bio-Rad, USA) electrophoresis tank (Bio-Rad, USA). The membrane was incubated at ±20°C on a shaker operating at 270 rpm (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA) for 60 min. The milk was discarded and 1:1000 anti-his antibody (Novex, UK) in PBS was added at a volume that covers the entire nitrocellulose membrane and incubated at ±20°C on a shaker (LABEX and LABCON shaking incubator, Air and Vacuum Technologies, SA) for 60 min. The membrane was washed 3 times for 5 min with PBS with 0.1% tween 20 (Sigma-Aldrich, USA) in amounts that covered the gel. The membrane was developed using horse-radish peroxide (HRP) until bands develop. Blue bands indicating histidine tag at expected band size in relation to the Kaleidoscope™ Precision Plus Protein™ standard (Bio-Rad, USA) determine a positive reaction. The gel was viewed and photographed using the Alpha imager instrument (AlphaImager®HP Alpha, Innotech, USA). The total band size was calculated to be the actual gene size and the amplified plasmid DNA (235 bp) region flanking the gene insert amplified by both the forward and reverse plasmid primers (Table 2.1).

2.11 Protein purification

Both soluble and insoluble recombinant proteins were purified using the 2000 Nickel ion-iminodiacetic acid (Ni-IDA) columns Protino® Ni-IDA kit (Protino® Ni-IDA, Macherey-Nagel, Germany) according to the manufacturer’s instructions (Macherey-

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Nagel, 2013). Recombinant proteins were allowed to bind to the LEW buffer (8 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl adjusted to pH 8.0) (Protino® Ni-IDA, Macherey-Nagel, Germany) equilibrated columns through poly-histidine tags and were washed twice with 2 mℓ LEW buffer (Protino® Ni-IDA, Macherey-Nagel, Germany). Recombinant proteins were eluted into each of the 5 microcentrifuge tubes of 1.5 mℓ (Eppendorf, USA) with 1 mℓ of elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole at pH 8.0 (Protino® Ni-IDA, Macherey-Nagel, Germany) per tube. The eluates were stored at -20°C for SDS-PAGE analysis. Purification buffer containing 8 M urea (Sigma-Aldrich, USA) was used for purification of insoluble protein and not for soluble proteins.

2.12 Protein precipitation, concentration and measurements

Purified soluble and insoluble recombinant proteins confirmed with western blotting analysis were precipitated using the acetone precipitation method to remove urea, concentration and to promote long-term storage (Simpson and Beynon, 2010). Recombinant proteins were mixed and divided into 200 µℓ volumes in 2 mℓ microcentrifuge tubes (Eppendorf, USA) into which 1 800 µℓ of ice-cold acetone was aliquoted and mixed. The mixture was incubated for 16 h at -20°C and centrifuged (Eppendorf 5604, USA) at 1 000 *g* for 10 min at 4°C. The supernatant was carefully discarded and the pellet was re-suspended in 70% ice-cold ethanol (Merk Millipore, SA) at 4°C and mixed. The suspension was centrifuged at 1 000 *g* for 30 min at 4°C. The supernatants were carefully discarded to prevent discarding the protein precipitates. The precipitates were air dried and stored at -20°C for long-term storage without resuspension. The procedure was carried out on ice. Determination of recombinant protein concentration after resuspension in PBS was executed with Pierce bicinchoninic acid (BCA) protein assay kit (Thermo scientific, USA) according to manufacturer's instructions. The results were analysed using a standard curve analysis and Microsoft excel 2010.

2.13 Experimental infection of animals

This study used cryopreserved PBMC that were isolated from pigs that formed part of a study titled: The role of *Ornithodoros* ticks in maintaining ASFV, previously approved by the Onderstepoort Veterinary Research (OVR) Animal Ethics Committee (AEC)

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(AEC 19.12), the Department of Agricultural Forestry and Fisheries (TADP-S-13/04) and the University of Pretoria (UP) AEC (ECO11-13). Ethical approval for the pig that was used as a control (Clean pig) has also been obtained from OVR AEC 10.12 under the project entitled Diagnosis of Transboundary Animal Diseases. The OVR AEC (AEC 18.15) as well as the UP AEC approved the current study.

Two ASFV naïve pigs were delivered to the stables at Agricultural Research Council (ARC)-OVR-Transboundary Animal Diseases (TAD) and allowed to acclimatise to the environment for seven days. Only pig 1 was sedated with an intramuscular injection of Azaperone (40 mg/mℓ; 1-2 mℓ /20 kg) on day zero before ASFV isolate RSA/12/15 positive *Ornithodoros porcinus* ticks were mounted to feed on the right-side hip of pig 1. The ticks were covered by a round plastic container (ADDIS, Makro, SA) with a 10 cm diameter on the hip of pig 1 to prevent ticks from spreading on the animal's body. Tick feeding on pig 1 was allowed to proceed for a maximum of 30 minutes. The second pig (pig 2) was cohabitated with pig 1 to allow for horizontal transmission of ASFV (Boshoff, 2017). Both pigs were euthanized on day 10 pi. A third pig obtained as part of routine diagnostics at TAD that was naïve to African swine fever (ASF) (pig 3) was used as a control pig. On day 10 pi (termination), about 500 mℓ blood from each of the three pigs were collected by the veterinarian into separate containers with anticoagulant (Heparin) and immediately sent to the laboratory for PBMC isolation.

2.14 Peripheral blood mononuclear cell isolation and cryopreservation

A 37°C Hanks balanced salt-EDTA (HBS-EDTA) solution (Sigma-Aldrich, USA) pre-warmed in a water bath (Memmert, Lasec, SA) was mixed 1:2 with the blood from which the PBMC were isolated. Thirty millilitres of this blood was gently overlaid onto 15 mℓ Ficoll-Histopaque (Sigma-Aldrich, USA) at an angle of 45° in a 50 mℓ centrifuge tube (Eppendorf, USA). The tube was centrifuged (Eppendorf 5424/5810R, USA) immediately at 900 *g* for 40 min at 20°C without brakes. Five millilitres of the white/gold interface (PBMC/ Ficoll interface) was aliquoted into each sterile 50 mℓ centrifuge tube. This was topped with Alsevers buffer (Sigma-Aldrich, USA), gently hand mixed and centrifuged (Eppendorf 5424/5810R, USA) at 600 *g* for 10 min with maximum brakes. The supernatant was discarded and the pelleted cells were re-suspended by gentle and brief vortexing (C-GEN Biotech Vortex mixer, UK). A millilitre of lysis buffer

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(Sigma-Aldrich, USA) was added to the re-suspended PBMC and gently hand mixed for 1 min to lyse the remnant red blood cells (RBC). Thereafter, the PBMC were washed twice with 50 mℓ of Alsevers solution (Sigma-Aldrich, USA) and centrifuged (Eppendorf 5424/5810R, USA) at 300 *g* for 10 min with maximum brakes. The pelleted cells were re-suspended in 1 mℓ of GIBCO® Roswell park memorial medium (RPMI) 1640 (1%) medium (Gibco, Thermo Scientific, USA) that contained 10% foetal calf serum (FCS) (Lonza, Switzerland) and 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA) for cryopreservation and 100 µg/mℓ of streptomycin antibiotic (Lonza, Switzerland) before the PBMCs were counted (Hatton *et al*., 2009). Isolated PBMCs were stored at -80°C at a concentration of $1X10^7$ cells/mℓ (Adopted from: Reimann *et al*., 2000).

2.15 PBMC thawing for immunological assays

The 2 mℓ cryo-vials (Polylab Plastic ware, China) containing PBMC were removed from the -80°C freezer and transfered to a 37°C water bath (Memmert, Lasec, SA). The 2 mℓ cryo-vials (Polylab Plastic ware, China) were occasionally gently flicked during the thawing process for effective thawing. When a small piece of ice remained, the cryo-vials (Polylab Plastic ware, China) were wiped with 70% ethanol (Merk Millipore, SA) and cells were slowly transferred to a 15 mℓ conical centrifuge tube (Eppendorf, USA) containing 1X RPMI 1640 (Gibco, Thermo Scientific, USA) with 10% foetal bovine serum (FBS) (Thermo Scientific, USA) and 1% pen-streptomycin antibiotic (Lonza, Switzerland) pre-warmed to 37°C in a water bath (Memmert, Lasec, SA) in a total of 2X volume of the thawed cells. The cells were centrifuged (Eppendorf 5424/5810R, USA) for 10 min at 300 *g* at ±20°C. The supernatants were discarded and the PBMC were re-suspended in 10 mℓ of PBS pre-warmed to 37°C in a water bath (Memmert, Lasec, SA) in order to thoroughly wash DMSO (Sigma-Aldrich, USA) from the cells. The suspension was centrifuged (Eppendorf 5424/5810R, USA) for 10 min at 300 *g* at ±20°C. The supernatants were discarded and the pelleted PBMC were re-suspended in 1 mℓ of RPMI 1640 (1%) Gibco, Thermo Scientific, USA) that contained 2 mM L-glutamine,10% HyClone foetal bovine serum (Thermo Scientific, USA) and 1% Gibco® pen-streptomycin antibiotic (Life technology, USA) pre-warmed to 37°C in a water bath (Memmert, Lasec, SA) (Reimann *et al*., 2000).

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2.16 Cytokine real time PCR for cytokine mRNA quantification

The cytokine quantitative real time PCR (qPCR) is an assay that measures the fold increase of specific cytokine messenger ribonucleic acid (mRNA) (Qiagen, Netherlands). Transcription is compared between antigen experienced and antigen un-experienced biological conditions in order to investigate the recall immune response experimentally. In this experiment, 2.5 x 10^5 thawed PBMC in a volume of 100 µl/well were stimulated, in a bio-safety cabinet (Labotec, SA), with 20 µg/mℓ of each purified recombinant protein and incubated (Formaz Scientific water-Jacked incubator, ThermoFisher, USA) for 24 h at 37°C in 5% carbon dioxide (CO_2) before isolating total ribonucleic acid (RNA) (Qiagen, Netherlands). Ribonucleic acid (RNA) from antigen stimulated PBMC was isolated using the RNAeasy Plus Universal mini kit (RNAeasy Plus Universal mini kit, Qiagen, Netherlands) with QIAzol lysis reagent (Qiagen, Netherlands) following the manufacturer's instructions (Qiagen, Netherlands). Concanavalin A (Con A) (Sigma-Aldrich, USA) was used as a positive control at 10 µg/well and 20 µg/mℓ of Beta (β)-galactosidase protein (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) was used as background control while RPMI 1640 (Gibco, Thermo Scientific, USA) was used as negative control.

The RNA concentration of the eluates was measured using a Spectrophotometer (NanoDrop™ 1000, ThermoFisher Scientific, USA) and stored at -80°C for Complementary Deoxy-ribonucleic acid (cDNA) conversion. The cDNA conversion was done using QIAGEN® One Step qPCR Kit (QIAGEN® One Step qPCR Kit, Qiagen, Netherlands) following the manufacturer's instructions.

The cytokine qPCR assay was carried out using custom RT² Profiler PCR Array kit (custom RT² Profiler PCR Array kit, Qiagen, USA). The genes coding for the following proteins were used as housekeeping genes (Qiagen) β-Actin, Ornithine decarboxylase antizyme 1 (*OAZ1*), Ribosomal protein L27 (*RPL27*) and Glyceraldehyde -3-phosphate dehydrogenase (*GAPDH*) (Qiagen, Netherlands). The assay investigated the transcription levels of cytokines IL-2, IL-4, IL-8, IL-10, IL-12, IFN-γ and IFN-α. The β-Actin was selected for normalising the data because it had the second lowest coefficient of variation to OAZ1 in comparison with other housekeeping genes. The OAZ1 was not selected for normalisation despite the low coefficient of variance

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because it had late threshold cycle (CT) values (around 30). The RNA from antigen stimulated PBMC's was isolated using RNAeasy Plus Universal mini kit (RNAeasy Plus Universal mini kit, Qiagen, Netherlands) with QIAzol lysis reagent (Qiagen, USA) following the manufacturer's instructions (Qiagen, Netherlands). The cDNA was used as a template for the qPCR assay with the RT^2 SYBR Green Master mix (RT^2 SYBR Green Master mix, Qiagen, Netherlands). The qPCR assay was carried out according to manufacturer's instructions (Qiagen, USA). The results were analysed using the delta delta threshold cycle (ΔΔCT) method (Rao *et al*., 2013).

ΔCT = Ct (cytokine) – Ct (housekeeping gene)

ΔΔCT = ΔCT (PBMC stimulated with antigen) – ΔCT (unstimulated PBMC)

The results were further analysed in Microsoft office 2010 excel by the student T-Test (1 tailed distribution, homoscedastic). Data generated from this study was analysed with tables, graphs, charts, pictures and ΔΔCT calculations method specifically for Custom Porcine Cytokine RT^2 PCR assay. The data was reported as cytokine fold change. Results were considered positive if the fold change induced by the ASFV protein was ≥ 2 times that induced by β-galactosidase control protein. Thereafter, a fold change of ≥ 2 and ≤ 2 to the control pig 3 value was considered as up-regulated and down-regulated, respectively. A result was statistically significant when $p \leq 0.05$ when pig 1 and 2 data was compared to β-galactosidase and/or pig 3 data. The ΔΔCT method is described online as RT^2 Profiler PCR Array Data Analysis Version 3.5 on the following web address:
http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php

2.17 The ASFV p72 antibodies detection enzyme linked immunosorbent assay

The ASF Enzyme linked immunosorbent assay (ELISA) was carried out using INGEZIM PPA Compac R.11.PPA.K3 kit according to the manufacturer's instructions (INGEZIM PPA Compac, Ukraine) (Pourquier and Lesceu, 2011). The INGEZIM PPA Compac R.11.PPA.K3 ELISA was used to screen for ASFV serologic immune response (INGEZIM PPA Compac, Ukraine). Blood was collected into BD Vacutainer™ serum tubes (Thermo Scientific, USA) from all three pigs used in the study on the following days post infection (dpi): 0 dpi, 5 dpi, 6 dpi, 8 dpi, 9 dpi and 10 dpi. The blood was centrifuged (Eppendorf 5604, USA) at 10 000 *g* for 10 min for

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serum. Serum was aliquoted into steile 2 mℓ cryo-vials (Polylab Plastic ware, China) and used in the ASF ELISA assay (INGEZIM PPA Compac, Ukraine). The plate was precoated with ASFV 72 and antibodies to ASFV p72 present in positive serum samples binds to the precoated antigen. The monoclonal antibody horseradish peroxidase (MAB-PO) conjugate is added to bind to p72 antigens that has not complexed with ASFV p72 antibodies in the case of negative samples thus blocking the antigen (INGEZIM PPA Compac R.11.PPA.K3 ELISA kit manual, Ukraine). In positive samples the MAB-PO is unable to bind because the antigen is blocked by serum antibodies to ASFV p72. The ASFV p72-MAB-PO complex is measured through a colorimetric reaction using spectrophotometer (BioTek, SA) after adding horseradish peroxidase (HRPO) substrate (INGEZIM PPA Compac R.11.PPA.K3 ELISA kit manual, Ukraine). Samples were considered positive when the optical density (OD) values were ≤ positive cut off and negative when theOD values were ≥ negative cut off. The OD values between positive cut-off and negative cut off were considered undetermined (Pourquier and Lesceu, 2011).

2.18 Porcine IFN-γ quantification ELISpot asssay

The enzyme linked immunospot (ELISpot) (Mabtetch, Sweden) was used to quantify the number of IFN-γ secreting cells (Porcine IFN-γ ELISpot ^{PLUS} (HRP) kit, Mabtetch, Sweden). The Porcine IFN-γ ELISpot^{PLUS} (HRP) kit was used to conduct the assay according to the manufacturer's instructions (Mabtetch, Sweden). The plate was pre-coated with anti-IFN-γ monoclonal antibodies (Mabtetch, Sweden). In this experiment, 4 x 10^5 PBMC was stimulated with 20 µg/mℓ of ASFV recombinant proteins and incubated (Formaz Scientific water-Jacked incubator, ThermoFisher, USA) for 48 h at 37°C in 5% CO_2. Ten micrograms of Con A (Sigma-Aldrich, USA) per well was used as a positive control and 20 µg/mℓ of β-galactosidase protein (Champion™ pET Directional TOPO® expression kit, Invitrogen, USA) was used as background. One-time RPMI 1640 growth medium 1640 (Gibco, Thermo Scientific, USA) that contained 2 mM L-glutamine (Lonza, Switzerland) and 10% HyClone FBS (Thermo Scientific, USA) and 1% Gibco® pen-streptomycin antibiotic (Life technology, USA) (Reimann *et al.*, 2000) was used as a solvent for the ASFV recombinant antigens as well as the culture medium in this assay. The wells were manually washed 5 times with 200 µℓ of filter sterilised PBS at ±20°C. Thereafter, the secondary biotinated monoclonal

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antibody (0.5 µg/mℓ PAN-biotin, 0.5% FBS (Thermo Scientific, USA) in PBS at ±20°C (mAb-biotin) was added and incubated for 2 h. After another 5 washes with 200 µℓ of wash buffer, 1:1000 Streptavidin-enzyme conjugate in PBS at ±20°C containing 0.5 % FCS (Lonza, Switzerland) was added and incubated (Formaz Scientific water-Jacked incubator, ThermoFisher, USA) for 1 h. The plate was washed as above and the Tetramethylbenzidine substrate (Mabetch, Sweden) added and incubated at ±20°C for at ±3 min until spots develop. The reaction was stopped by washing extensively with tap water. A positive result was visualised as spots (Mabetch, Sweden).

The spots per well in the plates were counted using the KS ELISPOT reader (Zeiss, Lebanon) and the Porcine ELISpot results were reported as number of spots per million cells (spmc). The results were analysed in Microsoft office 2010 excel using the student T-Test (one tailed distribution, homoscedastic) statistical tool. The proteins were tested in triplicates including the controls (RPMI and ConA) in both pig 1, pig 2 and pig 3. The corresponding triplicate values of unstimulated cells in complete RPMI 1640 medium (Gibco, Thermo Scientific, USA) were subtracted from the corresponding stimulant value. The *p* values for pig 1 and pig 2 were all calculated against pig 3 separately using the T-Test by inserting the formula = T. Test (array1, array2, tails, type) enter. Array 1, three values were selected as well as array 2. Un-stimulated PBMC were used as negative control and ASFV naïve pig 3 was used as a negative control while Con A was used as a positive control. Only a $p \leq 0.05$ was considered significant. The average of the three replicate values were used to plot a bar graph using both Microsoft Office 2010 word and excel. The negative and background control spots were counted and averaged, these were subtracted from the averaged spots counts of the test stimulants. Therefore, only the averaged spot counts of the stimulants that were 10 spots higher than the averaged negative and background controls were considered positive.

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Chapter 3

3. RESULTS

3.1 Introduction

The study aimed to identify African swine fever virus (ASFV) proteins that activate cellular and humoral immune cytokine responses. To achieve this, primers were designed for genotype II ASFV isolate MAL/11/02 gene amplification. The ASFV *KP177R, EP402R, CP2475L-F1, CP2475L-F2, CP2475L-F3, CP2475L-F4, S273R, A104R, E165R, F334L, K205R* and *L11L* genes were amplified using a conventional PCR assay under optimal conditions. These genes were ligated into the PET102 vectors and expressed in BL21 DE3 *E. coli* cells. The expressed proteins were purified and used as antigens to stimulate peripheral blood mononuclear cells (PBMC) that were isolated from two domestic pigs (*Sus scrofa domesticus*) previously experimentally infected with the virulent genotype XIX ASFV isolate RSA/12/15 (Boshoff, 2017). A third pig that was naïve, named pig 3, was used as a control for recall immune responses. The immunological characteristics of subject proteins were investigated using cytokine qPCR and ELISpot assays.

3.2 The symptoms of pigs post infection

On day six post tick feeding on pig 1 while co-habiting pig 1 and pig 2, both pig 1 and pig 2 were observed to be ASFV negative by African swine fever (ASF) clinical signs observations and ASFV viremia tests using ASF p72 gene PCR assay and ELISA (Table 3.1). On day 9 post infection, pig 1 and 2 showed an increase in rectal temperature above 40°C and pig 1 tested positive for ASFV viremia and pig 2 showed a weak positive band after the p72 PCR assay. On the 10th day post infection both pigs were PCR positive and the temperature of pig 1 and 2 was 41.4°C and 40.6°C respectively as shown in Table 3.1. Both pigs were euthanized on day 10 post infection (Boshoff, 2017).

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Table 3.1: The temperatures, ASF ELISA and ASF PCR results from 0 to 10 days post infection of pigs 1 and 2.

		0 Days post infection (dpi)	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
PIG 1	**Temperature (Temp)**	39.2	39.3	39.4	39.6	39.2	39.5	39.5	39.4	39.6	40.2	41.3
	ASF PCR	Negative (NEG)	-	-	-	-	NEG	NEG	-	NEG	**Positive (POS)**	POS
	ASF ELISA	NEG	-	-	-	-	NEG	NEG	-	NEG	NEG	NEG
PIG 2	**Temp**	39.3	39.4	39.9	39.5	39.3	39.3	39.8	39.2	39.3	40.0	40.6
	ASF PCR	NEG	-	-	-	-	NEG	NEG	-	NEG	**WEAK POS**	POS
	ASF ELISA	NEG	-	-	-	-	NEG	NEG	-	NEG	NEG	NEG

3.3 The ASFV genes amplification and cloning

The presence of ASFV DNA (section 2.2) in the total isolated genome from tissue culture for ASFV gene amplification was confirmed by amplification of a 478 bp region corresponding to the size of the central portion of the p72 gene (Figure 3.1 for RSA/12/15, lane 2; MAL/11/02, lane 3; UGA/2003/1, lane 4). Both the negative extraction control (lane 5) and PCR negative control (lane 6) had no visible bands on the gel.

Chemically competent TOP10 *E. coli* cells and BL21 DE3 *E. coli* cell lines were initially used for cloning and protein expression but because cloning failures were experienced they were later replaced with electro-competent cell lines. Similarly, chemically-competent transformations have shown a high cloning failure rate and low cloning efficiency in other studies (Joersbo and Brunsted, 1991; Oswald, 2016). The electro-competent transformation method was used to overcome this challenge as it was reported to have a high cloning efficiency and a high cloning success rate (Oswald, 2016).

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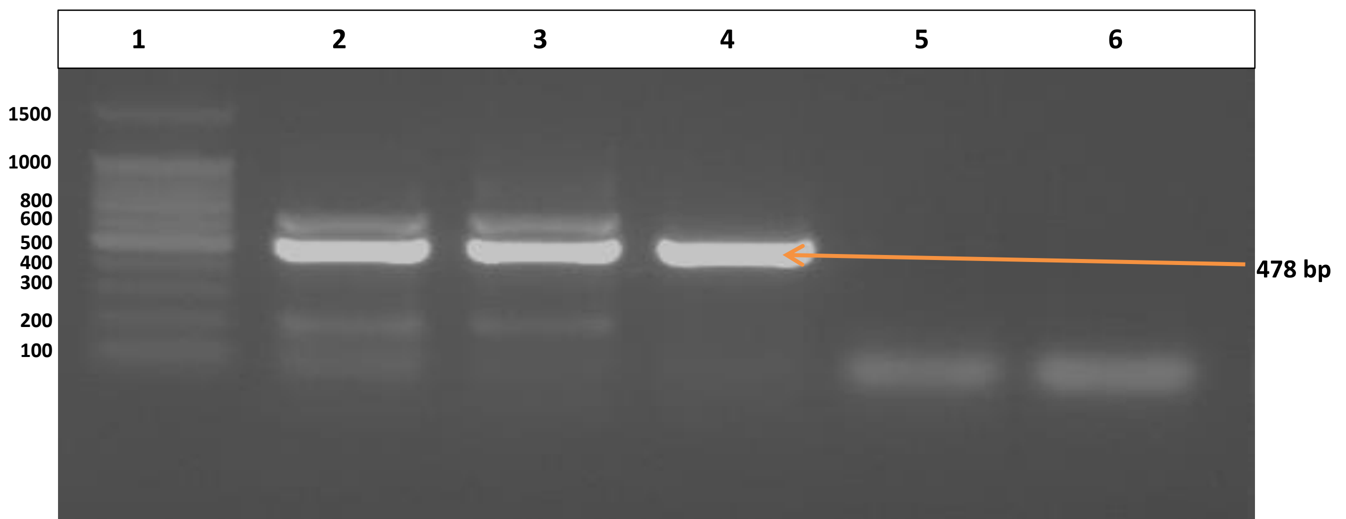

Figure 3.1: The PCR products of 478 bp were analysed by electrophoresis on a 1% agarose gel, containing Ethidium bromide and viewed under UV to confirm ASFV genome isolation from tissue culture stocks. Lane 1: GeneScript 100 bp DNA Ladder; Lane 2: RSA/12/15; Lane 3: MAL/11/02; Lane 4: UGA/2003/1; Lane 5: negative extraction control and Lane 6: PCR negative control.

The gene *CP2475L* coding for a polyprotein 220 (pp220) of 7 431 bp in size was divided into 4 fragments named *CP2475L-F1*, *CP2475L-F2, CP2475L-F3* and *CP2475L-F4* for the purposes of cloning efficiency. The fragments 1 to 3 were divided in such a way that each would have a size of 1 920 bp and fragment four of 1 850 bp long as depicted in Figure 3.2.

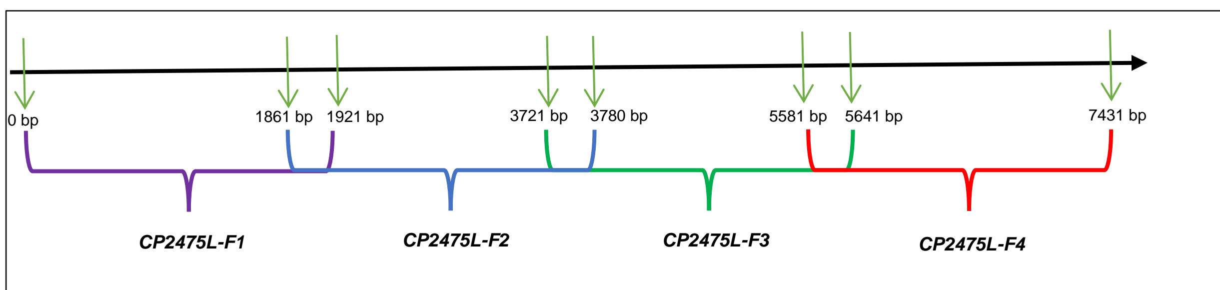

Figure 3.2: The gene *CP2475L* coding for a polyprotein 220 of 7 431 bp in size was divided into four fragments named *CP2475L F1, CP2475L F2, CP2475L F3* and *CP2475L F4.*

The primers were designed in such a way that pp220 F1-F4 of the polyprotein will have an overlap of 60 bp to guard against cutting an epitope. The ASFV genes (MAL/11/02 *KP177R, CP2475L-F1, CP2475L-F2, CP2475L-F3, CP2475L-F4, S273R, A104R, E165R, F334L, K205R* and *L11L*) were successfully amplified as shown in

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Figures 3.3 and 3.4, producing amplicons of the correct sizes as outlined in Table 3.2. Despite countless attempts to amplify the EP402R gene from the MAL/11/02 strain through modification of parameters such as working primer consentration re-preparation, same primer re-synthesis, template DNA concentration adjustments, annealing temperature modifications, elongation parameters modifications and final extension parameters modifications, the amplification of the EP402R gene proved to be problematic. The genotype II of the ASFV isolate MAL/11/02 strain sequence was not yet available and the ASFV Georgia 2007/1 strain sequence was used as the template to design the primers. This was solved when new primers were designed using the ASFV Pretoriuskop/96/4 (Accession no: AY261363) sequence as reference. After redesigning primers and successfully amplifying the EP402R gene of genotype II ASFV isolate MAL/11/02 strain (Figure 3.3), the sequence of the EP402R gene confirmed that the previously used forward primer was a mismatch at the priming region of the strain.

Table 3.2: The ASFV gene names, protein names and sizes.

GENE	SIZE (bp)	GENE SIZE WITH HIS-TAG (bp)	PROTEIN NAME	PROTEIN SIZE (kDa)	PROTEIN SIZE WITH HIS-TAG (kDa)
KP177R	569	804	p22	23	35
EP402R	1082	1317	CD2V	43	55
CP2475L-F1	1920	2155	F1	75	89
CP2475L-F2	1920	2155	F2	75	89
CP2475L-F3	1920	2155	F3	75	89
CP2475L-F4	1850	2085	pp220-F4	72	87
S273R	821	1056	pS273R	34	49
A104R	314	549	pA104R	15	30
E165R	497	732	pE165R	22	37
F334L	1004	1239	pF334L	40	55
K205R	617	852	pK205R	26	40
L11L	233	468	pL11L	12	26

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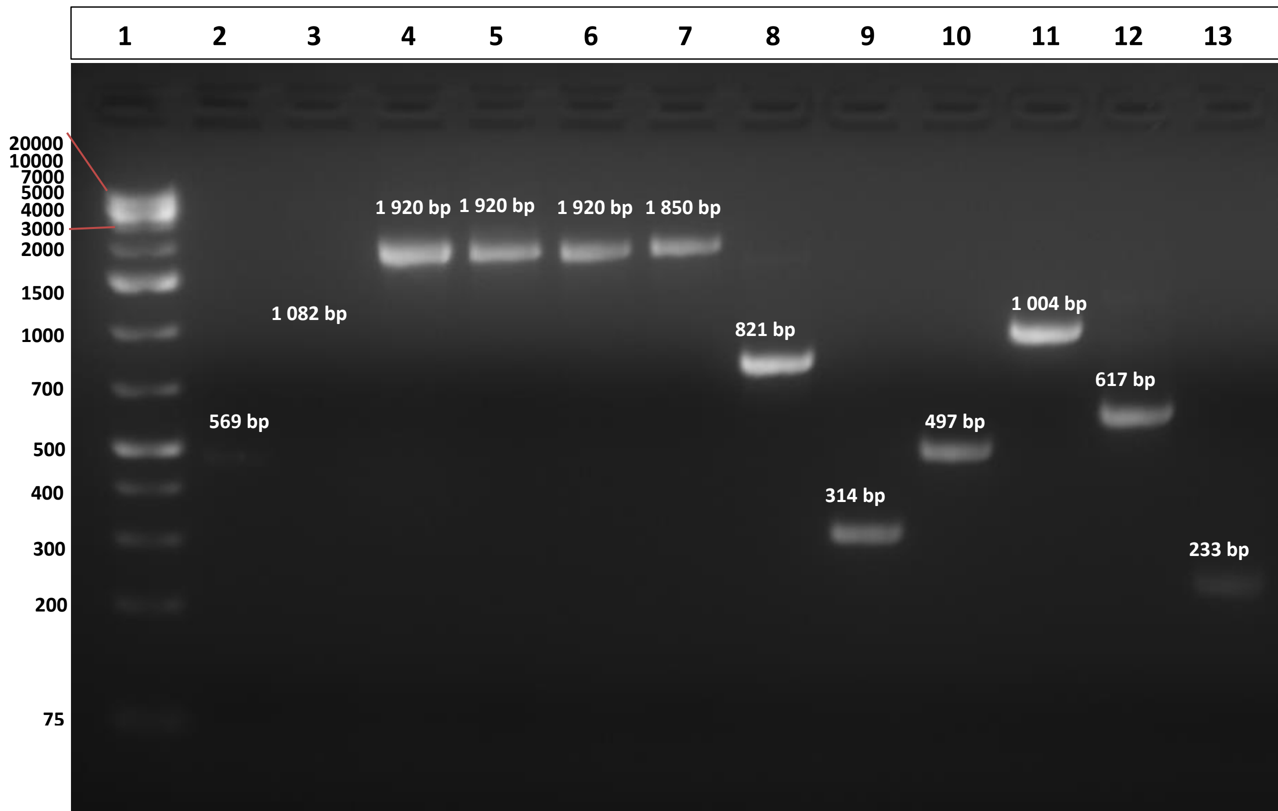

Figure 3.2: Agarose gel electrophoresis of purified PCR amplicons of ASFV isolate MAL/11/02 genes. The gel is labelled; Lane the 20 000 bp DNA Ladder; Lane 2: *KP177R*; Lane 3: *EP402R*; Lane 4: *CP2475L-F1*; Lane 5: *CP2475L-F2;* Lane 6: *CP2475L-F3*; Lane 7: *CP2475L-F4*; Lane 8: *S273R*; Lane 9: *A104R*; Lane 10: *E165R*, Lane 11: *F334L*; Lane 12: *K205R* and Lane 13: *L11L*.

No positive control was included because the genes were amplified for the first time (Figure 3.3. Although KP177R (15.47 ng/mℓ, lane 2), EP402R (5.5 ng/mℓ, lane 3) and L11L (28.85 ng/mℓ, lane 13) are faintly visible in Figure 3.3, the products were usable downstream. Colony PCR was conducted of the transformed TOP10 *E. coli* cells and agarose gel electrophoresis indicated successful preliminary ligation of all the ASFV genes (Figure 3.3). The RSA/12/15 KP177R of 804 bp (lane 13) clone was used as a positive control (Figure 3.4). Clones with correct sizes were isolated and samples with concentrations of ≥ 50 ng/mℓ were sent for sequencing.

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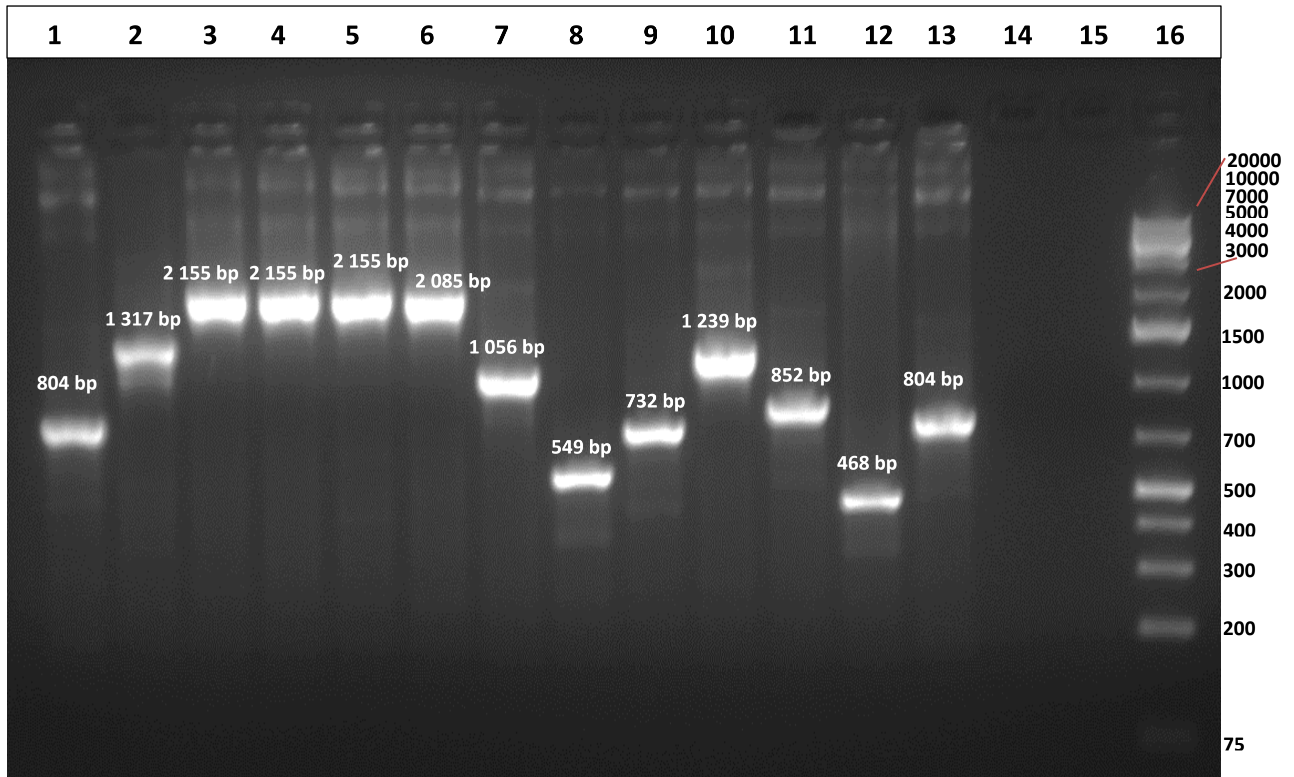

Figure 3.3: Agarose gel electrophoresis of the PET102 clone PCR amplicons of ASFV isolate MAL/11/02 genes. Lane 1: 804 bp *KP177R*; Lane 2: 1317 bp *EP402R*; Lane 3: 2155 bp *CP2475L-F1*; Lane 4: 2155 bp *CP2475L-F2*; Lane 5: 2155 bp *CP2475L-F3*; Lane 6: 2085 bp *CP2475L-F4*; Lane 7: 1056 bp *S273R*; Lane 8: 549 bp *A104R*; Lane 9:732 bp *E165R*; Lane 10:1239 bp *F334L*; Lane 11: 852 bp *K205R*; Lane 12: 468 bp *L11L*; Lane 13: 804 bp RSA/12/15 *KP177R* positive control, Lane 14: Negative Control; Lane 15: negative control and Lane 16: GeneRuler 20 000 bp DNA Ladder.

3.4 Recombinant protein expression

Nine of the study proteins were expressed in BL21 DE3 *E.coli* cells overnight without IPTG induction (p22, pA104R, pS273R, pE165R, pK205R, pA224L, pB385L, pB475R and pK196R of ASFV RSA/12/15 isolate) and six of them (p22, pp220-F1 to F4 and pS273R) were re-expressed with IPTG induction. Recombinant proteins, isolated from the cells using the Bug buster reagent, were expressed and of the correct size as indicated on SDS-Page gels and western blotting (Figures 3.5 to 3.9), with the exception of CD2V (Figures 3.5 and 3.7), pF334L, and pL11L (Figures 3.6, 3.8 and 3.9). These latter proteins were not observed on both SDS-PAGE gel and western blotting and therefore were not expressed (Table 3.2, Figures 3.5 to 3.9). However, the CD2V (*EP402R*) gene was successfully cloned and the resulting recombinant protein detected with dot blot (results not shown). Other recombinant proteins were expressed including β-Galactosidase (positive expression control) and were at the expected sizes (Table 3.2) as shown on the SDS-PAGE gels (Figures 3.5 and 3.6). There was background protein present on SDS-PAGE and western blots for all

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proteins expressed (Figures 3.5 to 3.8), regardless of attempts to reduce this by washing with a wash buffer containing 8 mM imidazole before eluting the recombinant protein with 250 mM imidazole (Results not shown). This helps to wash off column bound impurities and it does this by un-binding molecules that are weakly bound to the column matrix leaving only proteins with high affinity or strongly bound molecules (Macherey-Nagel, 2013). The background protein impurities could influence the concentration measurements and the immunological profile of the proteins during immunological assays and that is why a non-specific protein was included in the assays. While urea could affect the conformation of the protein (post-translational modifications) thereby hiding the six histidine tag from the column, hence preventing column binding (Macherey-Nagel, 2013; Rosano and Ceccareli, 2014), this was not experienced during protein purifications in the current study. Bovine serum albumin (BSA) protein of 125 mg/mℓ was loaded as concentration indicator (Figure 3.5).

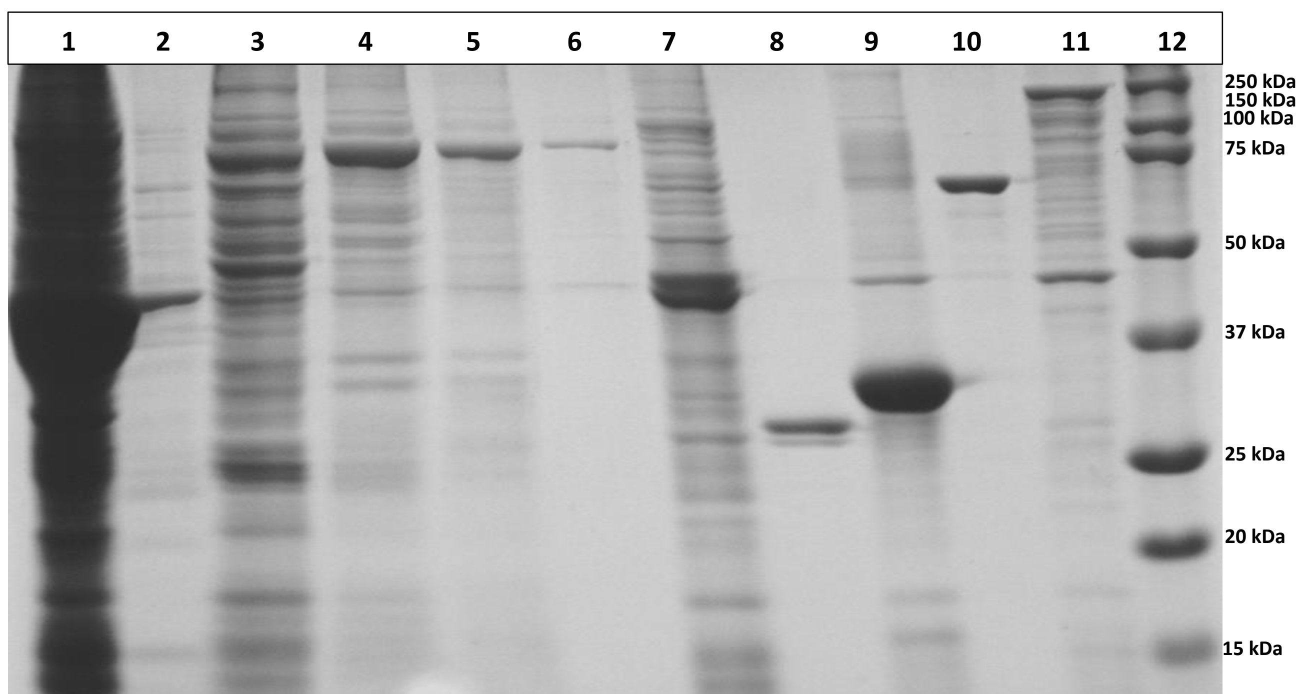

Figure 3.4: Coomassie blue stained 12% SDS-PAGE gel showing ASFV MAL/11/02 proteins that were expressed in bacterial cells and purified by Protino® Ni TED columns: Lane 1: p22 of 39 kDa; Lane 2: CD2V of 58 kDa; Lane 3: pp220-F1 of 89 kDa; Lane 4: pp220-F2 of 89 kDa;Lane 5: F3 of 89 kDa; Lane 6: pp220-F4 of 87 kDa; Lane 7: pS273R of 49 kDa; Lane 8: pA104R of 30 kDa; Lane 9: pE165R of 37 kDa; Lane 10: BSA of 67 kDa (125 µg/mℓ); Lane 11: β-Galactosidase 133 kDa; Lane 12: 250 kDa Precision Plus Protein™ Standards Kaleidoscope™. The F1 and F2 were expressed through IPTG induction. The p22 and pS273R that were IPTG induced were not loaded on this gel or used for immunological assays because un-induced versions of these proteins were available.

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It was evident that recombinant proteins p22, pK205R, pE184L, pB475R and pK196R had concentration higher than 125 mg/mℓ while protein pp220-F3, pp220-F4, pA104R, and pA224L had concentrations lower than 125 mg/mℓ. The p22 recombinant protein was expressed as an insoluble protein without IPTG induction. The PS273R was expressed in both soluble and insoluble form without IPTG induction and when IPTG induced, both fractions increased and the soluble fraction was isolated and purified. The PA104R protein was expressed in the insoluble fraction without IPTG induction; pE165R was expressed mostly in the insoluble fraction and without IPTG induction. The PK205R was expressed without IPTG induction in soluble form. The pp220-F1 to F4 was expressed in the insoluble fraction without IPTG induction and its concentration increased when IPTG induced. At high concentrations, this protein had a gelatinous consistency which was difficult to solubilise and therefore was not purified as it could not penetrate the purification column. Attempts to solubilise this protein with 8 mM urea was unsuccessful. The pp220-F1 toF4 was therefore used for immunological assays without being purified and excluded from further experiments.

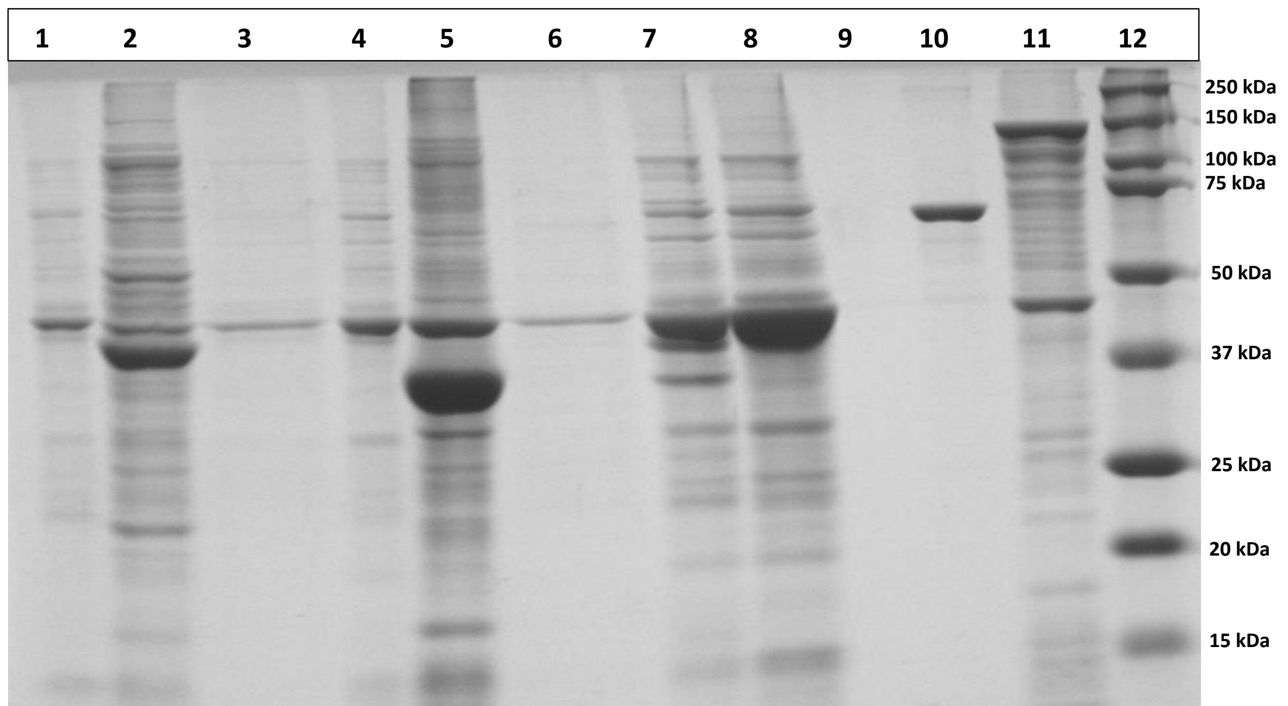

Figure 3.5: Coomassie blue stained 12% SDS-PAGE gel showing ASFV MAL/11/02 proteins that were expressed in bacterial cells and precipitated after purification by Protino® Ni TED columns. Lane 1: pF334L of 56 kDa; Lane 2: pK205R 41 kDa; Lane 3: pL11L 27 kDa; Lane 4: pA224L 43 kDa; Lane 5: ASFV isolate RSA/12/15 pE184L 39 kDa; Lane 6: pB385L 61 kDa; Lane 7: pB475R 71 kDa; Lane 8: ASFV isolate RSA/12/15 pK196R 40 kDa; Lane 9: Loading dye; Lane 10: BSA of 67 kDa (125 µg/mℓ); Lane 11: β-Galactosidase positive expression control of 133 kDa and in Lane 12: 250 kDa Precision Plus Protein™ Standards Kaleidoscope™. Expressed study proteins in this Figure were not IPTG induced.

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3.5 Confirmation of gene expression using western blotting.

Western blotting of both purified and un-purified proteins was done to confirm if the expressed proteins were the correct recombinant proteins using both size standard and binding of the anti-Histidine antibody to the Histidine tag of the expressed and isolated protein. Western blotting of recombinant proteins β-Galactosidase of 133 kDa, pL11L of 27 kDa, pK205R of 41 kDa, pA104R of 30 kDa and pS273R of 49 kDa is illustrated in Figure 3.8. The protein concentration loaded onto the gels improved the western blot image for all the proteins except that of *S273R*, which concentration was too low for detection on the blot (Figure 3.9).

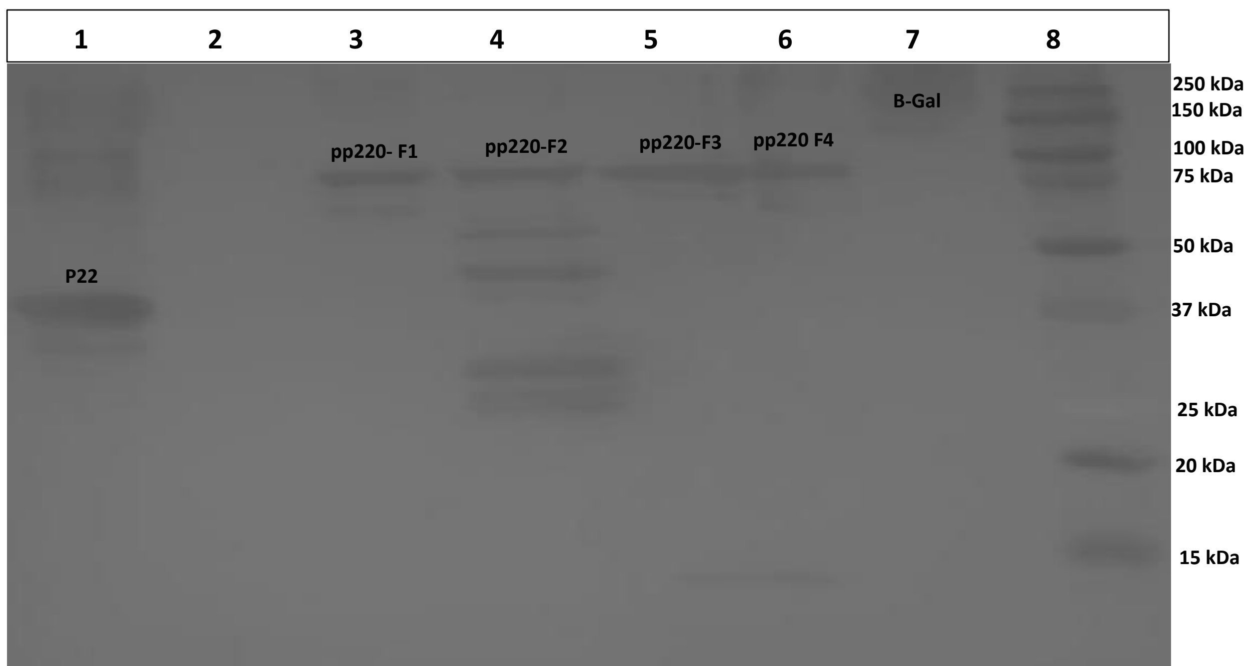

Figure 3.6: A western blot of a 12% SDS-PAGE gel showing re-suspended acetone precipitates of purified proteins of ASFV isolate MAL/11/02 proteins purified by Protino® Ni TED columns. Lane 1: p22 of 39 kDa; Lane 2: CD2V of 58 kDa; Lane 3: F1 of 89 kDa; Lane 4: F2 of 89 kDa; Lane 5: F3 of 89 kDa; Lane 6: pp220-F4 of 87 kDa; Lane 7: β-Galactosidase of 133 kDa and in lane 8: 250 kDa Precision Plus Protein™ Standards Kaleidoscope. Proteins in this figure were not IPTG induced.

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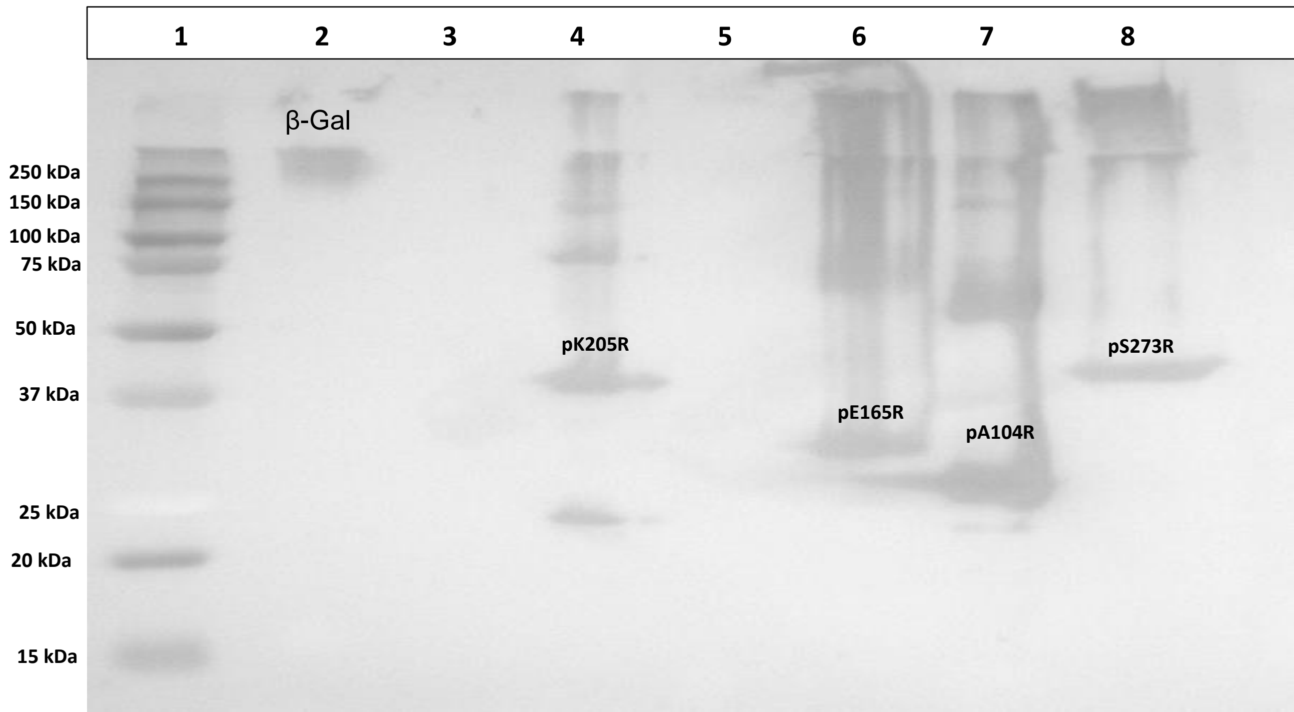

Figure 3.7: A western blot of a 12% SDS-PAGE gel showing re-suspended acetone precipitates of purified proteins of ASFV isolate MAL/11/02 proteins purified by Protino® Ni TED columns. Lane 1:250 kDa Precision Plus Protein™ Standards Kaleidoscope; Lane 2: β-Galactosidase of 133 kDa; Lane 3: pL11L of 27 kDa; Lane 4: pK205R of 41 kDa; Lane 5: pF334L of 56 kDa; Lane 6: pE165R of 37 kDa; Lane 7: pA104R of 30 kDa and lane 8: pS273R of 49 kDa. Proteins in this figure were not IPTG induced.

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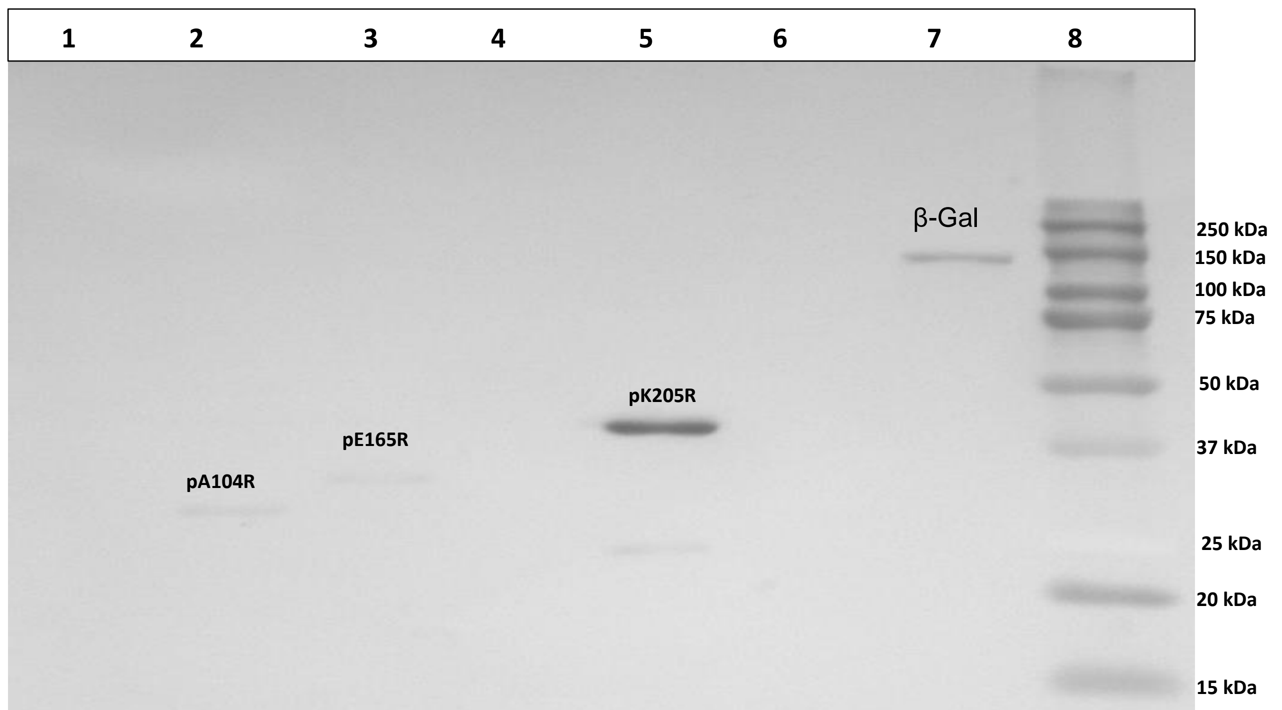

Figure 3.8: A western blot of a 12% SDS-PAGE gel showing re-suspended acetone precipitates of purified proteins of ASFV isolate MAL/11/02 proteins. Lane 1: pS273R of 49 kDa; Lane 2: pA104R of 30 kDa; Lane 3: pE165R of 37 kDa; Lane 4: pF334L of 56 kDa; Lane 5: pK205R of 41 kDa; Lane 6: pL11L of 27 kDa; Lane 7: β-Galactosidase of 133 kDa and in lane 8: 250 kDa Precision Plus Protein™ Standards Kaleidoscope.

Figure 3.9: Coomassie blue stained 12% SDS-PAGE gel showing Ni^{+} IDA column purified p22 recombinant protein of ASFV MAL/11/02. The recombinant protein was expressed without IPTG induction. Lane C: crude protein expression mixture, Lane F: column flow through, Lane W: wash, Lane E: elution and Lane M: 250 kDa Precision Plus Protein™ Standards Kaleidoscope™.

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3.6 Protein concentration determination with Pierce bicinchoninic acid (BCA) assay

The representatives of each protein's acetone precipitates were re-suspended and concentration measurements were conducted using the Pierce BCA protein assay. The standard curve was used to interpret the purified recombinant proteins concentration (Figure 3.10). The pp220 F2 had the lowest concentration of 4 µg/mℓ (Table 3.3). However, it was expressed in large quantities as shown in Figure 3.7 lane 6. The protein pS273R had the highest concentration of 474 µg/mℓ (Table 3.3).

Table 3.3: Protein concentration for ASFV recombinant proteins determined by the Pierce BCA protein assay after column purification, acetone precipitation and re-suspension.

GENES	PROTEIN	BCA CONCENTRATION (µg/mℓ)
KP177R	p22	121
CP2475L-F1	pp220-F1	38
CP2475L-F2	pp220-F2	4
CP2475L-F3	pp220-F3	210
CP2475L-F4	pp220-F4	100
S273R	pS273R	474
A104R	pA104R	80
E165R	pE165R	230
K205R	pK205R	276

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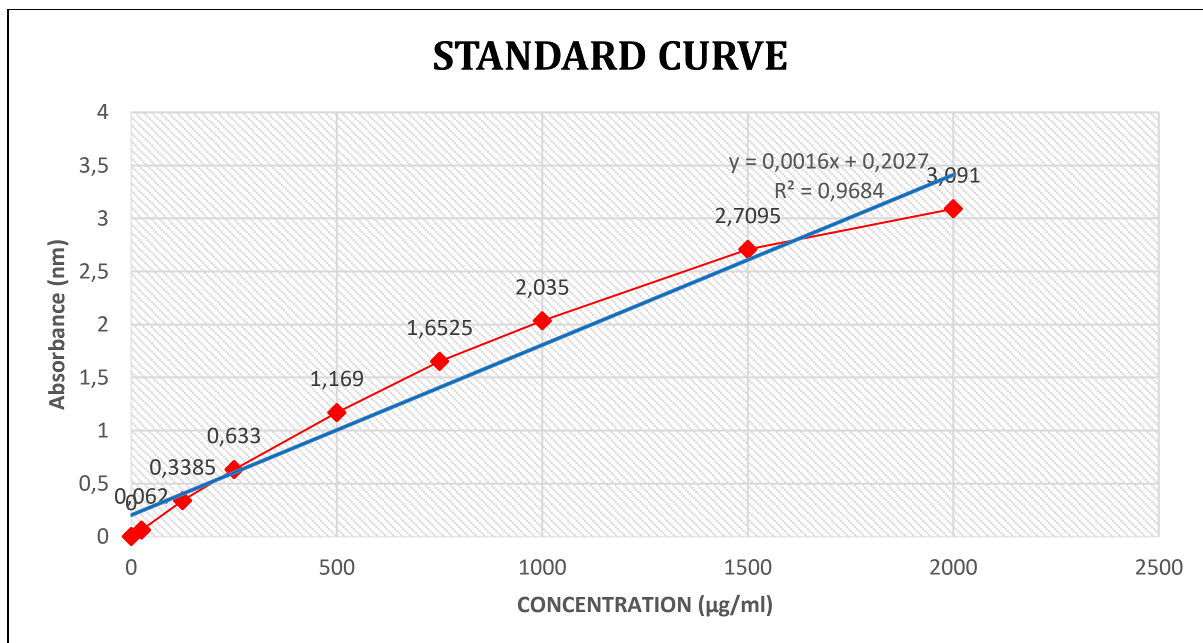

Figure 3.10: A standard curve representation of the average absorbance readings for BCA standards at 560 nanometres (nm). The x-axis shows the known standards concentrations in mg/mℓ. The y-axis shows the absorbance of the standard in nm. The blue line represents a function of the equation used to calculate recombinant protein concentrations.

3.7 Cytokine real time PCR for cytokine mRNA quantification

PBMC were stimulated with Con A, positive induction of IL-2, IL-4 and IFN-γ were observed in pig 1 and 3 (Table 3.4) but only IFN-α induction was found in pig 2. The assay worked considering that, Con A induced a positive fold increase of several cytokines (Katial *et al*., 1998). It was noted that β-galactosidase induced non-specific cytokine responses in all three pigs (Table 3.4). Therefore, fold changes induced by ASFV recombinant proteins, which were ≥ 2 than that induced by β-galactosidase, were considered positive and ASFV specific responses and indicated in blue, green and red in Table 3.4. Three ASFV recombinant proteins (p22, pS273R and pA104R) induced background 'positive' cytokine responses in the ASFV naïve pig 3. Therefore, a fold change of ≥ 2 and ≤ 2 to the control pig 3 value was considered as up-regulated and down-regulated, respectively.

In pig 1, p22 stimulated the highest positive and upregulated values for IL-2 and IL-4, IL8, IL10 and IFN-γ. Recombinant protein pp220-F4 up-regulated IL-4, IL-8, IL-12 and IFN-α, whereas pA104R and pE165R up-regulated IFN-α. The pS273R down-regulated IFN-α. In pig 2, p22 and pp220-F4 only up-regulated IFN-α.

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Table 3.4: The cytokine qPCR results after PBMC stimulation with ASFV recombinant proteins indicated as average fold increase statistically significant fold change values compared to pig 3 (P ≤ 0.05) are indicated with *.

= Positive ≥ 2 fold higher than β-galactosidase.
= Positive ≥ 2 fold higher than β-galactosidase and significantly up-regulated when compared to control pig 3.
= Positive ≥ 2 fold higher than β-galactosidase and significantly down-regulated when compared to control pig 3.

PIG 1											
CYTOKINES	CON A	B- GAL	P22	F1	F2	F3	Pp220-F4	PS273R	PA104R	PE165R	PK205R
IL-2	17.0*	NEG	7.3*	NEG	NEG	NEG		NEG	2.4	NEG	NEG
IL-4	2.5*	NEG	6.2*	NEG	NEG	NEG	3.4*	NEG	3.0	NEG	NEG
IL-8	0.7*	5.7*	30.4*	5.0*	3.4*	8.3*	14.4*	7.3*	8.8*	5.5*	7.5*
IL-10	1.3*	3.3	10.7*	2.0*	1.8	2.9*	4.5*	1.4	2.9*	1.4*	3.0*
IL-12	NEG	NEG	7.4	NEG	NEG	NEG	3.5*	0.8	3.8	NEG	NEG
IFN-γ	2.7*	0.8	5.1*	0.9*	0.8	NEG	1.5	0.3	1.2	NEG	NEG
IFN-α	0.6*	NEG	8.1	0.7*	NEG	NEG	3.5*	1.5*	8.4*	1.9*	NEG
PIG 2											
IL-2	NEG	NEG	1.9*	NEG	NEG	NEG	0.4*	NEG	NEG	NEG	NEG
IL-4	0.5	NEG	1.8*	NEG	NEG	NEG	0.4*	NEG	0.8	NEG	NEG
IL-8	NEG	41.3*	28.7*	9.5*	3.8*	9.7	0.0*	0.7*	0.3*	0.4*	4.9*
IL-10	0.9*	4.2	5.2*	1.3*	0.5	0.9	1.7*	0.8	NEG	NEG	0.4
IL-12	0.6*	0.7	3.7	NEG	NEG	0.3	0.4	NEG	NEG	NEG	NEG
IFN-γ	0.4*	1.1	2.8	0.9*	0.4*	1.0	1.5	0.8	1.4	NEG	NEG
IFN-α	2.3	0.9*	14.5*	0.6*	NEG	0.4	1.9*	1.1*	1.9	NEG	0.3*
PIG 3											
IL-2	25.3	NEG	2.7	NEG	NEG	NEG	NEG	7.6	NEG	NEG	NEG
IL-4	4.7	NEG	3.1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
IL-8	0.1	2.4	1.0	0.5	0.6	0.5	0.4	0.9	1.6	0.9	0.5
IL-10	0.3	3.0	1.5	0.7	0.8	0.8	0.5	0.7	1.1	NEG	NEG
IL-12	0.6	NEG	4.7	NEG	NEG	0.7	NEG	NEG	NEG	NEG	NEG
IFN-γ	18.0	1.5	2.6	1.8	1.4	1.6	1.2	3.6	NEG	NEG	NEG
IFN-α	0.9	1.6	7.2	1.4	0.5	2.4	NEG	15.6	4.3	NEG	NEG

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3.8 Porcine IFN-γ quantification ELISpot assay

The enzyme linked immunospot (ELISpot) assay was conducted to further investigate the recall immune responses induced by study proteins in pig 1 and pig 2. The spots per million cells (spmc) of Con A and β-galactosidase controls were found to be at the expected respective ranges which were ≥10 spmc for Con A and < 10 spmc for β-galactosidase. In pig 1 and 2, only fragment pp220-F4 induced a positive IFN-γ response (Figure 3.11). No response in this assay was found to be statistically significant due to a small sample size (*n*=1).

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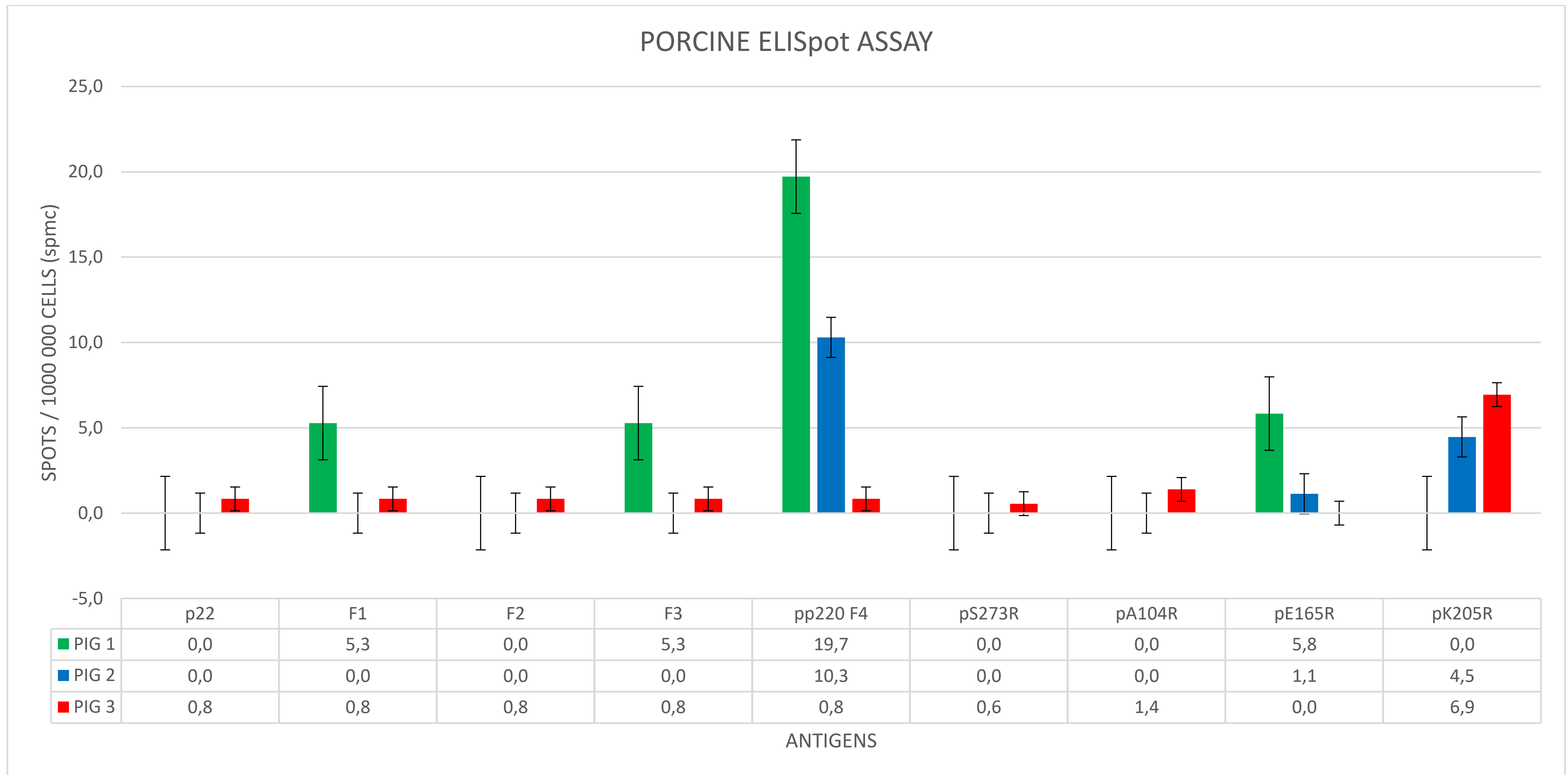

	p22	F1	F2	F3	pp220 F4	pS273R	pA104R	pE165R	pK205R
PIG 1	0,0	5,3	0,0	5,3	19,7	0,0	0,0	5,8	0,0
PIG 2	0,0	0,0	0,0	0,0	10,3	0,0	0,0	1,1	4,5
PIG 3	0,8	0,8	0,8	0,8	0,8	0,6	1,4	0,0	6,9

Figure 3.11: **The ability of ASFV expressed proteins p22, F1, F2, F3, pp220-F4, pS273R, pA104R, pE165R and pK205R to induce IFN-γ by PBMC from ASFV immunised pig 1, pig 2 and un-immunised control pig 3. Con A and RPMI 1640 (Gibco, Thermo Scientific, USA) used as positive and negative controls respectively were not included in this diagram for scaling purposes.**

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Chapter 4

4 DISCUSSION AND CONCLUSION

4.1 Discussion

The immune system is modulated and regulated by proteins called cytokines (Sivangala and Sumanlatha, 2015). Thus, knowledge of the cytokine profile in the environment of an infection is crucial for the development of a subunit vaccine aimed at inducing an effective protective immune response. The aim of this study was therefore to identify ASFV proteins that induce immune response cytokines during primary infection to assist in future vaccine development studies. When the immune response was investigated, p22, pp220-F4, pA104R and pE165R were observed to induce an immune response in PBMC isolated from domestic pig 1 previously infected with genotype II ASFV isolate MAL/11/02 by tick feeding. Cytokines that formed part of the current study were all significantly up-regulated in pig 1 by different recombinant proteins. The p22 was the only protein that induced up-regulation of IL-2, IL-10 and IFN-γ. The p22 and pp220-F4 were the only proteins that induced up-regulation of IL-4 and IL-8. The pp220-F4 was the only protein that induced up-regulation of IL-12. The pA104R, pp220-F4 and pE165R were found to significantly up-regulate IFN-α and pA104R induced the highest fold increase. The p22 induced the highest fold increase of cytokines IL-2, IL4, IL-8, IL-10 and IFN-γ. The pp220-F1 and pS273R induced significant down-regulation of IFN-α in pig 1.

In contrast, in the pig that was infected by horizontal transmission of ASFV, pig 2, IFN-α was significantly up-regulated by p22 and pp220-F4. The p22 induced the highest fold increase of IFN-α, but induced downregulation of IL-2 and IL-4. No study protein induced a down-regulation of IFN-α in pig 2. If proteins p22 and pp220-F4 are combined in a recombinant vaccine against ASFV they may induce a protective immune response characterised by the secretion of the following cytokines and their benefits: 1) IL-2 modulates the growth, proliferation, maturation and differentiation of naïve CD4, CD8 and B cells into effector cells (Waldmann and Tagaya, 1999); 2) IL-4 stimulates Th2 cell proliferation, naïve B cell differentiation into mature antibody

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producing cells (Moens and Tangye, 2014) and up-regulates major histo-compatibility complex (MHC) class II (Vazquez *et al.*, 2015); 3) IL-8 is a neutrophil chemo-attractant that plays a major role in the pro-inflammatory response and attracts neutrophils, NK and T cells to the site of infection (Fishbourne *et al.*, 2013); 4) IL-10 is an anti-inflammatory regulatory cytokine (Saxena *et al.*, 2015); 5) IFN-γ induces pathogen clearance and promotes cytolytic T lymphocyte, NK, and macrophage activity (Khan, 2008); 6) IFN-α an inhibitor of viral replication in infected cells by up-regulating MHC I and stimulatingcytotoxic lymphocytes and NK cells (Gil *et al.*, 2008).

The pS273R and pp220-F1 induced a down-regulation of IFN-α that may indicate that they may play a role in host immune evasion. This study may also suggest that IFN-α stimulation by pA104R is initially suppressed in the first few days of infection and later picks up as the disease progresses. This speculation is based on the fact that in pig 2, where the infection is relatively new in comparison to pig 1 (Boshoff, 2017), PA104R did not induce IFN-α. This observation may suggest that if domestic pigs are vaccinated with pA104R, the virus will not be able to suppress IFN-α after specific ASFV infection. This speculation can be strengthened by future studies that investigates the recall immune responses induced by pA104R in domestic pigs at different time points of infection.

The IL-8 is a pro-inflammatory cytokine that functions as a chemo-attractant for neutrophils, NK cells and T cells, which leads to swelling or inflammation, a known pathogenesis of ASF that if not tightly controlled may eventually lead to the death of the infected domestic pigs (Fishbourne *et al.*, 2013). Therefore, p22 that induced high IL-8 up-regulation observed in pig 1 may have epitopes that play a role in pathogenesis of the disease.

In PBMC from pigs 1 and 2, pp220-F4 induced positive IFN-γ responses (ELISpot assay). However, this response did not correlate with the cytokine qPCR results. Again, it is important to remember that the two assays measure different products, cytokine qPCR measures RNA transcription levels while ELISpot assay measures the number of IFN-γ secreting cells. There are also dynamics in the process from mRNA to protein as backed up by the literature that high number of mRNA does not necessarily imply a protein product, neither does it imply increased protein translation

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(Vogel and Marcotte, 2012). It is important to note that the ELISpot measures all IFN-γ produced within 48 h while for qPCR was measured at one time point (24 h). It is possible that RNA levels peaked at an earlier time point (2 to 6 h) as suggested in the literature (Carletti and Christenson, 2009). Furthermore, both ELISpot assay and qPCR needs to be optimised for each study protein to determine the optimal recombinant protein concentrations for PBMC stimulation (Kendrick, 2014). The results of ELISpot assay are more informative as this assay is a functional assay.

The immune response in pig 1 was stronger than the immune response in pig 2. This may highlight the difference in the immune response induced by the two routes of infection or indicate that pig 1 was infected longer than pig 2 because pig 2 was infected directly or indirectly by pig 1 (Boshoff, 2017). This is supported by the weak ASF PCR positive in pig 2 on day nine pi while pig 1 was clearly positive (Figure 3.1). Furthermore, the cytokine RT qPCR results for pp220-F4, p22, pA104R and pE165R response in pig 1 is higher than that in pig 2 (Figure 3.4) while the IFN-γ response in pig 1 on the ELISpot assay is almost double that in pig 2 for pp220-F4 (Figure 3.5). The variable results observed between the two pigs in the ability of the proteins to induce cytokines can also be attributed to the fact that the two pigs are outbred and may have different MHC alleles. The MHC typing also needs to be included in future studies to determine whether the proteins will induce immune responses in outbred population of pigs and thereby indicating the nature of the immune response observed (Shao *et al*., 2015).

It is important to note that it is difficult to regard the immune response in this study as recall since the infection period (10 days) was not enough for the domestic pigs to establish immunological memory. It is worth bearing in mind that the two pigs were infected differently (Paliard *et al*., 1988), this could explain why the response of both pigs to a similar antigen differed. However, only one-time point was analysed in this study and the antigen concentrations used are questionable although a uniform concentration of 20 µg/mℓ was used across the board amidst antigen dissolution challenges. Time points and proteins used in immunological assays in this study should be optimised in future studies.

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Furthermore, the study also investigated cross reactivity as the domestic pigs were infected with the haemadsorbing genotype XIX ASFV isolate from the republic of South Africa (RSA) RSA/12/15 while the recombinant protein antigens used to stimulate the PBMC were expressed from genotype II ASFV isolate from Malawi (MAL) MAL/11/02. The four recombinant proteins from the MAL strain shown to induce ASFV specific responses in the RSA strain immunised pigs indicates that the MAL strain proteins contain cross-reactive epitopes. This is important if a vaccine is to induce protective immunity to several strains.

The genotype XIX ASFV isolate RSA/12/15 strain used to infect pigs in this study is highly virulent and thus did not allow the animals to live long enough for the development of immune memory (Scheerlinck and Yen, 2010). Therefore, proteins were identified that induced cytokines during the infection phase of ASFV. In order to determine whether these proteins induce memory recall responses this study should be repeated using pigs that are infected with a less virulent ASFV that allows the pigs to survive long enough to develop ASFV specific immune memory that can be recalled. However, less virulent strains of ASFV have virulent genes deleted or mutated and this is therefore not favourable as it defies the main purpose of the study, which is to identify ASFV proteins that induce immune responses. Another alternative would be to isolate PBMC from warthogs experimentally infected with a virulent ASFV as they remain asymptomatic to virulent ASFV strains and thus could remain infected long enough for ASFV specific immune memory that can be recalled (Stanley, 2010). The disadvantage to this approach would be that the results obtained in warthogs would require re-testing in domestic pigs since warthog results cannot be extrapolated to domestic pigs.

Scientists have tried an approach where a chimera of p54 and p30 was used to immunise pigs (Jankovich *et al.,* 2018). The pigs developed neutralizing antibodies and the animals survived challenge with virulent ASFV (Barderas *et al.*, 2001). Also, when New Castle disease virus p72 (rNDV/p72) from a virus that was passaged ten times was used to immunise mice, a high titre of ASFV p72 protein specific IgG antibodies were induced together with T cell proliferation and the secretion of both IFN-γ and IL-4 and the IgG1 titres induced was more than the IgG2 titres (Chen *et al.*, 2016). Adenovirus-vectored ASFV multi-antigens research demonstrated both

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humoral and cellular immune responses (IFN-γ and CTL) against ASFV recombinant protein antigens p32, p54, pp62, p72, pA151R, pB119L, pB602L, pEP402RΔPRR, pB438L, pK205R and pA104R (Lokhandwala *et al.,* 2016; 2017). These examples indicate that single and multiple viral proteins can elicit immune responses against ASF. Researchers have recently shown that immune responses were induced against equine encephalitis virus using a multiagent DNA vaccine (Dupuy *et al*., 2018).

It is important to note that an amalgamation of ASFV P22, pp220-F4 and pA104R could induce all the studied cytokines. Furthermore, these structural proteins are most likely to interact with the immune system (Lorente *et al*., 2016) and ideal when they are expressed early in infection thus allowing early recognition by the immune surveillance thus leading to timeous immune response. These should therefore induce an early protective immune response against virulent ASFV strains within 10 dpi, the period within which the virulent ASFV kills the host. This study has provided new information on several ASFV proteins that induce a wide range of important cytokines and thus expanded previous knowledge, that may lead to the development of an improved effective ASFV vaccine.

4.2 Conclusion

The current study indicated that genotype II ASFV isolate from Malawi (MAL) MAL/11/02 virulent strain recombinant proteins p22, pp220-F4, pA104R and pE165R were able to induce immune response in PBMC isolated from a domestic pig that was experimentally infected (tick infected feeding) with haemadsorbing genotype XIX ASFV isolate from the republic of South Africa (RSA) RSA/12/15 virulent strain. The p22 induced IL-2, IL-4, IL-8, IL-10, IL-12, IFN-γ and IFN-α. The pp220-F4 induced IL-2, IL-4, IL-8, IL-12, IFN-γ and IFN-α. In addition, genotype II ASFV isolate MAL/11/02 strain recombinant proteins p22 and pp220 were able to induce immune response in PBMC isolated from a domestic pig that was experimentally infected via contact with haemadsorbing genotype XIX ASFV isolate RSA/12/15 virulent strain. The p22 induced IFN-α and pp220-F4 induced IL-2, Il-4 and IFN-α. Therefore, the combination of p22 and pp220-F4 may result in the up-regulation of all study cytokines in domestic pigs vaccinated with a chimera consisting of ASFV proteins that cross protect across different genotypes. The current study suggests that p220 F1 and pS273R plays a role

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in ASFV immune evasion based on their down-regulation of IFN-α. Furthermore it appears that ASFV suppresses IFN-γ, IL-12, IL-4 and IL-2 in domestic pigs. However, the suppression of these cytokines may be favourable for the pathogenesis of the virus in the early stages of infection as an immune evasion mechanism. The p22 and pp220-F4 were identified as proteins that may be protective across genotype II ASFV isolate MAL/11/02 and genotype XIX ASFV isolate RSA/12/15. In conclusion, the study revealed that ASFV recombinant proteins p22 and pp220-F4 induced all the study cytokines which are important in antiviral immunity. It also provided novel preliminary data on ASFV recombinant proteins that can be used as basis for future research and vaccine development.

4.3 Future research

Several aspects were identified during the investigation that could be inproved in future research. The protein product of genes *EP402R* (CD2V), pF334L and pL11L were not observed on both SDS-PAGE gel and western blotting. This implies that CD2V, pF334L and pL11L did not express. The clones could have been rejected or inserts rearranged as protein products could have been toxic to the bacteria (Kimelman *et al.*, 2012). The clones were however inspected by sequencing and were found to be correctly cloned. When the protein starts to interfere with the host cell physiology and homeostasis, cell toxicity occurs (Dong *et al.*, 1995). This could be prevented by using tighter expression control systems like *E. coli* BL21 (DE3) pLysS cell line (Miroux and Walker, 1996). Plasmid instability could be the reason behind proteins not expressing when using ampicillin as an antibiotic for selection (Rosano and Ceccarelli, 2014). This can happen because β-lactamase or hydrolization due to acid production in media can cause the destruction of ampicillin (Meena and Harish, 2001). In such cases, using carbenicillin instead of ampicillin has been shown to reduce plasmid instability (Meena and Harish, 2001). Ribonucleic acid degradation can occur before or during protein expression and this problem can be eliminated by cloning into a structured RNA vector and using an RNAse free host strain (Vogel *et al.*, 2011).

Recombinant proteins that did not require IPTG induction (p22, pA104R, pS273R, pE165R, pK205R, pA224L, pB385L, pB475R and pK196R of ASFV RSA/12/15 isolate) may also be linked to toxicity. Studies confirmed that the omission of IPTG

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from BL21 DE3 cells cultured in LB broth is an alternative method of producing membrane and secretory proteins (Zhang *et al*., 2015) implying that recombinant protein expression without IPTG induction is possible. The absence of IPTG decreased the recombinant protein concentration and may reduce the toxisity to the bacteria. These proteins could have been expressed in different hosts such as yeasts as yeasts have better tolerance.

Alternative expression systems that could be considered in future to express these recombinant proteins are the Vero cell line, recombinant baculoviruses (Rodriguez *et al*., 2004) and baculovirus transfer vector pBlueBac III (Neilan *et al*., 2004). Other than the Vero cells, cell lines such as the BL21-codonPlus expression competent cells (Rodriguez *et al*., 2004), Rosetta 2 (DE3) pLysS and Rosetta (DE3) pLacI2 host cells (Merk Millipore, 2017) can also be used. Biopharming, an agricultural plants system for the quick and easy production of useful molecules on a large scale can be used as well (Ahmad *et al*., 2012). Techniques such as auto induction system, a slow IPTG induction (Frangioni and Neel, 1993) in contrast to standard IPTG induction method can be utilised (Bujard *et al.,* 1987; Grabski *et al.,* 2005; Sreenath *et al*., 2005).

A subsequent study that investigates the immunological characteristics of all the recombinant proteins with emphasis on P22, pp220-F4, and pA104R, with at least 7 MHC typed domestic pigs that are infected with a less virulent strain is recommended. In parallel, an adenovirus vectored vaccine could be constructed using a chimera of P22, pp220-F4, and pA104R that could be tested in a group of three domestic pigs. Assays such as viral neutralization tests (VNT's) can be used to screen viral proteins for immunological activity. Cytokine ELISA can be used as well because cytokine transcription does not necessarily imply cytokine expression as was seen in this study with ELISpot. Cytotoxic T Lymphocyte (CTL) assays can also be included in future as a functional assay that indicates the ability of proteins to induce cytotoxic activity of T lymphocytes. The VNT's can be used to measure the efficiency of vaccines developed thereafter. Future studies with recombinant proteins from the same strain used to immunise the pigs in order to complement and expand the research findings to strain specific antigens are also recommended. Furthermore, the identified epitopes within the proteins of interest could be combined to formulate a chimera that could be tested in vaccine trials.

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Chapter 5

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APPENIX A: Materials and their sources

Bio-Rad	Agarose 12% Mini-PROTEAN® TGXTM precast SDS-PAGE gel SDS-PAGE loading buffer Stacking buffer Tris/SDS/glycine 10% SDS solution 30% Acrylamide solution Tetramethylethylenediamine (TEMED) solution 0.1% Tween 20
Elite	1% Milk powder
Invitrogen	Champion™ pET Directional TOPO expression kit
Invitek	Invisorb Spin Plasmid Mini Two
New England	Q5 Hot start 2X master mix
Novagen	Bug buster Benzonase Nuclease rLysozyme
Novex	Anti-His carboxyl-terminus (C-TERM) Horse radish peroxide (HRP) antibody 3, 3' 5, 5' –tetramethylbenzidine (TMB)
Mabtech	Porcine IFN- (PIFN-) ELISpot
Machery-Nagel	Protino® Ni^+ ted 2000 packed columns Protino® Ni^+ IDA 2000 packed columns
Merk Millipore	Acetone Ethanol
Pierce	BCA protein assay reagent kit
Promega	Ethidium Bromide stain Go Taq green 2X master mix
QIAGEN	RT^2 Profiler™ PCR Array for pig cytokine and chemokines RNeasy plus Universal Mini RT^2 First strand kit RT^2 SYBR Green master mix
Roche applied science	High pure PCR template preparation kit
Sigma-Aldrich	Ampicillin Isopropyl β D-1-thiogalactopryanoside (IPTG) Urea Concanavalin A Red blood cell lysis buffer Phosphate buffered saline (PBS) solution Hank's balanced salt solution Ficoll-histopaque® -1077 Alserver's solution 10% Dimethyl sulphoxide
Suppliers	Matetials and solutions
Thermo Scientific	PageBlue TM protein staining solution
Zymogen	ZymoGel DNA recovery kit

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APPENDIX B: Current Study's ARC-OVI animal ethics

AEC 18-15

APPROVED

ANIMAL ETHICS AT THE
ONDERSTEPOORT VETERINARY INSTITUTE

APPLICATION FOR MINOR MODIFICATIONS AND EXTENSIONS (M&E) TO A PREVIOUSLY APPROVED STUDY

Please note that major modifications and extensions to previously approved studies should rather be submitted as new applications on the correct form, whit a covering letter and termination report for the previously approved study.

NAME OF RESEARCHER: MR MABETLELA FREDDY MOKADI

DEPARTMENT: TADP

APPROVED STUDIS REFERENCE NUMBERS: AEC19.12 and AEC 10.12

PROJECT TITLE: Identification Of African Swine Fever Virus Proteins That Activates T-cell Immune Responses

NUMBER OF SPECIES OF ANIMALS ORIGINALLY APPROVED:	3	
NUMBER OF ADDITIONAL ANIMALS PREVIOUSLY ALLOCATED ON M&Es:	0	
NUMBER OF ANIMALS ALLOCATED TO THE PROJECT TO DATE:	3	
NUMBER OF ANIMALS USED TO DATE:	3	

SPICIFIC MODIFICATION / EXTENSION REQUESTED:

PBMC's isolated from the 2 pigs that forms part of AEC19.12 study and 1 pig that forms part of AEC 10.12 study to be used under the proposed research study entitled: Identification of African Swine Fever Virus Proteins That Activate Immune Responses under project number 30.01.V009. The PBMC's from this 3 animals will be stimulated with ASFV Ellsras 2012 and MAL2011/02 proteins that will be expressed and purified for immunological profiling using ELISpot and Custom Cytokine RT² Real Time Assays.

MOTIVATION FOR THE MODIFICATION / EXTENSION:

This will avoid re-infection of new pigs for the purpose of the proposed study will have financial implications. The proposed study is important as the study output may provide valuable information that can contribute towards developing ASFV vaccine.

DATE: 11-08-2015

SIGNATURE:

RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE

APPROVED

DATE: 17/09/2015

SIGNATURE:

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APPENDIX C: Current Study's UP animal ethics

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

14 April 2017

To whom it may concern
Study Administration
University of Pretoria

RE: Ethics Approval for Mr F Mabetlela (Student no 15402194)

Dear Sir/Madam

The letter serves to confirm that Mr F Mabetlela, under the supervision of Dr Mirinda van Kleef, has applied to the Office of Dean, Faculty of Veterinary Science) for exemption of the requirements to obtain ethics clearance for finalisation of his MSc project. After evaluating the documentation and discussion with senior academic personnel, a waiver from this requirement was granted as the candidate only made use of stored cells obtained from a study previously approved by the Animal Ethics Committee of the University of Pretoria.

This letter thus serves as confirmation that the candidate has complied with the ethical requirements for the conducting of research projects at the University of Pretoria.

If you have any concerns, please feel free to contact me.

Yours sincerely

Prof Vinny Naidoo
Deputy Dean: Research and Postgraduate Studies

Office of the Deputy Dean: Research and Postgraduate Studies
Room 6-7, Arnold Theiler Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8082
Fax +27 (0)12 529 8321
Email vinny.naidoo@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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APPENDIX D: Previous study's ARC-OVI animal ethics

APPROVED

AEC 19.12

ARC • LNR

Onderstepoort Veterinary Institute

Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

APPROVAL PERIOD: 2012 / 2013

PROJECT NUMBER:	OV24/01/P001			
PROJECT TITLE:	The role of *Ornithodoros* ticks in maintaining African swine fever virus			
PROJECT LEADER:	Carin Boshoff			
DIVISION:	Transboundary Animal Diseases Programme			
CATEGORY:	D			
	Domestic Pigs			
NUMBER OF ANIMALS:	3			
NOT APPROVED:				
APPROVED:	APPROVED			

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

SIGNATURE: Dr L. Lopez
CHAIRPERSON ANIMAL ETHICS COMMITTEE

DATE: 27-12-2012

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APPENDIX E: Previous Study's ARC-OVI Section 20

agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HerryG@daff.gov.za
Enquiries: Mr. Herry Gololo
Our Ref: 12/11/1/1
Your Ref No :

Carin Boshoff
Transboundary Animal Health Diseases Program
Private Bag X5
Onderstepoort
0110

Dear C. Boshoff

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 06 February 2013 requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa.
2. Ticks collected from the ASF control area of the country, must be sealed in individual sample jars with screw caps as primary packaging. These individual sample jars must then be placed in self-sealed labelled biohazardous bags as secondary packaging, and these bags placed in biohazardous safety boxes with silicone gasket and side locks as tertiary packaging;
3. Ticks collected from the ASF control area of the country, must be transported from the ASF control zone directly to OVI-TADP under Red cross Permit issued by the responsible State Veterinarian;
4. Only extracted DNA may be supplied by OVI-TADP to Inqaba biotech, Hatfield, Pretoria
5. If histopathology is to be conducted, only samples in formalin may leave OVI-TADP
6. As ASF is a controlled animal disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984), no information obtained during this study may be published or presented publically prior to approval by the Director Animal Health.

Title of research/study: "The role of *Ornithodoros* ticks in maintaining African swine fever virus. The aim of the study is to genetically characterise the genome of

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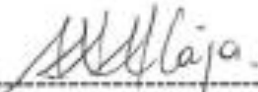
an ASFV occurring in a naturally infected tick and following its evolution by cycling the virus to a vertebrate host (the domestic pig) and back to a naïve uninfected tick, under experimental conditions. (TADP-S-13/04)"
Researcher (s): Carin Boshoff
Institution: Transboundary Animal Health Diseases Program
Your Ref./ Project Number: TADP-S-13/04
Our ref Number: 12/11/1/1

Kind regards



DIRECTOR: ANIMAL HEALTH

DATE: ---------------2013-04-22-------------------------

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

APPENDIC F: Previous study's UP animal ethics

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
ANIMAL ETHICS COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: EC011-13 (Revised)

8 April 2013

Dr L Heath
Onderstepoort Veterinary Institute
(heathl@arc.agric.za)

Dear Dr Heath

EC011-13 (Revised) **: The role of *Ornithodoros* ticks in maintaining African swine fever virus (*C Boshoff)***

Thank you for the revised application. The application for ethical approval dated 29 January 2013 is therefore approved by the Chairman of the Animal Ethics Committee on 5 April 2013.

Please provide the DAFF, Section 20 permit as soon as you have received it.

Kind regards

Elmarie Mostert

AEC Coordinator

Copy Mrs C Boshoff

Mrs A O'Niel

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APPENDIX G: Previous study's ARC-OVI animal ethics for control pig

ARC • LNR

APPROVED

Onderstepoort Veterinary Institute

Animal Ethics

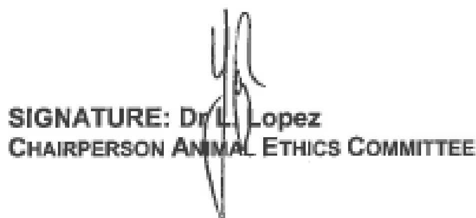
AEC 10.12

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

APPROVAL PERIOD: 20012/ March 2013

PROJECT NUMBER:	OV23/01/P001			
PROJECT TITLE:	Diagnosis of exotic diseases			
PROJECT LEADER:	Dr RM Dwarka			
DIVISION:	Transboundary Animal Diseases Programme			
CATEGORY:	D			
SPECIES OF ANIMAL:	Rabbits	Guinea pigs	Pigs	Lambs
NUMBER OF ANIMALS PER YEAR:	6	45	52	6
NOT APPROVED:				
APPROVED:				

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment



SIGNATURE: Dr L Lopez
CHAIRPERSON ANIMAL ETHICS COMMITTEE

DATE: 6/6/14

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