Evaluation of DNA vaccines against *Mycoplasma nasistruthionis* sp. nov. str. Ms03 infections in ostriches and the production of IgA heavy chain proteins

by
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Declaration

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Abstract

Ostrich products have internationally become very popular with South Africa being the world leader in this industry. An increase in the demand for ostrich products has influenced production strategies by intensifying rearing conditions through the use of feedlot systems. Intensive rearing creates ideal conditions for the spread of pathogens such as mycoplasmas, which is associated with a respiratory disease syndrome amongst feedlot ostriches. The three ostrich-infecting mycoplasmas, Ms01, Ms02 and Ms03, together with other secondary pathogens result in reduced production. Since there are no vaccines available against these ostrich-infecting mycoplasmas, three DNA vaccines have been developed in this laboratory. Each vaccine consisted of a eukaryotic expression vector (pCI-neo, VR1020 or VR1012) containing the Ms03 *oppA* gene as antigen.

The first objective of this study was to re-evaluate the anti-OppA immune response elicited by the pCI-neo and VR1020vaccines in ostriches. A vaccination trial was conducted and both vaccines were administered intramuscularly at 100, 600 and 1200 µg/ml doses followed by a booster vaccination. The anti-OppA immune response elicited by these vaccines in the ostriches was measured by means of the ELISA technique. All of the VR1020 vaccine doses as well as the 100 and 600 µg/ml doses of the pCI-neo vaccine were able to elicit a statistically significant anti-OppA immune response after administration of a booster vaccination.

Since mycoplasmas target the respiratory system of ostriches a mucosally administered vaccine should also be considered. Opposed to the intramuscular route of vaccination, which results in humoral immunity represented predominantly by IgG, mucosal administration would result in mucosal immunity represented by IgA production. For the measurement of IgA production, the ELISA requires secondary anit-IgA antibodies. Although the whole antibody is typically used for the production of secondary antibodies, it is possible to use only the region representing the heavy chain constant region. This can then be produced as a recombinant protein that will allow an easy reproducible source for the production of secondary antibodies. The second objective of this study was therefore to evaluate the baculovirus-insect expression system for the production of the ostrich IgA heavy chain constant region (IgA_H) protein, as this system will allow glycosylation of the protein product. The *IgA_H* gene was inserted into the pAB-6xHis™ transfer plasmid and together with the ProFold™-ER1 baculovirus used to co-transfect Sf9 insect cells for the production of ProFold™-ER1_*IgA_H* by means of homologous recombination. The IgA_H protein was successfully expressed as confirmed by using HisProbe-HRP as well as previously produced rabbit anti-ostrich IgA antibodies during western blot analysis.

Opsomming

Volstruisprodukte het internasionaal baie gewild geword en Suid-Afrika is die wêreld leier in hierdie industrie. Die verhoogde aanvraag na volstruisprodukte het 'n invloed op produksie strategieë deur intesivisering van grootmaak kondisies deur die gebruik van voerkrale. Intensiewe grootmaak kondisies skep ideale toestande vir die verspreiding van patogene soos mikoplasmas wat geassosieer word met ‘n respiratoriese siektesindroom in voerkraalvolstruise. Drie volstruis-infekterende mikoplasmas, Ms01, Ms02 en Ms03, tesame met ander sekondêre patogene het 'n afname in produksie tot gevolg. Aangesien daar geen entstof teen hierdie volstruis-infekterende mikoplasmas beskikbaar is nie, is daar drie DNS entstowwe in hierdie laboratorium ontwikkel. Elke entsof bestaan uit ‘n eukariotiese ekspressievektor (pCI-neo, VR1020 of VR1012) wat die Ms03 *oppA* geen bevat wat dien as antigeen.

Die eerste doelwit van hierdie studie was om die anti-OppA immuunreaksie, soos ontlok deur die pCI-neo en VR1020 entstowwe, te herevalueer. 'n Inentingsproef was deurgevoer waar beide entstowwe binnespiers toegedien was aan volstruise met onderskeidelik 'n 100, 600 en 1200 µg/ml dosis, gevolg deur 'n skraagdosis. Die gevolglike anti-OppA immuunreaksie was gemeet deur die ELISA tegniek. Alle VR1020 entstof dosisse asook die pCI-neo entstof se 100 en 600 µg/ml dosisse was daartoe in staat om 'n anti-OppA immuunreaksie te ontlok na die skraagdosis toediening.

Aangesien mikoplasmas die respiratoriese sisteem van volstruise teiken, moet die ondersoek na 'n mukosale entstof oorweeg word. In vergelyking met binnespierse toediening van ‘n entstof wat sal lei tot humorale immuniteit wat oorwegend verteenwoordig word deur IgG, sal mukosale toediening lei tot mukosale immuniteit wat verteenwoordig word deur IgA produksie. Vir die meting van IgA produksie benodig die ELISA sekondêre anti-IgA teenliggaampies. Alhoewel die teenliggampie normaalweg in geheel gebruik word vir die produksie van sekondêre teenliggaampies, is dit moontlik om slegs die gedeelte wat die swaarketting konstante area verteenwoordig te gebruik. Dit kan dan as ‘n rekombinante proteïen geproduseer word wat sal dien as 'n maklike reproduseerbare bron vir die produksie van sekondêre teenliggaampies. Die tweede doelwit van hierdie studie was dus om die baculovirus-insek ekspressiesisteem te evalueer vir die produksie van die volstruis IgA swaarketting konstante area (IgA_H) proteïen aangesien die sisteem glikosilering van die proteïen toelaat. Die *IgA_H* geen was ingevoeg in die pAB-6xHis™ oordragplasmied en tesame met die ProFold™-ER1 baculovirus gebruik om Sf9 insekselle te infekteer vir die produksie van ProFold™-ER1_ *IgA_H* deur middel van homoloë rekombinasie. Die IgA_H proteïen was suksesvol uitgedruk soos bevestig deur HisProbe_HRP en voorheen geproduseerde konyn anti-volstruis IgA teenliggampies deur middel van 'n western-klad.

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Abbreviations

%C	amount of cross-linker
%T	acrylamide present
ABC	ATP-binding cassette
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AcMNPV	*Autographa californica* multicapsid nucleopolyhedrovirus
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
ATP	adenosine triphosphate
BALT	bronchus-associated lymphoid tissue
BDB	bisdiazobenzidine
BGH	bovine growth hormone
BLAST	basic local alignment search tool
BmNPV	*Bombyx mori* nucleopolyhedrovirus
BSA	bovine serum albumin
BV	budded virus
CAF	central analytical facility
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CUT	carbohydrate uptake transporter
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
Fc	fragment crystallisable region
GALT	gut-associated lymphoid tissue
GC	green control
GFP	green-fluorescent protein
GST	glutathione S-transferase
HMW	high-molecular weight
HOAc	acetic acid
HRP	horseradish peroxidase
ID	intradermal
IM	intramuscular
IDT	integrated DNA technologies
IFN-γ	interferon gamma
Ig	immunoglobulin
IgA	immunoglobulin A
IgA_H	immunoglulubin A heavy chain constant region
IgD	immunoglobulin D
IgG	immunoglobulin G
IL	interleukin
IPTG	isopropyl β-D-1-thiogalactopyranoside
IV	intravenous
L	liter
LB	Luria Bertani
LPS	lipopolysaccharide
LSD	least significant difference
m/v	mass per volume ratio
MALT	mucosa-associated lymphoid tissue
MetOH	methanol
MG	*Mycoplasma gallisepticum*
MHC I	major histocompatibility complex class I

MHC II	major histocompatibility complex class II
Ms01	*Mycoplasma struthionis* sp. nov. str. Ms01
Ms02	*Mycoplasma* sp. Ms02
Ms03	*Mycoplasma nasistruthionis* sp. nov. str. Ms03
NC	no control
NCBI	national centre for biotechnology information
NK	natural killer cells
OB	occlusion body
OD	optical density
ODV	occlusion derived virus
OPD	*o*-phenylenediamine
Opp	oligopeptide permease
OppA	oligopeptide ABC transporter substrate-binding domain
OppB	oligopeptide ABC transporter subunit, membrane component, B
OppC	oligopeptide ABC transporter subunit, membrane component, C
OppD	oligopeptide ABC transporter subunit, ATP-binding component, D
OppF	oligopeptide ABC transporter subunit, ATP-binding component, F
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline tween
PCR	polymerase chain reaction
pDNA	plasmid deoxyribonucleic acid
pNPP	*p*-nitrophenyl phosphate
poly (A)	polyadenylation
PTP	protein tyrosine phosphatase
RE	restriction enzyme
rpm	resolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SAP	shrimp alkaline phosphatase
SC	subcutaneous
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-S	disulfide bond
SV40	simian virus 40
TB	terrific broth
TEMED	tetramethylethylenediamine
Th	T helper cell
TLR	Toll-like receptors
TMB	3,3',5,5' -tetramethylbenzidine
TPA	tissue plasminogen activator
UT	untransfected
UV	ultra violet
v/v	volume per volume ratio
VPN	veterinary procedural notice
WASP	Wiscott-Aldrich syndrome protein

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1. General introduction

The ostrich industry in South Africa is the world leader with a 75% global market share. This industry dominates in the Klein Karoo region of the Western Cape as well as regions of the Southern Cape that are associated with dryer climates and winter rainfall. Oudtshoorn, in the Klein Karoo region, is known as the ostrich capital due to the amount of ostriches reared and slaughtered there. This industry generates 20 000 jobs in South Africa and export of ostrich products contributes R1.2 billion annually to the economy (DAFF, 2014).

The three main products produced from ostriches include meat, leather and feathers. Ostrich meat is considered healthy and highly nutritive and there has been an increased demand for ostrich meat from especially the European Union as they are the biggest consumer of South African exported meat. As the demand for ostrich products, and specifically meat, increased over time, the rearing of ostriches became more intensive with the resulting use of feedlot systems also referred to as feeding camps. These intensive rearing systems create ideal conditions for the spread of pathogens with resulting disease outbreaks. One of the pathogens found to be problematic amongst feedlot ostriches is mycoplasmas (Botes et al., 2005; Poławska et al., 2011; DAFF 2014). Three ostrich-infecting *Mycoplasma* species were identified in South Africa by Botes et al. (2005) which were provisionally named Ms01, Ms02 and Ms03. In 2009 Langer isolated mycoplasmas from Namibian ostriches with 16S rRNA sequences that matched that of Ms01 and Ms03. Langer (2009) named Ms01 *Mycoplasma struthionis* sp. nov. as this strain was isolated from the lungs of the ostriches and Ms03 *Mycoplasma nasistruthionis* sp. nov. as this strain was isolated from the nasal cavity of the ostrich. The description by Langer (2009) has not yet been published and therefore not formally accepted. For the sake of simplicity the ostrich-infecting mycoplasmas will be referred to as Ms01, Ms02 and Ms03 in the rest of this thesis.

In ostriches mycoplasmas are associated with upper respiratory tract infections which are mostly observed during seasonal changes and can be exacerbated due to stress and environmental conditions (Verwoerd 2000; Botes 2004). Ostrich-infecting mycoplasmas together with other pathogens can results in the loss of ostrich stocks, a reduced growth rate with resulting reduced production and downgrading of carcasses. Due to the impact of mycoplasma infections on ostrich production, strategies need to be developed to manage these organisms (Botes, 2004; Botes et al., 2005; Verwoerd, 2000). Mycoplasma infections can be managed with good biosecurity practices, however, some ostriches might appear to be healthy although they are carriers of the organism. Medication such as antibiotics is used to treat existing infections but antibiotics do not eliminate the mycoplasmas. Antibiotics are also expensive and long periods of antibiotic treatment can result in

traces of antibiotic residues in the meat which makes the meat unsuitable for export (Kleven, 2008; Olivier, 2006; Stipkovits and Kempf, 1996). Hence, other approaches are needed to control mycoplasma infections such as vaccination.

Several vaccines are commercially available for use against mycoplasma species in different animal species. These include vaccines such as Stellamune One (Pfizer Animal Health), Suvaxyn® MH One (Zoetis) and Respisure® (Zoetis). These formulations have been developed for different routes of administration and the route of administration does not necessarily correlate with the route of infection. For example Myco-Bac® B (Texas Vet alb) is administered subcutaneously in the side of the neck but is for the prevention of respiratory disease in cattle. Other examples include Ingevac 3FLEX (Boehringer) which aids in prevention of respiratory disease in pigs but is administered intramuscularly.

Currently there is no registered vaccine available for use against mycoplasma infections in ostriches therefore the development of a vaccine that can be administered either intramuscularly, intradermally or mucosally should be investigated. The main types of vaccines that could be considered for development included live attenuated, inactivated whole cell, subunit vaccines and DNA vaccines. Live attenuated and inactivated whole cell vaccines, however, require cultivation of the organism in large quantities and subunit vaccines require an adjuvant. As ostrich-infecting mycoplasmas are difficult to cultivate and some subunit vaccines were shown to aggravate the symptoms of infections, these vaccines were eliminated as options. Attention was subsequently turned to DNA vaccines which have become more popular as they do not require the cultivation of the organism in large quantities. They are also inexpensive, regarded as easy to manufacture and safe. Similar to live attenuated vaccines they also have the ability to stimulate both humoral and cellular immunity (Bellamy and Freedman, 2005; Bosch and Frey, 2003; Ingolotti et al., 2010; Kumaragurubaran and Kaliaperumal, 2013).

1.1 Evaluation of DNA vaccines

In a previous study by Wium (2015) three DNA vaccines against Ms03 were developed and evaluated. The *oppA* gene, which codes for part of an ABC peptide transporter, was selected as the immunogenic gene and cloned into the pCI-neo, VR1020 and VR1012 vaccine plasmids. A field trial was conducted in which a single dose of all three vaccines was respectively administered intramuscularly to ostriches, but the trial had to be prematurely terminated due to an avian influenza outbreak. Despite this, preliminary results indicated that an immune response was elicited by both the pCI-neo_*oppA* and VR1020_*oppA* vaccines and therefore the use of DNA vaccines in ostriches

appears to be a viable approach. However, in a study done by De Wet (2015) on vaccination of ostriches against Ms01 infections, it was found that the dose as used by Wium (2015) was not necessarily sufficient. The first objective of this study was therefore to re-evaluate the ability of the pCI-neo_*oppA* and VR1020_*oppA* vaccines developed against Ms03 to elicit an anti-OppA antibody response in ostriches at different doses. To achieve this objective, the following aims were set:

- To perform a vaccination trial in ostriches with the pCI-neo_*oppA* and VR1020_*oppA* DNA vaccines at three different doses including a booster vaccination.
- To analyse the anti-OppA antibody response using the enzyme-linked immunosorbent assay (ELISA).
- To monitor mycoplasma infections during the trial using PCR-analysis.

1.2 Production of the IgA heavy chain constant region protein

Given that mycoplasmas target the respiratory system of ostriches, the future development of a mucosal vaccine should also be considered. Compared to an intramuscular route of vaccination that results in the production of mainly IgG, a mucosally administered vaccine would result in the production of a different antibody class as the mucosal surfaces are protected by IgA antibodies. Therefore the ELISA developed to measure humoral antibodies against the OppA antigen of Ms03 would have to be adapted to detect anti-OppA IgA antibodies. For this purpose, secondary anti-IgA antibodies would be required. Previous isolation of IgA from ostrich nasal samples for the production of secondary antibodies proved to be largely unsuccessful yielding low quantities of this protein. Instead of isolating the whole antibody, the protein representing the IgA heavy chain constant region (IgA_H) can be produced *in vitro* and subsequently used for the production of secondary antibodies. The IgA_H contains three glycosylation sites which plays a role in their function as well as in prevention from degradation. Therefore using a eukaryotic expression system capable of post-translational modifications would be preferable (Huang et al., 2012; Schroeder and Cavacini, 2010). The second objective of this study was therefore to evaluate the use of a baculovirus-insect expression system for the production of the IgA_H protein. The aims set to achieve this objective were as follows:

- To sub-clone the *IgA_H* gene into the pAB-6xHis™ transfer vector.
- To generate a recombinant baculovirus by means of homologous recombination.
- To transfect an insect cell line and produce the IgA_H protein.
- To verify the expression of the IgA_H protein.

1.3 Thesis layout

A literature review relevant to the first and second objective of this study will be presented in Chapter 2 which includes an overview of mycoplasma metabolism, pathogenesis and control. Mycoplasmas specifically infecting the ostrich are discussed as well as management of these organisms using vaccines and the measurement of immune responses. The IgA antibody structure and baculovirus-insect expression system for production of proteins is also discussed in this chapter.

Results obtained from the evaluation of two DNA vaccines during a vaccination trial are then presented in Chapter 3 followed by the results obtained from the production of the protein representing the IgA_H in a baculovirus-insect expression system as described in Chapter 4.

Finally the conclusions and future perspectives of this study are given in Chapter 5 followed by the reference list. At the end of this thesis are Addenda containing the results of the statistical analysis of the ELISA data as well as data used for the analysis (Addendum A), an alignment of the ostrich genome and the *IgA_H* gene (Addendum B) and lastly an alignment of the IgA_H secretory and transmembrane proteins with an indication of the domains (Addendum C).

2. Literature Review

2.1 Introduction

The first objective of this study was to evaluate the immune response, elicited by two DNA vaccines against mycoplasma infections in ostriches. The literature review therefore includes a brief overview of the immune system as well as a discussion of different kinds of vaccines that can be used to elicit immune responses with a specific focus on DNA vaccines as was used in this study. An overview is then given of the enzyme-linked immunosorbent assay (ELISA) as measurement technique for the evaluation of vaccine induced immune responses. The second objective of this study was to produce a protein representing the constant region of the IgA antibody which would allow adapting the ELISA for the detection of mucosal immunity. An overview is therefore given of the structure of antibodies and specifically IgA followed by the baculovirus-insect expression system as a method for the production of immunoglobulin proteins. The literature review is concluded with a discussion on mycoplasmas, their pathogenicity, the control of ostrich-infecting mycoplasmas and DNA vaccine strategies previously developed in this laboratory.

2.2 The immune system

As background for later discussions on mycoplasma pathogenicity and specifically the mode of action of vaccines as a control measure against these organisms, it is important to understand how the immune system of a vertebrate host functions to provide protection against pathogens. To this end an overview of innate and adaptive immunity is given.

2.2.1 Innate Immunity

Innate immunity provides initial protection against infections. It was previously believed that innate immunity is nonspecific, weak and ineffective regarding its ability to protect against infections. It is now known that the innate immune system targets microbes specifically and is an effective early defence mechanism able to control and eliminate infections before the activation of the adaptive immunity (Abbas et al., 2014).

For the innate immune system to function effectively it consists of components that have different but complimenting roles, including the prevention of microbes to enter the host as well as eliminating those that succeed to enter the host. The components responsible for these functions are epithelial barriers, phagocytes (neutrophils and monocytes/macrophages), dendritic cells, natural killer (NK) cells, cytokines and plasma proteins (including compliment system proteins) (Abbas et al., 2014).

The innate immune system becomes activated by recognising conserved features of the invading pathogen (Alberts et al., 2002). In order to indicate that these conserved features, mentioned above, are shared by microbes of the same type they are referred to as pathogen-associated molecular patterns (PAMPs). One example is bacterial lipopolysaccharides (LPS), also known as endotoxins, which are present in the cell wall of Gram-negative bacteria species but mammalian cells do not produce them. Similarly, bacteria contain surface glycoproteins with a terminal mannose residue which is absent in mammalian surface glycoproteins. Double-stranded RNA present in many viruses, as well as unmethylated CpG oligonucleotides, found in microbial DNA, are not found/abundant in mammalian cells and are therefore recognised by phagocytes (Abbas et al., 2014).

The innate immune system comprises of receptors that bind specifically to microbial molecules or PAMPs. A major class of these receptors is known as toll-like receptors (TLRs) which are able to recognise different microbial products such as nucleic acids or bacterial cell wall. Some TLRs are situated on the cell surface of the host cells and others are found in endosomes. Binding of TLRs with microbial products results in the expression of certain genes due to activation of transcription factors. The expressed genes encoding cytokines and enzymes aids activated phagocytes and dendritic cells to fulfil their function which is to terminate micro-organisms. Other receptors such as the cell surface receptor for *N*-formyl methionine and the mannose receptors to name a few, also form a part of the innate immune system (Abbas et al., 2014).

2.2.2 Adaptive immunity

Although the function of the innate immune system entails early defence against infections, it also provides secondary signals which lead to the activation of the adaptive immune response. Compared to innate immunity, adaptive immunity is known as the slower process of defence against infections but it mediates a more specific and thereby effective defence in comparison to the innate immune system (Abbas et al., 2014).

The components of the adaptive immune system that will defend the host against microbes consist of lymphocytes. The lymphocytes can be sub-divided into B-lymphocytes (B-cells), NK cells and T-lymphocytes (T-cells). T-cells can further be activated into T-helper cells (Th) and cytotoxic T-lymphocytes (CTL) (Abbas et al., 2014).

In order for the adaptive immune system to be effective against infections, it has several properties including specificity, diversity, clonal and memory expansion. The functional significance of the specificity of the adaptive immune system is that a distinct antigen will be able to elicit a specific

response. However the functional significance of diversity is the ability of the adaptive immune system to elicit an immune response against a variety of antigens. For the adaptive immune system to keep up with the rapidly dividing pathogens or microbes, the activated lymphocytes must proliferate rapidly resulting in numerous clonal progeny cells. All of the cells are specific for the same antigen and this process is known as clonal expansion (Abbas et al., 2014).

The adaptive immune response is initiated in compartments in which the T- and B-cells are matured and these compartments include the peripheral lymph nodes, the spleen and the mucosal lymphoid system. The lymph nodes and spleen responds to antigens that have entered the tissues and/or blood whereas the mucosal lymphoid system respond to pathogens that have breached the epithelial layer of mucosal surfaces (Abbas et al., 2014; Janeway et al., 2001).

The adaptive immune system is able to recognise and differentiate between many antigens or portions of antigens and consists of two types, humoral and cellular immunity. The humoral immune response (mediated by B-cells) forms a very important part of the adaptive immune system as it is able to respond to different types of molecules, whereas the cellular immune response (mediated by T-cells) recognises and respond only to protein antigens. Each type of adaptive immunity is mediated by different cells and molecules for defence against microbes in the blood, and microbes inside infected cells. Protection is mediated by the humoral immune response also known as the production of antibodies. Protection against intracellular infections is mediated by cellular immunity or the cell-mediated response. For some diseases either a humoral or a cellular immune response would be sufficient for protection against a pathogen but for other diseases both humoral and cellular responses are required (Abbas et al., 2014; Gurunathan et al., 2000).

The primary immune response is the first response elicited, after exposure to an antigen for the first time and naive B-cells or T-cells are then activated to give a primary immune response. A small part of the activated B-cells or T-cells do not differentiate into antibody secretion cells (plasma cells) or effector T-cells and become known as memory cells. These cells circulate in the blood and are found in various tissues and survive for months to years until the immune system is exposed to the same antigen again. A repeated encounter with the same antigen will result in a more rapid and larger immune response than the primary immune response and is facilitated by the memory cells. This is known as the secondary immune response (Abbas et al., 2014). The memory feature allows the immune system to react in a more effective manner against antigens that it encounters repeatedly. This is illustrated by the immune response pattern of B-cells as shown in Figure 2.1.

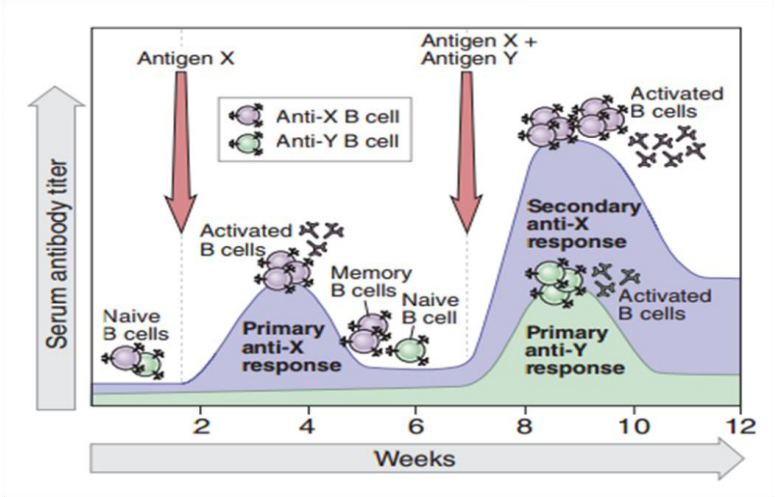

Figure 2.1 Memory feature of the adaptive immune response results in a primary response with the first encounter of an antigen X and a more rapid secondary response for the second encounter of the same antigen (Abbas et al., 2014).

2.2.2.1 Humoral Immunity

Humoral immunity is mediated by B-cell produced antibodies able to neutralise and eliminate invading pathogens. B-cells are activated by epitopes/antigenic determinants (the part of the antigen that the immune system is able to recognise) that bind to the B-cell antigen receptors (antibodies) and initiate an immune response (Abbas et al., 2014).

Some B-cells differentiate into plasma cells which secrete antibodies containing the same antigen binding sites as those that originally recognised the antigen. Activated B-cells are able to secrete different classes of antibodies after differentiating into plasma cells, for example IgM, IgG, IgE etc. The different antibody classes secreted by the plasma cells each possess their own distinct functions. The humoral immune response is able to prevent infections by targeting microbes for phagocytosis, by neutrophils and macrophages and/or by coating the microbes with antibodies and thereby eliminating the microbes (Abbas et al., 2014).

Globular protein epitopes are not able to bind to many antigen receptors and in order for a full B-cell response to be elicited Th cells are required. The B-cells therefore have to phagocytose the protein, degrade and display the peptides bound to Major Histocompatibility Complex (MHC) class II molecules for the Th cells and its $CD4^+$ co-receptor to recognise the globular proteins. Once the Th cells recognise the peptides presented by the B-cells, the Th cells expresses cytokines and cell surface proteins which activate the B-cells (Abbas et al., 2014).

2.2.2.2 Cellular Immunity

Cellular immunity is responsible for the elimination of intracellular microbes which is mediated by T-cells. These intracellular microbes may be phagocyte ingested microbes or microbes that have infected non-phagocytic cells. In the case of ingested microbes the intracellular antigens will be processed by the cell and presented on the surface of the cell in combination with MHC II molecules and in the case of infected cells, processed antigen will be displayed in combination with MHC I molecules. T-cells have antigen receptors that mainly recognise processed antigens bound to MHC molecules on the surface of these antigen presenting cells (APCs) (Abbas et al., 2014; Luckheeram et al., 2012).

Naive $CD4^+$ Th cells and naive $CD8^+$ CTLs are activated upon recognition of antigen peptides bound to MHC class II and I complexes respectively. Activation is initiated with the help of costimulator signals from antigens and cytokines (Abbas et al., 2014; Luckheeram et al., 2012; Murphy et al., 2012).

Th cells promote antibody production as well as removal of antigen. As Th cells produce cytokines and cell surface molecules that are able to bind to receptors on B-cells and macrophages they promote the production of B-cell antibodies or activation of macrophages. Th cells also have the ability to activate neutrophils resulting in the elimination of microbes. The Th cells may differentiate into subdivisions known as Th-1, Th-2 and Th-17 cells. Each produces different cytokines which distinguishes the groups from one another. IFN-γ, produced by Th-1 cells, is a potent activator of macrophages and influences the production of antibody. These antibodies will further enhance phagocytosis of microbes, as the antibodies bind directly to phagocyte Fc receptors. Th-2 cells produce IL-4, IL-5 and IL3. The function of IL-4 includes differentiation of Th cells into Th-2 cells as well as stimulation of non-compliment activating IgG subclasses and IgE production where IgE combats parasitic worms. IL-5 supports growth and differentiation of activated B-cells and bone marrow derived granulocytes whereas IL-3 promotes the expansion of immature bone marrow progenitors of all blood types. Th-17 cells aids in the protection against bacterial and fungal infections as they secrete cytokines such as IL-17 and IL-22 which are responsible for recruiting neutrophils as well as monocytes to sites where antigens was recognised (Abbas et al., 2014; Gurunathan et al., 2000; Takatsu and Nakajima, 2008).

Initiation of the activation of naive $CD8^+$ CTL requires cross presentation of cytoplasmic antigens by dendritic cells (DCs) which is a somewhat unusual feature. Another unusual feature is that differentiation of naive $CD8^+$ CTLs may require the accompanying activation of the $CD4^+$ Th. The

function of CTLs is to remove infections by killing infected cells and therefore eliminating microbes that are concealed in the infected cells cytoplasm. By completely eliminating the infected cells the source of the infection is removed. CTLs that have been generated in the lymphoid organ may target the sites where the antigen or microbe is present by migrating into the blood. At sights of infection the differentiated CTL cells are activated, by the antigen present, and execute the wanted function of eliminating microbes (Abbas et al., 2014).

2.2.2.3 The adaptive immune system in the mucosal lymphoid system

The mucosal immune system has several functions including protection of the mucous membrane, by preventing invasion and colonisation of microbes, and preventing the uptake of antigens i.e. foreign proteins ingested from food and airborne matter amongst others (Holmgren and Czerkinsky, 2005). Pathogens generally enter the host through a barrier, consisting of the external located epithelium and the underlying connective tissue, separating the internal environment from the external environment (Fellah et al., 2008). The surface layers of the eyes, respiratory, digestive and urogenital tracts are lined with epithelial cells. The epithelial layer together with the underlying tissue are known as the mucosa, mucosal membrane or the mucosal surface (Lundgren, 2004). These surfaces are protected by the innate and adaptive mucosal immune system against infections from the environment.

The respiratory, gastrointestinal and genitourinary tracts are guarded immunologically by mucosa-associated lymphoid tissues (MALT) which includes the gut-associated lymphoid tissue (GALT) and the bronchial-associated lymphoid tissues (BALT) (Fellah et al., 2008). MALT is a separate immunologic secretory system mediated by sub-epithelial clusters of lymphoid tissue. The MALT system immerses the mucosal surfaces with protective IgA antibodies produced by B-cells and thereby provide protection. Therefore the mucosal immune system is the main defense mechanism at the mucosal surface and protects these surfaces by means of secretory IgA through the mucosal immune system (Rabson et al., 2005).

2.3 Vaccines

In order to control infections and subsequent diseases the immune response needs to be stimulated. This can be done by means of vaccination as this process is the most effective way to control diseases, and uses non-pathogenic forms of microbes for the stimulation of the adaptive immune response (Abbas et al., 2014). Vaccines stimulate adaptive immunity allowing the host to fend off pathogens and thereby minimising diseases on farms which results in an increased productivity (Mäkelä, 2000; Marangon and Busani, 2006).

In the 17th century, Edward Jenner introduced the concept of vaccination when he was able to show that a previous exposure to cowpox prevented infection by smallpox as reviewed in Gurunathan et al. (2000). About 80 years elapsed before Louis Pasteur discovered that he could attenuate a bacterium for vaccine purposes. Killed or inactivated vaccines were developed by Daniel Salmon and Theobald Smith and used for vaccination against typhoid, cholera and plague (Plotkin and Plotkin, 2011). The germ theory was established by Pasteur, Koch, Ramon, and Mérieux and they developed the vaccines known as live attenuated, inactivated or killed pathogens, and inactivated toxins. These developments led to the first golden age of vaccines as these vaccines protected against a number of illnesses including rabies, tuberculosis in infants, diphtheria and many more (Delany et al., 2014).

In the 20th century the main types of vaccines in use are live attenuated, inactivated whole-cell, toxoid, subunit and DNA vaccines. Live attenuated vaccines are prepared by passaging or culturing. The weakened pathogens are, however, still able to infect and replicate within their hosts as they contain bacteria or viruses with reduced virulence. These vaccines are able to elicit both humoral and cellular immunity and although they are able to produce long term immunity they impose a risk of reverting back to their pathogenic form. Culturing and inactivating some pathogens proved to be a tedious tasks and in some cases it is not possible (Bellamy and Freedman, 2005; Hasson et al., 2015; Ingolotti et al., 2010; Kumaragurubaran and Kaliaperumal, 2013).

Inactivated whole cell vaccines consist of micro-organisms that have been inactivated through physical (γ-rays) or chemical means. These inactivated vaccines are still able to evoke an immune response although they are not able to infect or replicate within the host. They therefore require multiple doses (boosters) or adjuvants in order for them to produce long-term immunity. This results in higher production costs and as these vaccines might require adjuvants they are more expensive to manufacture. They are also only able to elicit a humoral immune response. Inactivated whole cell vaccines do not hold any risk for reverting back to its pathogenic form unlike live attenuated vaccines (Bellamy and Freedman, 2005; Ingolotti et al., 2010; Meeusen et al., 2007).

Subunit vaccines consist of antigenic proteins of pathogens including flagella or surface proteins. Manufacturing of these vaccines can be on large scale although they require repeated administration with adjuvants and are only able to induce a humoral immune response (Bellamy and Freedman, 2005; Ingolotti et al., 2010).

Toxoids are bacterial toxins that have been made non-toxic or are inactivated, but are still immunogenic. Although they are able to induce a humoral immune response they require boosters

and also have possible adverse side-effects. Manufacturing of these vaccines also holds obstacles regarding the detoxification process (Bellamy and Freedman, 2005; Ingolotti et al., 2010).

DNA vaccines consist of a DNA sequence encoding the desired antigen that is molecularly cloned into a eukaryotic plasmid expression vector. These vaccines are non-live vaccines and therefore they do not hold the risk of reverting back to a pathogenic form. Manufacturing of DNA vaccines can be done on large scale with ease. DNA vaccines are also able to elicit both a cellular and humoral immune response, however, a drawback of DNA vaccines is the possible need for adjuvants due to reduced immunogenicity (Hasson et al., 2015; Kutzler and Weiner, 2008). A summary of the main types of poultry vaccines is given in Table 2.1.

Table 2.1 Overview of the main type of vaccines used for poultry purposes.

Type of vaccine	Composition	Immunity Induced	Example
Live attenuated vaccines	Contain viruses or bacteria that have been made avirulent	Humoral and cellular immunity	Nobilis® IB Ma5 * (prevents infectious bronchitis in poultry)
Inactivated whole cell vaccines	Inactivation of micro-organisms by means of physical or chemical methods	Humoral immunity	MG bacterin (prevention of clinical signs of *Mycoplasma gallisepticum* in poultry)
Subunit vaccines	Antigenic proteins of micro-organisms (for example flagella)	Humoral immunity	CoxAbic® * (prevention of coccidiosis in poultry)
Toxoids	Bacterial toxins that have been inactivated	Humoral immunity	NetVax (prevention of Necrotic enteritis in poultry)
DNA vaccines	Expression vector containing the gene encoding the desired immunogen	Humoral and cellular immunity	West Nile-Innovator® (Prevention of West Nile Virus infections in horses)

* Available in South Africa.
Compiled from Bellamy and Freedman (2005), Belli et al. (2009), Crouch et al. (2010), Kjærup et al. (2014), Kumarangurubaran and Kaliapernumal (2013), Pereira et al. (2014) and Ferguson-Noel and Williams (2015).

2.3.1 DNA vaccines

In the 1990's, the public's attention was turned to DNA vaccines as Tang and Johnston attempted to deliver human growth hormone, as gene therapy, into mice. DNA vaccines are known as the simplest vaccines as they consist of genes encoding the antigen rather than consisting of the antigen itself (Donnelly et al., 2005). It has been shown in the past decade and a half that this non-live vaccine approach is a simple way to induce an immune response, evoking a humoral and cellular immune response (Hasson et al., 2015; Kutzler and Weiner, 2008).

DNA vaccines are the simplest yet safest approach to use as they overcome safety concerns associated with live vaccines as well as the risks involved in the manufacturing of killed vaccines (Gurunathan et al., 2000; Kutzler and Weiner, 2008; Pereira et al., 2014). In order to be able to produce a vaccine the vaccine plasmid containing the immunogenic gene of interest needs to be amplified therefore the plasmid is transformed into bacteria. The bacteria are grown leading to amplification of the plasmid, followed by purification of the plasmid from the bacterial culture. The purified vaccine plasmid can then be inoculated into the animal where cells will take up the DNA and produce the antigenic protein (Gurunathan et al., 2000).

Proteins that are synthesized in the cells after DNA vaccination have the ability to access pathways for presentation by both class I and II MHC molecules. Similar to live-attenuated vaccines, DNA-vaccines are able to induce both the humoral and cellular immune response but, unlike live-attenuated vaccines, DNA vaccines only encode selected components of the pathogen and therefore there is no risk for reverting back to a pathogenic form and thereby infecting the host (Gurunathan et al., 2000). Although no DNA vaccines for human use have been licensed, there are a small number of licensed DNA vaccines for veterinary use as listed in Table 2.2

Table 2.2 Licensed veterinary DNA vaccines.

Product name	Species	Target
Apex-IHN®	Salmon	Infectious Haematopoietic necrosis virus (IHNV)
LifeTide®- SW5	Swine	Growth hormone releasing hormone (GHRH)
Oncept™	Dog	Melanoma
West Nile-Innovator®	Horses	West Nile Virus

Adapted from Pereira et al. (2014)

2.3.2 Characteristics of DNA vaccines

2.3.2.1 Characteristics of the expression vector

DNA vaccine vectors combine prokaryotic and eukaryotic regions enabling the propagation of the DNA vaccine in bacterial cultures such as *Escherichia coli* as well as the expression of the gene of interest in the eukaryotic host (Williams, 2013). For a DNA vaccine to be functional it should contain the following:

Firstly, transcriptional control elements for expression of the gene of interest in eukaryotic cells. For the production of the protein from the DNA vaccine, the plasmid should contain highly active transcriptional control elements. Most vaccine plasmids contain sequences from the immediate early region of the cytomegalovirus (CMV) as enhancer and promoter areas. The presence of CMV intron A in the upstream promoter sequence of the desired plasmid is effective for expression of

microbial cDNA's and would likely provide transcripts with signals allowing the transcripts to be processed and transported as eukaryotic mRNA (Gurunathan et al., 2000; Robinson and Torres, 1997).

Secondly, a polyadenylation sequence allowing for stabilisation of the mRNA transcripts. Polyadenylation, the addition of a poly (A) tail to mRNA transcripts, is of significant importance for nuclear export as well as translation of the mRNA transcript. A gene sequence from bovine growth hormone (BGH) is known as the most frequently used sequence for polyadenylation but simian virus 40 (SV 40) sequences can also be used (Gurunathan et al., 2000).

Thirdly, an origin of replication for multiplication of the plasmid in bacterial cells. The origin of replication allows for multiplication and maintenance of plasmids in host cells and for stable plasmid production during bacterial growth. The goal of the origin of replication is the production of a substantial number of copy DNA plasmids as well as high purification yields in a short time (Pereira et al., 2014).

Fourthly, a selectable marker usually a bacterial antibiotic resistance gene allowing for selection of the DNA vaccine during bacterial culture. Bacterial antibiotic resistance genes ensure stable production of the plasmid of interest and some of the most widely used antibiotic resistance genes are the kanamycin and ampicillin genes (Gurunathan et al., 2000; Pereira et al., 2014; Williams, 2013).

2.3.2.2 Characteristics of vaccine candidate genes

A vaccine target should not contain homology with any protein of the host as the host can respond on this in two ways. Firstly, an autoimmune response can be induced in the host due to the vaccine which can result in autoimmune disease and secondly the host can be made tolerant to the vaccine hence no immune response would be induced (Flower and Perrie, 2013).

The antigen should not be able to mutate rapidly (hypermutable) nor should it be involved in antigen variation. The epitope or antigenic determinant should not be masked and thereby prevent an effective immune response (Liu, 2011; Rueckert and Guzmàn, 2012). Neutralisation, by vaccination, of factors that enable the pathogen to access the host as well to survive in the host may offer protection. Therefore, genes that are essential for the viability of the pathogen will make a good vaccine target (Allan and Wren, 2003).

2.3.3 Mode of action of DNA vaccines

The ability of a DNA vaccine to elicit an efficient immune response can be influenced by the concentration and by the type of APCs transfected. DNA vaccination is able to induce a B-cell response, Th responses as well as CTL responses. Depending on the route of vaccination, the injected DNA enters the nucleus of, for example, myocytes, keratinocytes or APCs. Genes encoded on the plasmid are transcribed and translated for the generation of the protein of interest (Donnelly et al., 2000; Gurunathan et al., 2000; Mäkelä, 2000). In order for a DNA vaccine to elicit an immune response the antigen encoded by the plasmid DNA is processed and transiently presented by at least three different mechanisms:.

I. Direct transfection of myocytes, keratinocytes or any MHC class II-negative cells: injection of DNA into the muscle or skin of the organism will most probably result in transfection of somatic cells. The somatic cells will present produced and processed antigenic proteins via the MHC class I pathway to CTLs (Gurunathan et al. 2000).

II. Direct transfection of professional APCs: due to intramuscular injection, a small amount of APCs can be transfected which are able to present produced and processed antigenic proteins via both the MHC class I and II pathways to CTLs and Th respectively (Gurunathan et al., 2000).

III. Cross-priming: antigenic proteins secreted by somatic and/or professional APCs transfected with plasmid DNA, that is then taken up by other professional APCs, processed and presented via both the MHC class I and II pathways to CTLs and Th respectively (Gurunathan et al., 2000).

APCs transfected with the antigen migrate to the draining lymph nodes where they activate naïve T cells by presenting the antigen via both MHC class I and II molecules. Costimulatory signals are released and trigger an immune response including more CTLs and Th. In the lymph node the Th secrete cytokines and APCs secrete some of the antigen which activates the B-cells and induce the production of antibodies. The activated T- and B-cells can migrate to the tissues transfected with the vaccines and be reactivated where the CTLs are able to lyse the infected myocytes presenting antigens which result in increased release of antigens. Several factors, however, can influence the immune responses elicited by the DNA vaccine such as the route and method of administration and the dose of vaccine administered (Ingolotti et al., 2010; Kutzler and Weiner, 2008; McCluskie et al., 1999).

2.3.4 Routes of administration and immunogenic response

DNA vaccines may be administered in many ways such intramuscular (IM) injection, intradermal (ID) injection, subcutaneous (SC) injection, intravenous injection (IV) and intranasal (IN) delivery amongst others (McCluskie et al., 1999). The most popular routes are the IM and ID injection. Expression of the protein following ID and IM injection occurs in different cell types namely the keratinocytes and skeletal muscle (myocytes) respectively (Dunham, 2002).

New age methods for delivering the DNA vaccines intradermally and intramuscularly include the use of a gene gun, electroporation and a needle-free method. With the use of a gene gun the skin is bombarded with DNA-coated gold beads by using compressed helium to accelerate the beads. As the DNA is delivered directly into the cell, only small amounts of DNA are necessary for the induction of an immune response (Doria-Rose and Haigwood, 2003; Dunham, 2002; Okuda et al., 2014). Electroporation makes use of electrical impulses to produce pores in the cells, improving transfection. This method, however, has the disadvantage of prolonged exposure to high voltage electricity. Therefore animals will have to be sedated which will be impractical for large scale vaccination (Cole et al., 2015; Liu, 2011; Okuda et al., 2014; Saade and Petrovsky, 2012; Van Drunen Littel-van den Hurk et al., 2008). The needle-free method uses the CO_2-powerd Biojector® device to spray the DNA vaccine through the skin for direct transfection of cells. This method did not show an increase of immune response in comparison to the use of the conventional needle approach on non-human primates and clinical trials using this device are still ongoing (Okuda et al., 2014; Saade and Petrovsky, 2012)

DNA delivery is, however, most commonly performed by the use of a hypodermic needle for both ID and IM delivery (Okuda et al., 2014). Typically a Th1 response will be elicited as a result of needle injection delivery as the DNA is delivered in the extracellular spaces of the cell. This promotes antibody production and therefore both cellular and humoral immunity is activated. Only the gene gun method is capable of a balanced Th1/Th2 response whereas both electroporation and the needle free method result in a Th2 response. As the needle injection results in both cellular and humoral immunity and is the most affordable it is the most popular method of administration. Factors that have an influence on delivery of DNA vaccines by means of needle injections include the needle type, muscle type injected and animal age (Okuda et al., 2014).

2.3.5 Dose requirement

Many factors have an impact on the dose required in order to elicit or induce an effective immune response and these include route of delivery as well as age of the animals amongst others. Different

doses administered to different animals are shown in Table 2.3. The dose administered to mice can be as low as 10-100 µg, where higher doses are required for larger animals and humans ranging from 500 to 2500 µg (Dunham, 2002; Gurunathan et al., 2000; Oshop et al., 2002). The total dose given to ducks and chickens, as seen in published studies, ranges from 0.5 to 1500 µg per bird administered over time. Only a few studies used DNA vaccine concentrations above 500 µg although in one published study a concentration of 10 mg was used per injection against infectious bursal disease in chickens (Meunier et al., 2016; Oshop et al., 2002).

According to Dunham (2002) less DNA is required when using a gene gun due to the fact that the DNA is coated onto golden beads minimising degradation of the DNA. In mice it was shown that needle injections required 2-20 µg DNA in order to induce an immune response whereas with gene gun delivery only 1-3 µg DNA was required for an effective immune response (Dunham, 2002). DNA is also delivered directly into the cell compared to using a needle which will result in introduction of the DNA outside of the cells (Robinson and Torres, 1997). Although the use of the gene gun will result in the use of a smaller dose, multiple injections several times are required as only a small amount of DNA can be put on the gold beads. The stability of the DNA on the beads is also questionable and can complicate the results obtained by using this technique (Dunham, 2002; Liu, 2011).

Not all the DNA injected into the animal will enter the cell; some is degraded before it enters the nucleus. About 90% of DNA never enters the cytoplasm. Of the remaining 10% DNA, less than 1% enters the nucleus (Babiuk et al., 2003). It was shown that after DNA inoculation of poultry (such as chickens and turkeys) that antigen was produced in small amounts in the picogram to nanogram range but was still able to induce an immune response (Meunier et al., 2016; Oshop et al., 2002).

In some studies a dose related response could be observed, but in the case of poultry there was not a good response seen up until a booster vaccine was administered. It is not yet clear if this phenomenon is due to an immature immune system of the birds or if the time frame needs to be adjusted (Meunier et al., 2016; Oshop et al., 2002).

Table 2.3 Dose range and schedule of booster DNA administration to different animals.

Animal	Organism/disease	Dose	Booster	Route	Reference
Mice	*Taxoplasma gondi*	100 µg	(Every 2 weeks) x 2	IM	(Yuan et al., 2011)
Mice	H5N1 influenza virus	10 µg	Week 2	IM	(Luke et al., 2011)
Chickens	*Emeria tenella*	100 µg	Week 2	IM	(Xu et al., 2008)
Chickens	Infectious bronchitis virus	150 µg	Week 2	IM	(Tian et al., 2008)
Chickens	Infectious bronchitis virus	100 µg	Week 1, 2 and 5	IM	(Yan et al., 2013)
Chickens	Infectious bursal disease virus	400 µg, 1000 µg-10 000 µg	(Every week) x 2	IM	(Hsieh et al., 2010)
Turkey	*Chlamydophila psittaci*	100 µg - 500 µg	Week 2	IM	(Verminnen et al., 2010)
Turkey	Turkey coronavirus	750 µg	Week 2	IM	(Chen et al. 2013)
Ducks	Duck plague virus	50 µg- 200 µg	-	IM/gene gun	(Lian et al., 2011)
Ducks	Hepatitis B virus	400 µg	Week 3 and 7	IM	(Saade et al., 2008)
Cattle	Bovine viral diarrhea virus	1500 µg	(Every 6 weeks) x 2	IM	(Van Drunen Littel-Van Den Hurk et al., 2013)
Cattle	*Mycobacterium bovis*	1000 µg	Week 3	IM	(Skinner et al., 2003)
Monkeys	Alzheimer's disease	200 µg -2000 µg	Weeks 2, 6 and 26	IM	(Evans et al., 2014)
Monkeys	Ebola virus	1000 µg	(Every 4 weeks) x 2	IM	(Sullivan et al., 2000)
Monkeys	Simian immunodeficiency virus	1500 µg -10 000 µg	(Every 4 weeks) x 2	IM	(Schadeck et al., 2006)
Dogs	*Trypanosoma cruzi*	500 µg	Week 2	IM	(Arce-Fonseca et al., 2013)
Sheep	*Toxoplasma gondii*	1000 µg	Week 4	IM	(Hiszczynska-Sawicka et al., 2011)
Pigs	Influenza A virus	200 µg - 1972 µg	-	Needle free	(Borggren et al., 2016)

2.3.6 DNA vaccines: Advantages and concerns

DNA vaccines can be manipulated to circumvent many safety issues and still retain specificity. DNA vaccines are not associated with problems regarding protein production such as improper folding of the protein of interest or high purification costs. There is also not the risk associated with live-attenuated vaccines since DNA vaccines are not able to spread or replicate and are not living, resulting in little risk of secondary infections or of reversion to an infecting form (Coban et al., 2008; Kutzler and Weiner, 2008).

Some concerns regarding the safety of DNA vaccines have however been raised and include the potential of the DNA vaccine to integrate into cellular DNA. This can have devastating results as for example instable chromosomes, mutagenesis and also malfunction of tumour suppressor genes. Integration, however, will occur at rates much lower than the spontaneous mutation frequency (Kumar et al., 2013; Kumaragurubaran and Kaliaperumal, 2013; Kutzler and Weiner, 2008). Another concern is the development of autoimmunity but early studies in both non-primates and humans showed that autoimmune disorders are unlikely (Hasson et al., 2015; Kutzler and Weiner, 2008).

Another concern regarding DNA vaccines is that they typically contain antibiotic resistance markers for selection and it is possible that these resistance genes can be horizontally transferred resulting in antibiotic resistant pathogenic bacteria. Kanamycin antibiotic resistance genes commonly used in DNA vaccine vectors, however, are not commonly used in the treatment of infections (Kutzler and Weiner, 2008; Vandermeulen et al., 2011). Although this is a concern, new antibiotic free selection systems have been developed and includes mechanisms such as complementation of auxotrophic complementing markers, RNA based selection markers and toxin-antitoxin-based strategies amongst others (Mignon et al., 2015; Vandermeulen et al., 2011). These systems, however, can be expensive as they require development of mutant host strains and defined media and therefore, if used in DNA vaccines, would result in an increase in production costs (Dong et al., 2010; Peubez et al., 2010).

Other advantages of DNA vaccines includes their ability to encode multiple types of genes including viral and bacterial, as well as immunological and biological proteins and are therefore immensely flexible. They are also highly stable, and can be stored and transported with ease and are produced effortlessly on large scale (Kutzler & Weiner 2008; Ghanem et al. 2013; Kumaragurubaran & Kaliaperumal 2013). Furthermore, both arms of the adaptive immune system can be induced by DNA vaccines, and additionally the immune response can be directed to either the humoral or cellular immune response (Hasson et al., 2015; Khan, 2013).

2.3.7 Evaluation of antibody responses

Humoral and mucosal immune responses to infection or vaccination can be measured by a variety of techniques including radioimmunoassay (RIA) and enzyme immunoassays. Enzyme immunoassays can be divided into two types known as heterogeneous and homogeneous enzyme immunoassays. Enzyme-linked immunosorbent assays (ELISA) are included in the heterogeneous type as they consist of both a solid and liquid phase whereas the homogeneous type has no solid phase. Since 1971, as termed by Engvall and Perlmann and by Weenmen and Schuurs (Clark et al., 1986), ELISA has become standard use in the scientific world. ELISAs use enzyme amplification of antibody binding followed by conversion of colorimetric substrates to measure this. The ELISA technique was derived from the RIA, described by Berson and Yalow (Yalow and Berson, 1960). RIA however made use of radioactive substances and, as this can be a safety hazard, the technique was modified by replacing the radioisotopes with enzymes. The ELISA technique is very specific and easy to execute, sensitive as well as highly versatile requiring very little equipment and reagents are readily available (Hornbeck, 1991). To date there are different types of ELISAs including the sandwich, indirect and competitive ELISAs to name a few (Butler, 2000; Gan and Patel, 2013). For this study the focus will be on the indirect ELISA.

This ELISA is often used in studies on infectious diseases and allows for the measurement of antibody levels. The first step of the indirect ELISA is to immobilise or adhere the antigen onto the solid phase which in this case is a pre-treated 96 well microtitre plate by means of passive adsorption (Gan and Patel, 2013; Voller et al., 1978). This is followed by the addition of a non-reacting protein as that will block all areas of the well which was not coated with antigen and therefore prevent non-specific binding. This is followed by a wash step to remove the excess non-reacting protein that did not bind to the wells. The primary antibody is then added to the wells and incubated in order for the primary antibodies to bind to the antigen. After incubation, excess unbound serum components are washed away, followed by the addition of an enzyme-conjugated secondary antibody (Burry, 2011; Gan and Patel, 2013). Depending on whether humoral or mucosal antibody levels are being measured this secondary antibody can either be anti-Ig, anti-IgG or anti-IgA antibodies. Several detection and amplification methods for subsequent steps are available but in this study the biotin/avidin detection method was used, therefore biotin was covalently attached to the secondary antibodies by an *N*-hydroxysuccinimide ester of the biotin. The biotin-linked secondary antibody is incubated with the antigen-primary antibody complex in order for the biotin-linked secondary antibody to adhere to the primary antibody. The unbound or excess biotin-linked secondary antibody is removed by a wash step after incubation. The wash steps that are performed between all steps will minimise the background of the ELISA results (Guesdon et al., 1979).

This is followed by the addition of avidin or streptavidin. Avidin or streptavidin has a high affinity for biotin and this ensures that the complex is very stable through pH changes, presence of chaotropes as well as multiple washings. Avidin has four binding sites per molecule and therefore four biotin moieties can bind to one molecule of avidin. However, all the avidin sites are not always occupied by biotin but the signal is increased as more biotin molecules bind to the avidin (Diamandis and Christpoloulos, 1991). As avidin is an egg-white glycoprotein which is highly alkaline this can result in non-specific binding. Therefore the use of a bacterial analogue streptavidin, isolated form *Streptomyces avidinii*, is preferred as it is a neutral non-glycosylated protein resulting in less non-specific binding (Bayer and Wilchek, 1990). The streptavidin is labelled with horse radish peroxidase (HRP) or alkaline phosphatase in order to detect the biotinylated secondary antibody by means of a colour reaction which is caused by the addition of a substrate. The colour change can be measured by means of a spectrophotometer and the colour reaction measured can be interpreted as being proportional to the amount of primary antibody present in the well (Gavrilova et al., 2014; Voller et al., 1978).

Most ELISA assays contain marker enzymes such as β-galactosidase, alkaline phosphatase or horse radish peroxidase (HRP) and each has an appropriate substrate available for use in the ELISA. Some of the substrates include 2,2 - azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS), *o*-phenylenediamine (OPD), 3,3',5,5' -tetramethylbenzidine (TMB), *p*-nitrophenyl phosphate (pNPP) and bisdiazobenzidine (BDB) amongst others. The appropriate substrates for the alkaline phosphatase enzyme includes OPD and pNPP resulting in an orange and yellow colour respectively. The appropriate substrates for HRP include TMB which results in a yellow colour, BDB resulting in a purple/reddish colour or ABTS which results in a green colour (Butler, 2000; Espina et al., 2004; Frey et al., 2000). In this study the ELISA marker enzyme used was HRP and ABTS was used as a substrate which resulted in a green product of which the absorbance can be measured at 405 nm (Butler, 2000)

2.4 Secondary antibody production for detection of mucosal immunity

Mucosal surfaces are mainly protected by means of secretory IgA through the mucosal immune system or mucosa-associated lymphoid tissues (MALT) (Rabson et al., 2005). Measurement of mucosal immunity implies that the amount of IgA produced is measured. Using a technique such as ELISA requires the production of secondary anti-IgA antibodies and therefore the isolation of IgA antibodies. Alternatively only a portion of the IgA antibody could be produced *in vitro* and used for the production of the secondary anti-IgA antibodies. To understand which part of the IgA antibody would be most suitable for this purpose, the structure of antibodies first needs to be discussed.

2.4.1 Antibody structure

As shown in Figure 2.2 antibodies consists of two identical large polypeptide chains known as the heavy chains (H) as well as two identical small polypeptide chains known as the light chains (L). Each of the H and L chains contain a variable region (V) as well as a constant region (C) and the constant regions are the same in antibodies of the same class. The four chains are assembled to form a Y-shaped molecule as each of the light chains are attached to a heavy chain and the heavy chains are attached to each other by disulfide bonds. There are five types of heavy chains known as μ, α, δ, ε and γ which all differ in their constant region. In mammals the five classes are known as IgM, IgA, IgD, IgE and IgG, but only three classes are described in avian species namely IgM, IgA and IgY, which is the homologue of IgG in birds (Abbas et al., 2014; Huang et al., 2012; Zhao et al., 2006). Immunoglobulin H chains consist of an N-terminal variable domain and 3-4 constant domains. All of the mammalian Ig types have three H chain constant region domains except for IgM and IgE which have four constant region domains whereas the IgA, IgM and IgY of birds have four constant region domains (Higgins and Warr, 1993; Kaetzel, 2014; Zhao et al., 2006)

All of the H chains of the different classes of immunoglobulins can be produced in two forms namely the membrane-bound form or secreted form. The difference is that the membrane bound form has a hydrophobic transmembrane region which is coded for by two exons known as the membrane coding segment (Figure 2.3 indicated in yellow). These exons code for of roughly 25 amino acid residues at the carboxy terminus of the H chain that anchor it to the B-cell surface. The secreted form lacks this domain and instead has a hydrophilic secretory tail (Figure 2.3 indicated in orange) that is coded for by a different exon known as the secretory-coding sequence. The two forms can only be produced by alternative RNA processing during the processing of the initial transcript. The primary transcript encoding the H chain gene has two potential polyadenylation (pA) sites indicated as pAm and pAs in Figure 2.3. Splicing between the 4th exon of the constant region and the secretory-coding sequence (indicated as SC in Figure 2.3) as well as at a second site downstream of the membrane coding segment results in the loss of the hydrophilic secretory tail and the transmembrane immunoglobulin is formed. The secreted form is produced if splicing occurs at the pAs site resulting in the membrane encoding segment being removed. This process of RNA splicing occurs in the same way for all classes of immunoglobulins (Janeway et al., 2001; Murphy et al., 2012).

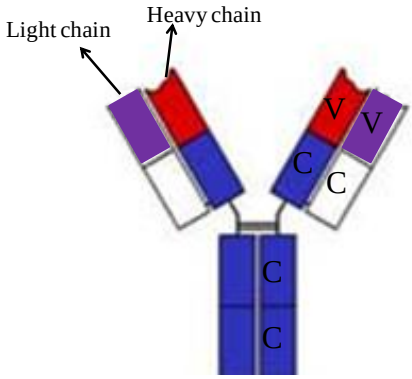

Figure 2.2 Illustration of an immunoglobulin molecule. C=constant region and V=variable region of the heavy and light chains respectively. Adapted from Murphy et al. 2012.

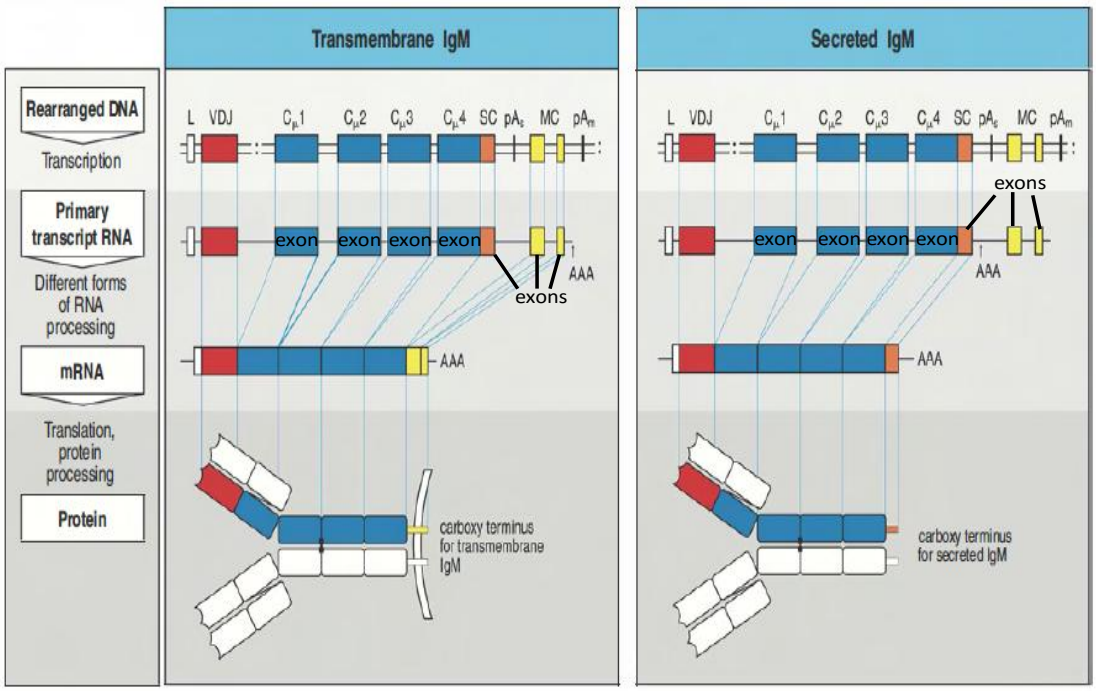

Figure 2.3 Process of RNA splicing of immunoglobulin heavy chain genes to produce the transmembrane and secreted forms of immunoglobulins. MC= membrane coding sequence, SC= secretion-coding sequence, pA_m= ployadenilation site resulting in the transmembrane form of the antibody, pA_s= polyadenylation site resulting in the secretory form of the antibody (Murphy et al., 2012).

2.4.2 IgA heavy chain structure

The ostrich IgA H chain protein consists of four constant region domains (IgA_H) that shares 44% DNA sequence similarity with chickens and 66% with ducks. The IgM and IgA genes are positioned in reverse orientation on duck, chicken and goose chromosomes. The *IgA_H* genes of ducks, chickens, goose and ostriches have a close genetic relationship to one another, it is therefore assumed that the *IgA_H* gene of the ostrich is also positioned in the reverse orientation (Guo et al., 2014; Huang et al., 2012).

Huang et al (2012) showed that the ostrich genome contains a single *IgA* H chain gene that consists of 4 exons that each code for a constant domain in the final protein product (Addendum B). The hydrophobic secretory tail is contained within the C-terminal end of the 4th domain and compared to other species the translated protein contains 10 conserved cysteine residues. The constant region also contains three N-linked glycosylation sites (N-165, N-221 and N-419) in 2, 3 and 4 with the third being situated in the canonical secretory tail. These are all conserved in birds (Huang et al., 2012).

2.4.3 Antibody region suitable for production of secondary antibodies

The IgA_H domains of the goose have similar antigenicity to natural IgA as suggested by western blot analysis (Guo et al., 2014; Huang et al., 2012) and since the goose IgA_H is closely related to that of the ostrich the same would be expected for the ostrich IgA_H. Furthermore, the antibody class is determined by the sequence and function of the heavy chain constant region which implies that this region within IgA can be used for the reproducible production of secondary anti-IgA antibodies (Huang et al., 2012; Karu et al., 1995).

The IgA_H protein can be produced *in vitro* and as the IgA_H protein has three glycosylation sites it is important to use an expression system that will allow post-translational modifications such as glycosylation. The baculovirus-insect expression system is capable of post-translational modifications such as glycosylation and is able of producing a greater amount of proteins more rapidly compared to mammalian cell lines. Carayannopoloulos et al. (1994) showed that insect cells were able to assemble and secrete recombinant human IgA successfully in the baculovirus-insect expression system therefore this system can potentially be effective for the production of ostrich IgA_H protein which in turn can be used for the production of secondary anti-ostrich IgA_H antibodies (Carayannopoulos et al., 1994; Hasemann and Capra, 1990; Huang et al., 2012)

2.5 The Baculovirus

The *Baculoviridae* family is a large family of viruses capable of infecting arthropods (mainly insects), and contains a relatively large genome consisting of double stranded circular DNA. The genome of these viruses is packaged into rod-shaped nucleocapsids (Figure 2.4) and they replicate in the nucleus of the infected insect cell. Baculoviruses are used to control insect pests for example *Cydia pomonella*, on apple, the velvet bean caterpillar and the cotton bollworm to name a few (Jarvis, 2009; Van Oers, 2011).

2.5.1 The baculovirus-insect cell expression system

The use of the baculovirus-insect cell expression system for production of recombinant proteins dates back to the 1980's when the first report regarding the virus as an expression vector was published. This system is known for providing high levels of production and eukaryotic protein processing capabilities. These two characteristics are usually associated with bacterial and mammalian systems respectively and make the virus suitable for foreign gene expression. It is regularly stated that a baculovirus-insect cell system has the ability to process a protein with eukaryotic modifications. Therefore high levels of recombinant protein with posttranslational modifications can be produced by means of a baculovirus vector in combination with insect cells or larvae. Although the insect cell possesses the ability of eukaryotic modifications, it is not equivalent to those of higher eukaryotes (Kost et al., 2005).

The different genera of the family *Baculoviridae* are capable of infecting different orders of insect classes. The *Alphabaculoviruses*, as well as *Betabaculoviruses*, infect the Lepidoptera order whereas *Gammabaculoviruses* are specific for hymenopteran insects and *Deltabaculoviruses* infect dipteran insects. In the *Alphabaculoviruses* the GP64 protein is known as the budded virus (BV) envelope fusion protein which is essential for entrance of baculovirus particles into susceptible insect cells. The same function is however achieved by the non-homologous F protein in the *Betabaculoviruses* and *Deltabaculoviruses*. The *Gammabaculovirus*, however, does not contain a BV envelope fusion protein (Van Oers, 2011).

Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) and, to a lesser extent, *Bombyx mori* NPV (BmNPV) are the two baculovirus species most extensively studied and utilised in biotechnology as vectors for production of recombinant proteins in insect cells (Jarvis, 2009; Van Oers, 2011). These two viruses form part of the *Alphabaculovirus* genus. The insects cells generally used for the baculovirus-insect expression system are known as lepidopteran derived insect cells which are easy growing cells that can easily be up scaled (Van Oers, 2011).

2.5.2 Replication cycle of baculoviruses

Baculoviruses have two different infective forms, also known as virions (Figure 2.4). The first virion is the occlusion derived virus (ODV) which is stable outside the insect host as it is embedded in an occlusion body (OB). The OB's are responsible for horizontal transmission as they enter the insect larvae gut by ingestion and release the ODV. The second virion is known as the budded virus (BV) which is responsible for vertical transmission as they are able to spread the infection from cell to cell within the host insect and is not occluded as in the case of the ODV (Rohrmann, 2013). The

BVs and the ODV differ in their morphological characteristics and are formed by the early and late phases of replication respectively (Figure 2.5), which resulted in the development of the baculovirus-insect expression system (Luque and O'Reilly, 1999).

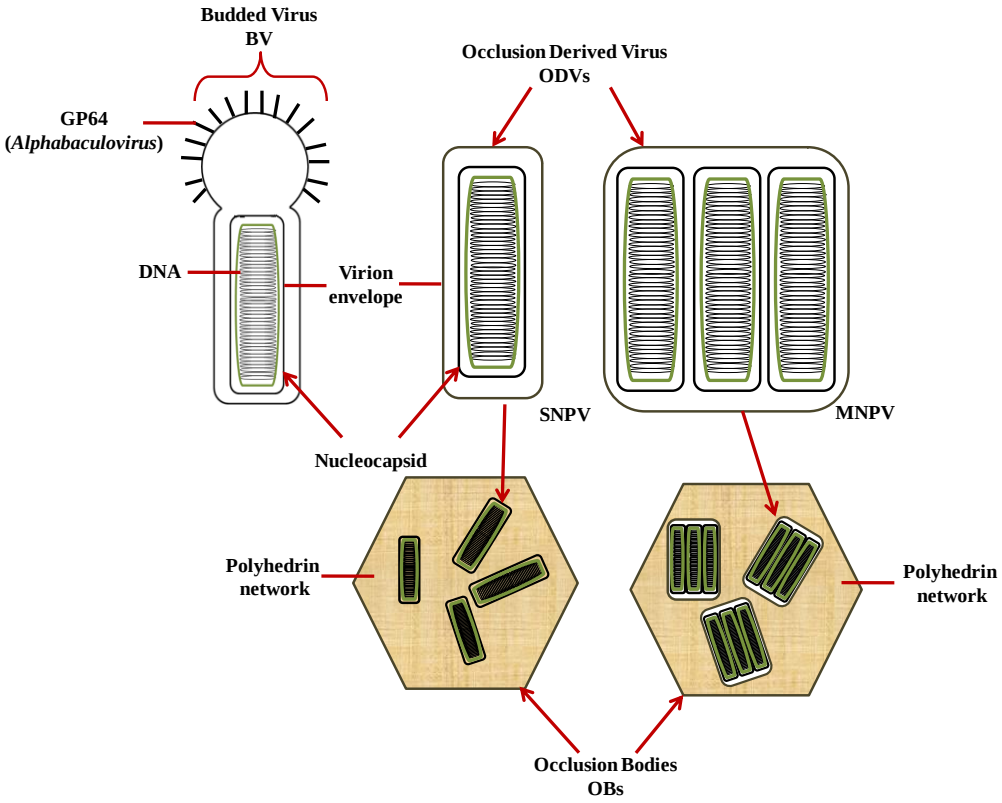

Figure 2.4 Two virion types of the baculovirus namely the BV and ODV occluded in OB's. MNPV= Multicapsid nucleopolyhedrovirus, SNPV= Singlecapsid nucleopolyhedrovirus (Larramendy and Soloneski, 2012).

Studies suggest that the early and late phases are subdivided and the baculovirus therefore expresses genes in four phases during the infection process. This phenomenon is known as the replication cycle and the four phases of this cycle are referred to as the immediate early, early, late and very late phases (Figure 2.5) (van Oers 2011; Chen et al. 2013). During the immediate early phase the OB is ingested by the insect larvae followed by dissolution of the OB by the alkaline conditions of the midgut of the larvae whereby the virus is released and is now known as ODV (Rohrmann, 2013). ODV infects the epithelial cells of the midgut through binding of the virus to receptors on the epithelial cells. These receptors mediate binding of the virus and virus envelope proteins, allowing fusion with the host cell membrane. The ODV enters the midgut epithelial cells by the enzymatic activity of some proteins present in the envelope allowing fusion with the cell (Rohrmann, 2013).

According to Rohrmann (2013) the ODV envelope fuses with the membrane of the epithelial cell of the midgut resulting in the release of nucleocapsids which are transported to the nuclear membrane

and through the nuclear pores. During the early phase the double stranded DNA (dsDNA) of the nucleocapsids is replicated. The late phase entails exiting of the nucleocapsids from the nuclear membrane. Envelope proteins are required for virus budding and allow the replicated nucleocapsids to bud from the plasma membrane which is then known as the BV. The BV is responsible for systemic infection of neighboring and other susceptible cells in the insect. During the very late phase polyhedrin protein production starts which is responsible for the formation of the matrix of OBs and which protect the virions from harsh environmental conditions outside the insect. The release of BV is terminated during this phase and nucleocapsids accumulate in the nucleus of the midgut epithelial cell. The singlecapsid nucleopolyhedrovirus (SNPV) or multicapsid nucleopolyhedrovirus (MNPV) will obtain an envelope from the nucleus of the infected epithelial midgut cells and be known as ODV. The produced polyhedrin accumulates in the nucleus and at some point forms a crystallized structure around the ODV which is then known as OBs and released from the larvae (Rohrmann, 2013; Van Oers, 2011).

Polyhedra are large particles, consisting of BV embedded within a protective paracrystalline array, that appear in the nuclei of infected insect cells. A single virus-encoded protein known as polyhedrin is responsible for the paracrystalline array structure of the OB's. The very late phase, however, includes the production of both polyhedrin and a 10 kDa protein (P10) in high concentrations (van Oers 2011; Chen et al. 2013). The function of P10 however is not yet fully understood but plays a role towards the end of infection by releasing the OBs from the cell nucleus (van Oers 2011; Chen et al. 2013).

Viral cathepsin-like protease and chitinase enzymes are responsible for liquefying the larvae and in this process OBs are released from the cell (Clem and Passarelli, 2013; Juliant et al., 2013). Infected larvae become hyperactive and have an increase in their locomotory activity due to the baculovirus as it is able to modify the behavior of its host. This abnormal behavior is known as 'Wipfelkrankheit' or 'tree top disease' and is brought about by the expression of the protein tyrosine phophatase. By modifying the host behavior the baculovirus ensures the spread of OBs over large areas of plants as larvae will move to the top of plants before they liquefy. The next larvae will ingest the OBs while ingesting the plant and be infected (Clem and Passarelli, 2013; Van Houte et al., 2012).

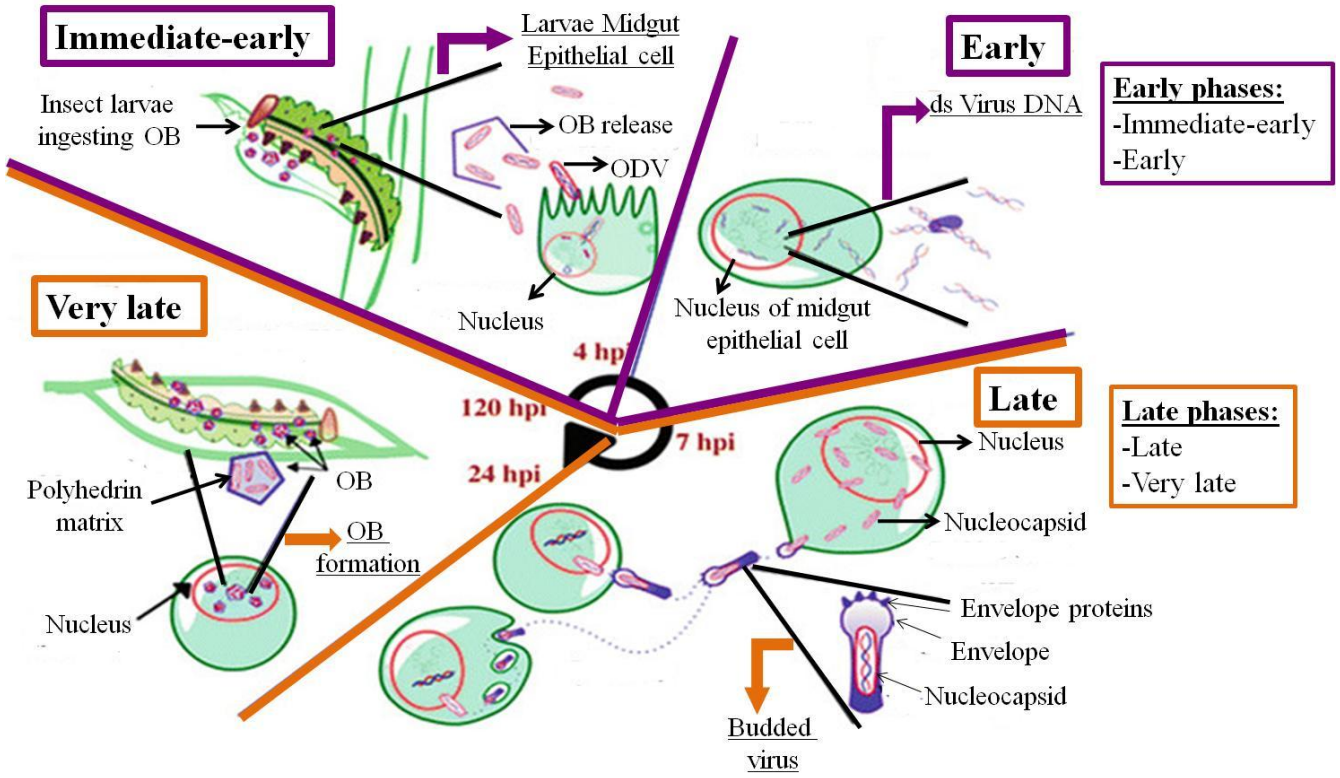

Figure 2.5 Replication cycle of the Baculovirus consisting of four phases: The immediate-early phase- where the OB is ingested and released ODV are transported to the nucleus of the larvae midgut epithelial cells. The early phase- Replication of baculovirus DNA in the nucleus of the larvae midgut epithelial cell. The late phase- Release of the budded virus resulting in secondary infection of the larvae cells. The very late phase- where polyhedrin is produced for the formation of OBs and OB release (Al-Rubeai, 2015).

2.6 The development of the baculovirus as expression vector

For cell culture purposes the BV is required since it is responsible for cell to cell infection. Since OBs are responsible for horizontal transmission, they are not required in a cell culture system and therefore polyhedrin production is also not necessary *in vitro*. Hence the polyhedrin gene can be replaced with foreign DNA or a gene of interest. The baculovirus contains strong promoters in order to produce large amounts of polyhedrin. If the polyhedrin gene is replaced by another gene of interest for expression, the viruses would not be able to form occlusion bodies as the formation of the occlusion body matrix is dependent on polyhedrin. Instead, the gene of interest will be expressed in high levels due to the control of the powerful polyhedrin promoter (Luque and O'Reilly, 1999).

The lack of polyhedrin production will cause vertical transmission to continue since the viruses containing a gene of interest are still able to form BV's and can be cultured. The approach of replacing the polyhedrin gene with the gene of interest has been modified over time, for example, by using other promoters, producing occlusion-positive recombinant viruses as well as the expression of more than one foreign gene simultaneously (Luque and O'Reilly, 1999).

2.6.1 Baculovirus transfer plasmid design

As the bacoluvirus vector itself has a relatively large genome it is frequently not possible to insert a foreign gene into the baculovirus genome by means of direct cloning. Therefore, homologous recombination between the viral genome and a transfer plasmid, containing the gene of interest, is used in an approach to produce recombinant viral progenies (Van Oers, 2011).

A general method used to achieve homologous recombination includes the construction of a "transfer" plasmid as well as the process of co-transfection. Recombinant baculovirus transfer plasmids are constructed to contain the polyhedrin promoter and to be able to express chimeric genes as they would also contain the foreign gene of interest. The constructed plasmid should contain the gene of interest flanked by up- and downstream sequences from the polyhedrin region of the baculovirus genome in order for homologous recombination to occur (Figure 2.6). The recombinant baculovirus vector is then generated by co-transfecting the cells with the transfer plasmid and genomic baculovirus DNA (Figure 2.7) (Jarvis, 2009).

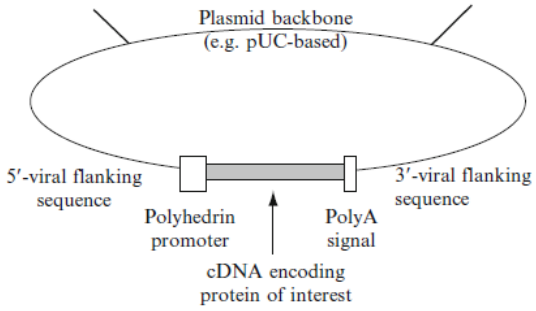

Figure 2.6. Transfer plasmid containing cDNA encoding for the protein of interest, flanked by polyhedrin sequences (Jarvis, 2009).

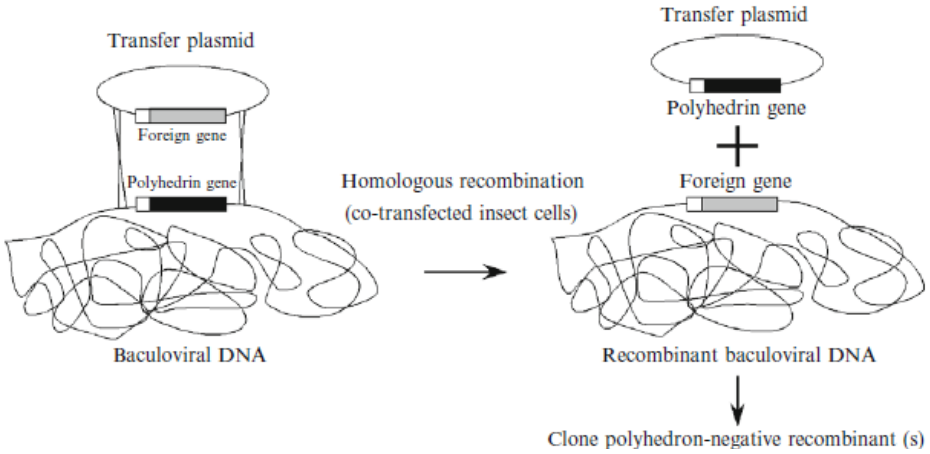

Figure 2.7. Generation of a recombinant baculovirus by means of homologous recombination (Jarvis, 2009).

2.6.2 Modifications to the baculovirus vector

Recombinant baculovirus DNA molecules are produced in co-transfected insect cells as a result of homologous recombination. In order for the polyhedrin gene to be eliminated and the foreign gene to be inserted in the baculovirus genome a double cross over recombination event is necessary. This event however does not occur often with a frequency of about 0.1% (Jarvis, 2009).

As only 0.1% of the produced viruses will be recombinant, separation of recombinant viruses from the majority of parental viral progenies is necessary and can be achieved by baculoviral plaque assays. The produced viruses could be distinguished from one another as parental viral progeny produced polyhedrin positive plaques whereas the recombinant viral progeny produce polyhedrin negative plaques since the recombinant viral progeny does not contain the polyhedrin gene. Hence separation of parental and recombinant viral progenies, by an investigator, could be achieved by using a visual screen. The investigator, however, should have a trained eye and many investigators fail to separate the plaques (Jarvis, 2009; Van Oers, 2011). Around 1990 it became clear that the system needs improvement to be able to isolate recombinant viral progeny. Modifications were developed that entailed two categories: modifications of the transfer plasmid and modifications to the parental baculovirus genome to create a baculovirus vector.

Modification of the transfer plasmid was done to simplify the recombinant expression and purification. These modifications included incorporation of secretory signal peptide sequences and purification tags, replacing the polyhedrin promoter with baculovirus promoters that vary as well as promoters responsible for simultaneous drive of expression of more than one recombinant protein (Jarvis, 2009). The purpose of modifications to the parental baculovirus genome was to isolate recombinant baculoviruses and to increase the recombination efficiency of the baculovirus.

The incorporation of the *lacZ* gene into the transfer plasmid which encodes β-galactosidase, was used to refine the technique used to purify recombinant viruses as it was recognisable by visual screening of plaque assays. This technique was however not infallible as it could not identify double homologous crossovers nor single homologous crossovers, which are recurrent. Single homologous crossover recombination results in viral recombinant genomes which contain the transfer plasmid as a whole as well as the bacterial replicon, at some loci (Jarvis, 2009; Van Oers, 2011).

Increasing homologous recombination efficiency was of the essence as the natural process resulted in less than 1% success. This was also a time consuming process hence modifications were made which resulted in the first baculovirus vector with a linearisable DNA genome. Introducing a *Bsu36I* site into the baculovirus genome by means of a novel sequence allowed for linearisation of

the genome at that specific site by means of *Bsu36I*. This approach resulted in more than 30% recombinant viral progenies. An important feature of this linear baculovirus vector was that it was not able to replicate. In order to improve the homologous recombination the *E. coli lacZ* gene was introduced into the genome up- and downstream of the viral genes. The *lacZ* gene contributed another *Bsu36I* site together with the other two *Bsu36I* sites introduced by the novel sequence in the polyhedrin site. This allowed triple digestion of the baculoviral genome and in this process the *orf1629* gene is lost. A wiscott-Aldrich syndrome protein (WASP), phosphoprotein PP78/83, is encoded by the *orf1629* gene. This protein is responsible for the nuclear actin filament formation during baculovirus infection. Hence the *orf1629* gene is an essential gene required for replication of the virus in insect cells. The *orf1629* gene is restored upon recombination and results in 90% recombinant baculovirus production. This is as a result of the deletion of the *orf1629* gene from linearised baculoviral genomes used, thus parental baculovirus vector would not be able to replicate on its own (Jarvis, 2009; Van Oers, 2011).

2.6.3 Advantages of the baculovirus-insect cell expression system

An advantageous feature of the baculovirus includes its narrow host range. The baculovirus is generally restricted to infect only specific species of invertebrates and as this narrow range only includes insects, it would be safe for vertebrates, plants and humans (Jarvis, 2009; Szewczyk et al., 2009). The baculovirus-insect expression system holds eukaryotic protein processing abilities including phosphorylation, N- and O-glycosylation, correct signal peptide cleavage and proteolytic processing amongst others. Proper folding of the protein as well as S-S bond formation can be accomplished by the baculovirus-insect expression system whereas this is not possible in the reducing environment of the *E. coli* cytoplasma. In contrast prokaryotic expression systems are able to produce high levels of recombinant proteins but are not capable of posttranslational modifications (Demain and Vaishnav, 2009; Jarvis, 2009). The baculovirus-insect expression system can be easily up scaled and as the virus will use the polyhedrin promoter in order to express the foreign protein, high levels of the foreign protein will be expressed. There is also no limit on the size of the protein of interest and multiple genes can be expressed simultaneously (Demain and Vaishnav, 2009).

2.6.4 Disadvantages of the baculovirus-insect cell expression system

It is known that promised results concerning recombinant protein production may differ from the results gained when actually producing recombinant proteins. Producing the recombinant protein as well as the solubility levels achieved is dependent on the protein which is being studied. This is an indication that although the baculovirus-insect expression system should theoretically be able to

produce high levels of the foreign protein as well as a soluble protein, it is not always that simple (Jarvis, 2009; Van Oers, 2011). Although the baculovirus-insect expression system has eukaryotic processing abilities as they infect lepidopteran insects, which is the host for baculovirus vectors, these processing abilities are different from those of higher eukaryotes. Another consideration to take into account is that the baculovirus infections of the host cell can have a unfavorable effect on the host protein processing capabilities (Jarvis, 2009)

2.7 Choice of insect cells, baculovirus vector and transfer plasmid

2.7.1 Characteristics of insect cells as host for the baculovirus

For the production of baculovirus, virus-like particles and recombinant proteins to name a few, over 400 cell lines derived from 100 insect species have been used as hosts. Insect cells have a diameter of 10 to 20 µm and can be spherical or fibroblast-like in shape. Lepidoptera insect cells are the most commonly used in the baculovirus-insect expression system and were primarily selected for production of biopesticides. Today engineered insect cells are able to mimic mammalian glycosylation patterns and produce high yields of recombinant proteins (Contreras-Gómez et al., 2014).

Three host cell lines are the most popular and frequently used for recombinant protein production. These include the Sf21 and Sf9 (a clonal isolate of the Sf21 cells) cell lines which were derived from the pupal ovarian tissue of the fall army worm *Spodoptera frugipedra*. The third host cell line is the BTI-Tn-5B1-4 or High-Five™ cell line derived from adult ovarian tissue of the cabbage looper *Trichoplusia ni* (Contreras-Gómez et al., 2014). Cell lines that are not used frequently include the Bm5 and Tn368 cell lines. These cell lines are able to produce large amounts of recombinant protein but do not grow well in suspension culture and are therefore not popular. Sf9, Sf21 and High-Five™ cells on the other hand, do well in adherent cultures as well as suspension cultures (Contreras-Gómez et al., 2014). High-Five™ cells are able to produce higher yields of recombinant proteins than Sf9 cells, they have rapid doubling time and allow quick adaptation to suspension culture. Although High-Five™ insect cells are able to express proteins up to 25-fold higher than Sf9 cells the level of expression of a protein mostly depends on the protein being expressed (Contreras-Gómez et al., 2014). Sf9 and Sf21 cells however are preferred for the propagation of virus stocks as they are able to produce high-titre viral stocks as a result of higher transfection efficiency (Frenzel et al., 2013).

2.7.2 Characteristics of the baculovirus vector and transfer plasmid

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is able to infect many insect cells and as a result numerous expression vectors based on this baculovirus have been developed. Other baculovirus vectors derived from *Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) are available but the AcMNPV is the most popular and widely used baculovirus vector for the expression of recombinant proteins in insect cells (Contreras-Gómez et al., 2014).

Different baculovirus vectors are available and recommended for different types of proteins such as cytoplasmic proteins, transmembrane proteins, cystein-rich proteins and glycosylated proteins. As immunoglobulins are glycoproteins the focus will be on the vectors recommended for glycoprotein expression. Generally, functional secreted proteins such as glycoproteins as well as membrane-bound proteins are expressed at lower levels in comparison to nonsecreted cytoplasmic proteins. As mentioned in section 2.5.2 the baculovirus encodes for viral cathepsin-like protease and chitinase enzymes which can compromise glycoprotein export machinery as chitinase accumulates in the ER (Van Oers et al., 2015). Increased expression levels of some glycoproteins can be accomplished by adding various DNA elements to baculovirus vectors. One of the elements known to increase production of glycoproteins is chaperones which aid in folding of the protein. Although insect cells synthesize endoplasmic reticulum (ER) molecular chaperones it is only at levels required for insect cell functions and as the target protein will be produced at much higher levels the chaperones produced by the insect cells are not sufficient to facilitate folding of the protein of interest. Some target proteins are therefore misfolded and accumulate as inclusion bodies or are degraded (Vembar and Brodsky, 2008). Hence, protein production can be improved by co-transfection of the insect cells with a baculovirus vector expressing not only the protein of interest but also chaperone proteins (Contreras-Gómez et al., 2014).

A variety of transfer plasmids are compatible with the different baculovirus vectors for different purification requirements. These include expression of a target protein with a GST, His or GFP tag to name a few. The multiple cloning site (MCS) region of each transfer plasmid is flanked by baculovirus derived sequences which include the strong polyhedrin promoter upstream of the MCS region and a polyadenylation and essential baculovirus *orf 1629* gene also present in the baculovirus vector. This allows for homologous recombination resulting in a circular baculovirus vector containing the gene of interest. The transfer plasmids contain an antibiotic resistance gene as well as a bacterial origin of replication for propagation of the plasmid in *E. coli* (AB Vector, 2014).

2.8 Mycoplasmas

2.8.1 Metabolism of mycoplasmas

Mycoplasmas are a group of bacteria and members of the prokaryotic class known as the Mollicutes. They are known for their reduced genome size and also as the smallest self-replicating organisms. Characteristics of mycoplasmas which make them unique include the lack of a cell wall, low GC content and a minimal set of organelles. As a result of their small genome these organisms do not contain an extensive range of metabolic activities (Henrich et al., 1999; Razin, 1978; Razin et al., 1998).

Mycoplasmas are divided into fermentative and nonfermentative organisms based on their carbohydrate metabolizing abilities. Fermentative mycoplasmas gain energy by the use of the pyruvate dehydrogenase pathway by producing acids from carbohydrates. Non-fermentative mycoplasmas, however, gain energy by ATP, ornithine, CO_2 and ammonia production by means of the arginine dihydrolase pathway. A complete tricarboxylic acid cycle is absent in mycoplasmas and they have no quinones or cytochromes and therefore they cannot use oxidative phosphorylation as an ATP- generating mechanism. As a result, low ATP yields and a substantial amount of metabolic end products are produced. Metabolic activities of the mycoplasma are, however, primarily associated with energy generation rather than provision of substrates for synthetic pathways. They consequently do not have the ability to synthesize products such as fatty acids, cholesterol, some amino acids, purines and pyrimidines. Consequently, they are obligate parasites as they depend on a host for these products (Henrich et al., 1999; Razin, 1978; Razin et al., 1998).
To acquire these essential molecules from their host, mycoplasmas use different types of transport systems such as the phosphoenolpyruvate-dependent sugar phosphotransferase transport systems (PTS) for transporting sugars and ABC transporters for transporting amongst other, peptides and proteins (Adamu et al., 2013; Henrich et al., 1999; Razin et al., 1998). Of these, the ABC transporters are the most prominent within mycoplasma genomes and the interest in this transporter has increased (Garmory and Titball, 2004; Nicolás et al., 2007).

2.8.2 ABC transporters in mycoplasmas

ATP-binding cassette transporters, more commonly known as ABC transporters, make up one of the largest protein families in mycoplasmas. These transporters are known for the coupling of adenosine triphosphate (ATP) hydrolisation to the movement of molecules across the membrane. ABC transporters are widespread and evolutionary related in different species, however they have different functions and roles (Beis, 2015; Garmory and Titball, 2004). By disrupting ABC

transporter function Garmory and Titball (2004) could demonstrate that these transporters played a role in bacterial virulence. Adding to this is the location of these transporters (in the membrane) as well as the surface exposure of some of the proteins which increase the probability of immunogenicity (Garmory and Titball, 2004). It was concluded that ABC transporter proteins may be satisfactory targets for the development of antimicrobials and antibacterial vaccines (Garmory and Titball, 2004; Nicolás et al., 2007).

These transporters were originally characterised as nutrient uptake systems although it is now clear that they consist of two groups namely the importers and the exporters. Exporters are found in all eukaryotic and prokaryotic organisms and are responsible for the export of proteases, hemolysin and polysaccharides out of the cell whereas the importers are found only in archea, prokaryotes and plants. The importers mediate uptake of molecules such as sugar, inorganic ions and oligopeptides (Beis, 2015; Davidson and Maloney, 2007; Eitinger et al., 2011; Henrich et al., 1999; Higgins, 2001).

Genome sequencing of both *Mycoplasma pneumoniae* and *Mycoplasma genitalium* resulted in the discovery of an ABC transporter in the group of importers which is responsible for the import of oligopeptides. This importer is also known as the oligopeptide permease (Opp) ABC transporter (Henrich et al., 1999; Wium et al., 2015). The OppABCDF system of mycoplasmas consists of five core domains in which each of the domains is encoded by a single gene which is polycistronically organised within an operon. The domains consist of the homologous integral membrane proteins, OppB and OppC; the homologous nucleotide-binding proteins, OppD and OppF found in the cytoplasm and lastly, the extracellular substrate-binding domain OppA which is a lipoprotein anchored to the membrane (Berntsson et al., 2009; Henrich et al., 1999; Hopfe et al., 2011). Gram-negative bacteria contain periplasmic substrate-binding proteins whereas Gram-positive bacteria contain substrate-binding proteins anchored to the cell membrane (Berntsson et al., 2009; Nicolás et al., 2007). A pore is formed by the homologous integral membrane proteins as both proteins, OppB and OppC, cross the membrane six times with 12 transmembrane segments in total. This was predicted from the sequences of these domains as well as the requirement of the 12 transmembrane segments for substrate transport through the membrane (Henrich et al., 1999). The OppD and OppF domains bind ATP and hydrolyse the ATP which fuels the transport of the substrate through the membrane whereas OppA is responsible for the binding of the substrate which results in the import of oligopeptides into the cell (Berntsson et al., 2009; Wium et al., 2015).

As the OppA protein has the function of binding substrates it is regarded as an essential protein and is well recognised in bacteria. The OppA protein in mycoplasmas is expected to be essential for

survival as these organisms are not able to produce oligopeptides. Staats et al. (2007) found that only two out of eleven Mollicutes genomes which were available at the time contained the *oppA* gene. It was therefore questioned if the OppA protein is essential for survival of mycoplasmas. The genomes of 39 mycoplasmas were studied by Wium et al. (2015) to determine whether these mycoplasmas do contain the *oppA* gene and therefore their possible significance for survival. It was illustrated that all mycoplasmas which contained annotated OppABCDF operons (not including hemoplasmas) do contain an *oppA* gene which suggests the importance of the OppA protein as part of the Opp transport in mycoplasmas (Wium et al., 2015). Therefore the OppA protein can be considered as a possible target for vaccines and antimicrobials against mycoplasmas.

2.8.3 Pathogenicity of mycoplasmas

2.8.3.1 Adhesion of the mycoplasma to its host

Infection of a host cell is established by the adherence of the mycoplasma to the host tissue. With this being the first step to colonisation it is regarded as a major virulence factor. Adherence and colonisation of a host holds nutritional advantages such as a higher concentration of nutrients and the availability and utilisation of fatty acids and cholesterol of the host membrane. Mycoplasmas are dependent on their host for nutritional substances therefore they usually adhere to and colonise epithelial linings of respiratory and urogenital tracts of infected animals and can be considered surface parasites. The attachment of the mycoplasmas to the host cells will not be disrupted by ciliated epithelium or urine as they attach firmly to the host cell (Bradbury, 2005; Kleven, 1998; Razin, 1978; Thompson et al., 2011). Mycoplasmas can also adhere to a variety of other surfaces including erythrocytes, tracheal epithelium, spermatozoa as well as surfaces such as glass and plastic but are particularly known for their adhesion and colonisation of epithelial linings (Razin, 1978).

Fimbriae and pili are structures used in a large number of bacteria for adherence to the host cell. Mycoplasmas, however, lack these structures and therefore externally exposed membrane bound components, mediate binding to the host cell and are collectively known as adhesins (Hopfe et al., 2011; Le Roux and Hoosen, 2010; Razin et al., 1998). *M. pneumoniae* and *M. genitalium* have the most defined and studied adhesins (Razin et al., 1998). The major adhesin proteins P1 of *M. pneumoniae* and MgPa of *M. genitalium* play a crucial role in cytoadhesion, are similar in structure, function in host attachment, and are exposed membrane proteins (Le Roux and Hoosen, 2010; Razin et al., 1998).

A variety of mycoplasmas have unique features such as a terminal organelle, also known as the tip organelle which enables them to adhere to the host cell in order to colonise and obtain nutrients.

The adhesins are concentrated in the tip organelle which also contains accessory proteins (Chaundhry et al., 2007; Seto et al., 2001). Both *M. pneumoniae* and *M. genitalium* contain these tip organelles. The tip organelle of *M. pneumoniae* consists of P1 and P30 proteins which provide polarity to the cytoaherence event whereas the tip structure in *M. genitalium* contains MgPa and P32 proteins (Browning et al., 2011; Rottem, 2003). For *M. pneumoniae* the process of adhesion does not rely on the adhesin proteins alone but it also includes the accessory proteins HMW1, HMW2, HMW3, A, B and C. The accessory proteins involved in adhesion of *M. genitalium* include MG312, MG317 and MG320. The accessory proteins together with cytoskeletal elements are responsible for the assistance in lateral movement and the location/distribution of the adhesin molecules at the tip organelle. The cytoskeleton-like structure has multiple functions which include adjusting cell shape, gliding motility and localisation of adhesins and are therefore of significant importance (Razin et al., 1998).

The tip organelle is not found in all mycoplasma species. Homologous structures are only found in the mycoplasmas which are in the same phylogenetic group as *M. pneumoniae* and *M. gallisepticum* (Browning et al., 2011). The mycoplasmas which are not in the same phylogenetic group depend on unrelated proteins and lipoproteins for adhesion to the host cell. Species such as *M. hominis* lack the tip organelle and are dependent on their surface lipoproteins which play a role in cytoadherence such as the variable-adherence-associated (Vaa) or P50 protein as well as the OppA protein (Brown et al., 2014; Browning et al., 2011; Henrich et al., 1999).

2.8.3.2 Invasion of the host cell

It is seen in numerous bacteria that when there is contact between the bacterial pathogen and the host cell, bacterial internalisation is made easier as rearrangements in the host cytoskeleton are triggered. As mycoplasmas only contain a cell membrane and no cell wall, direct contact is made with the host cell which could facilitate invasion of the host cell (Rottem, 2003).

It was believed for a considerable time that mycoplasmas do not invade the host cell but rather stay attached to epithelial cells. However, some mycoplasmas have evolved the ability to enter non-phagocytic cells of the host and this forms an important part of their pathogenicity (Rottem, 2003). The mycoplasmas able to invade their host cells have a unique advantage as they will be able to resist the host defenses, which will enable them to establish chronic and in some cases systemic infections (Rosengarten et al., 2000). One of the mycoplasma species able to invade their host cells is known as *M. penetrans* which was isolated from a patient with acquired immunodeficiency syndrome (AIDS). Winner et al. (2000) showed that mycoplasma invasion is not restricted to

human pathogens such as *M. penetrans*, *M. fermentans*, *M. pneumoniae* and *M. genitalium* because these properties could also be seen in *M. gallisepticum*, an avian pathogen.

The mechanism by which the mycoplasma is able to invade the host cell is not fully understood although it was proposed that the mycoplasmas generate 'uptake signals'. The signals would then cause assembly of cytoskeletal structures which include actin filaments, tubulin and α-actin in the host cell (Rottem and Naot, 1998). The invasion mechanisms of mycoplasmas are similarly to the clathrin-coated pits mechanism of endocytosis observed in *Chlamydia trachomatis*. In the case of *M. genitalium*, after it has entered the cell it seems to be situated in membrane-bound vacuoles close to the host cell nucleus (Le Roux and Hoosen, 2010).

2.8.3.3 Evasion of the host's immune system

Some mycoplasmas survive and persist without being affected by the host immune system by locating themselves intracellularly. As survival of mycoplasmas entails avoiding the immune system but still remaining in contact with the host cell, these organisms developed mechanisms allowing them to circumvent recognition by the immune system (Bradbury, 2005; Le Roux and Hoosen, 2010). The host usually targets the Mollicute's surface, as the epitopes recognised by the host immune system are the membrane proteins expressed on the surface of the Mollicutes. In order to survive they change their surface antigenic repertoire to avoid detection by the hosts immune system (Le Roux and Hoosen, 2010; Rottem, 2003).

Molecular mimicry and phenotypic plasticity are two of the most studied mechanisms which enable the mycoplasmas to avoid recognition by the host's immune system. Sequence or structural similarities of antigenic determinants or self antigens that are found in both mycoplasmas and the host cell are known as molecular mimicry (Cusick et al., 2012; Rottem, 2003). Mycoplasmas are also able to change the variation of one genotype's nucleotide sequence by DNA arrangement which leads to the production of more than one alternative form of the phenotype. This phenomenon is known as phenotypic plasticity and can change the morphology, response to environmental conditions and the physiology of a trait of the mycoplasma (Bradbury, 2005; Le Roux and Hoosen, 2010).

Bacterial pathogens have a survival strategy whereby they gain or lose surface components at a high frequency. This method is known as antigenic variation and is the common way to achieve phenotypic plasticity. Some mycoplasmas possess multigene families encoding cell surface lipoproteins, playing an important role in the generation of antigenic variation and switching. This

allows the mycoplasma to evade the host's immune system (Bradbury, 2005; Le Roux and Hoosen, 2010).

2.8.4 Managing mycoplasma infections

2.8.4.1 Prevention

In poultry, mycoplasmas can be transmitted from generation to generation, which is known as vertical transmission. Some progress has been made regarding the elimination of this vertical transmission by dipping eggs in antibiotic solutions or heating the eggs to 46 ºC for 12-14 h. A combination of egg dipping and injection with antibiotics can also be used as preventative method (Kleven, 2008; Nascimento et al., 2005; Swayne et al., 2013).

In previous studies it has been shown that mycoplasmas are able to survive up to 6 days on human hair and skin. They can also survive on materials such as cotton and rubber (Christensen et al., 1994). As the mycoplasmas are able to survive in the environment on different materials, they can be transmitted indirectly via wild birds, drinking water, breeding materials on clothes, footwear or contaminated equipment (Marois et al., 2000; Scholtz, 2014).

To detect mycoplasma infections in the early stages would be advantageous as this could prevent spread of infections to other flocks and therefore flocks need to be monitored consistently. However, it should be kept in mind that diagnosis can sometimes result in false negative detection of mycoplasma infections as mycoplasmas are able to conceal themselves in affected tissues. Another preventative measure is an effective biosecurity program which entails following certain methods in order to prevent infections or spread of infections. Small geographical areas, however, then contain a greater number of poultry resulting in a higher risk for exposure to infections. Therefore it is crucial that all personal on the farm follow the procedures to insure that there is no lapses in the program which can lead to infection outbreaks (Kleven, 2008; Nascimento et al., 2005).

2.8.4.2 Medication

Poultry mycoplasmas can be treated with antimicrobials or antibiotics which will decrease manifestations as well as the risk of vertical transmission. However, antibiotics that target the cell wall of microbes will not have an effect on mycoplasmas as they lack a cell wall. Therefore mycoplasmas are resistant to antibiotics like penicillins or cephalosporins but they are sensitive to antibiotics such as macrolides and tetracyclines which inhibits protein synthesis and fluoroquinolones which inhibits DNA synthesis (Kleven, 2008).

Enrofloxacin and tiamulin are the preferred drugs to use against *M. synoviae* and *M. gallisepticum* infections in chickens and turkeys. Commercially available enrofloxin is a fluoroquinolone with antimicrobial activity against most pathogens such as Gram-negative bacteria and *Mycoplasma* spp. Tiamulin known as a pleuromitilin antibiotic has been used routinely against *M. gallisepticum* infections in poultry such as chickens and turkeys. These drugs accumulate in high concentrations in mucosal membranes of the respiratory and reproductive tracts where the mycoplasmas are usually found (De Lucas et al., 2013; Li et al., 2010; Nascimento et al., 2005; Stipkovits and Kempf, 1996).

A factor to consider in the treatment of mycoplasma infections is that secondary bacterial infections can also occur in conjunction with mycoplasma infections. Therefore antibiotics that will target both the secondary infection and the mycoplasma infection need to be selected (Stipkovits and Kempf, 1996). Introduction of antibiotics to birds can be accomplished through their drinking water, feed or injection. However, the use of antibiotics as well as an immune response can have an influence on diagnosis of mycoplasma infections as they are able to evade or conceal themselves in affected tissue. Thereby they protect themselves from the host immune system and antibiotic treatments. Residing intracellularly, encourages the establishment of latent infections resulting in ineffective drug therapies (Liu et al., 2007; Nascimento et al., 2005; Razin et al., 1998).

Although antibiotic medication can reduce vertical transmission and clinical signs it cannot eliminate infection from a flock. As a result, the antibiotic treatment has to be repeated but this could lead to the development of antibiotic resistance (Stipkovits and Kempf, 1996). Using antibiotics in food producing animals can lead to accumulation of these residues in meat and milk amongst others. The antibiotic residues can lead to numerous problems in humans such as transfer of antibiotic resistance and immunopathological effects to name a few (Darwish et al., 2013). Although it is useful in prevention of economic losses, antibiotics are therefore not viewed to be a long-term solution (Kleven, 2008; Stipkovits and Kempf, 1996).

2.8.4.3 Vaccines

The use of veterinary vaccines instead of antibiotics and drugs for the treatment or prevention of diseases holds many advantages and has become regular in farmed animals. It is also more cost effective and antibiotic resistance is avoided (Sharma and Hinds, 2012). A few factors should, however, be taken into consideration such as administration of the vaccine, the type of vaccine as well as costs of the vaccine and vaccination process. Although vaccines provide a good solution for the control of diseases, good biosecurity and management practices should still be a priority and vaccination should not be regarded as a replacement (Kleven, 2008; Marangon and Busani, 2006).

A few live attenuated vaccines against *M. gallisepticum* (MG) in chickens have been developed including the F strain, 6/85 and ts-11, with only 6/85 and ts-11 being commercially available in South Africa (Armour et al., 2013; Kleven, 2008). However, currently there are no vaccines available against mycoplasma infections in ostriches.

2.8.5 Ostrich-infecting mycoplasmas

Mycoplasmas that infect ostriches are associated with a respiratory disease syndrome where they infect the upper respiratory tract of ostriches leading to inflammation in the eye (conjunctivitis), nose (rhinitis) and air sack (airsacculitis) (Botes et al., 2005; Huchzermeyer, 2002). Clinical signs of this syndrome include swollen sinuses, eye infections, watery discharge from the nose and rattle sounds in the throat. This is observed mostly during cold and dusty conditions in feedlot ostriches and predominantly during seasonal changes. Mycoplasma infections can however be increased due to stress, environmental conditions and other opportunistic infections caused by pathogens such as *E. coli* and *Pasteuralla* species to name a few (Botes et al., 2005). The result of this respiratory syndrome is a loss in production, downgrading of carcasses and reduced growth rate (Botes, 2004; Botes et al., 2005; Verwoerd, 2000).

Botes et al. (2005) isolated three ostrich-infecting mycoplasma species referred to as Ms01, Ms02 and Ms03. Langer (2009) isolated Ms01 and Ms03 from Namibian ostriches and described them as non-motile, non-helical and fried egg forming colonies. The cells of both are able to be filtered through a 0.22 µm filter and they both require serum and cholesterol for growth (Langer, 2009).

The three ostrich-infecting mycoplasma species Ms01, Ms02 and Ms03 were found to be quite different from each other and phylogenetically fall into two mycoplasma groupings. This was shown by the analysis of the 16S rRNA gene sequences which revealed that Ms01 grouped in a different clade to Ms02 and Ms03 but all form part of the hominis group as can be seen in Figure 2.8 (Botes et al., 2005; Wium et al., 2015).

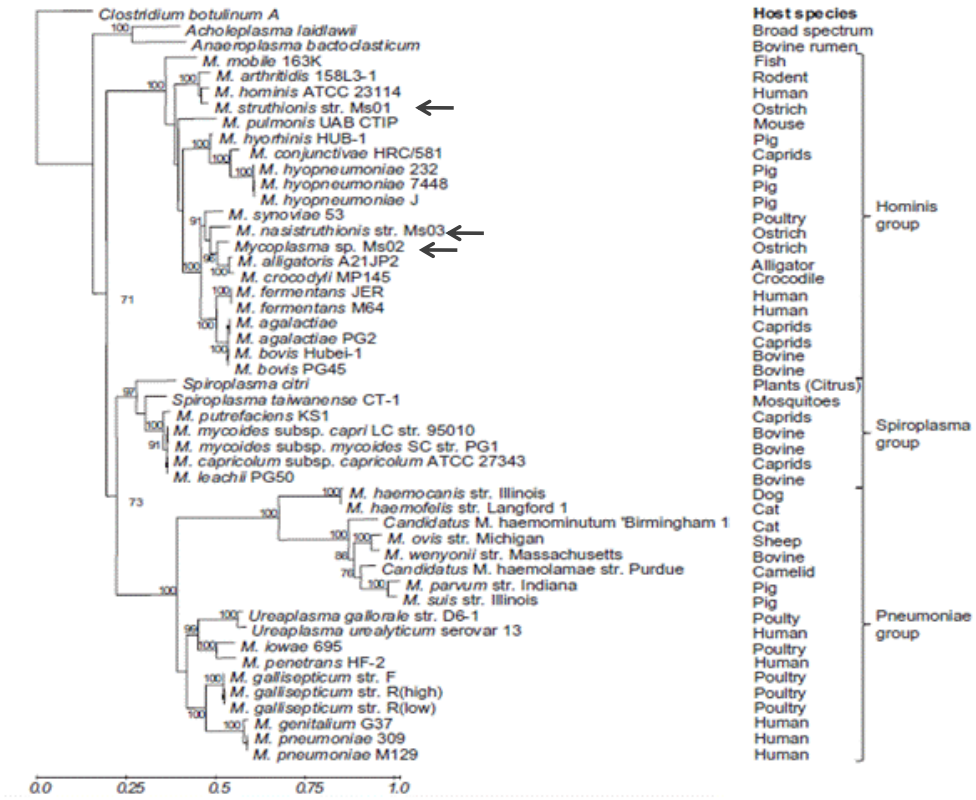

Figure 2.8 Phylogeny of mycoplasma species based on 16S rRNA sequences. All three ostrich-infecting mycoplasmas (indicated by the arrows) fall within the hominis group (Adapted from Wium et al. 2015).

2.8.5.1 Control of ostrich-infecting mycoplasmas

Intense rearing of ostrich chicks has resulted in increased population densities and concomitant stress which results in a greater risk of mycoplasma infections. Medication or antibiotics typically used against mycoplasma infections in ostriches are Doxycycline, Tylosin and Oxytetracycline amongst others. As antibiotics are not able to eradicate the mycoplasmas but only reduce or contain them, some ostriches will not have symptoms associated with mycoplasma infections but will be a carrier of the infection. Symptoms of mycoplasma infections can be identified early by inspecting ostriches in the morning for clinical signs of the disease and taking note of disapproved ostrich numbers at abattoirs due to infections. Ostriches that show clinical symptoms as mentioned in section 2.8.5 should be removed from the flock. Monitoring and early identification of mycoplasma infections can determine the type and time period of treatment (Olivier, 2006). Due to the ineffectiveness of medication, infections should be managed and controlled within commercial ostrich farms by means of biosecurity systems (Olivier, 2006).

Biosecurity systems include the reduction of factors causing stress, regular cleaning of water troughs, monitoring of feed intake and weight gain as well as shelter from wind, dust and rain for all ostriches. Vehicles, implements and protective clothing should be disinfected when arriving at ostrich farms as well as when departing, in order to keep the production areas sanitary as the

pathogens can stay viable in soil for long periods. Another preventative measure is the use of footbaths although this can also become a possible infection site therefore the footbaths needs to be disinfected regularly (Kleven, 2008; Mather and Marshall, 2011).

Maintaining biosecurity systems has become more difficult to implement as rearing of ostriches became intensive (Kleven, 2008; Mather and Marshall, 2011). The Veterinary Procedural Notice (VPN) VNP/04/2012-01 (Revision 6.0) 2012 provides biosecurity measures for ostrich production in South Africa. These regulations were implemented after the first outbreak of avian influenza in 2004 and all ostrich farms must adhere to these regulations to avoid any form of disease outbreaks. Although these measures were put in place to prevent avian influenza outbreaks they can also be of use to prevent spread of mycoplasma infections. As mycoplasmas are opportunistic pathogens, preventing spread of other pathogens might also prevent mycoplasma infections. Included in the VPN is that all water sources accessible to ostriches should be fenced off to prevent or minimise contact between the ostriches and wild birds. No pigs or other poultry may be kept on ostrich farms and water troughs should be disinfected regularly. A factor to consider is that biosecurity will only work with well trained and committed personnel (Scholtz, 2014).

As medication or antibiotic treatment against mycoplasma infections in ostriches is expensive and does not eradicate the mycoplasmas, alternative methods for controlling of mycoplasma infections by vaccination were investigated in previous studies. Pretorius (2009) found that the commercially available vaccines MS-Bac and MG-Bac against *Mycoplasma synoviae* and *Mycoplasma gallesepticum* respectively which infect chickens had no effect on the ostrich-infecting mycoplasmas. Currently there are no vaccines available against the ostrich-infecting mycoplasmas and successful cultivation of mycoplasmas is problematic (Botes et al., 2005). The development of DNA vaccines will circumvent the cultivation problem and can therefore provide a possible alternative to the development of vaccines against mycoplasma infections in ostriches.

2.8.6 Development of DNA vaccines against ostrich-infecting mycoplasmas

Selection of DNA vaccine vectors

The VR1020 and pCI-neo vectors were selected in a previous study for use in DNA vaccine trials against Ms03 in ostriches (Wium, 2015). The vectors were chosen as they were used for DNA vaccine studies in poultry such as chickens, ducks and turkeys (Lee et al., 2003; McCutchan et al., 2004; Meunier et al., 2016). The pCI-neo vector is a mammalian expression vector that contains a human enhancer/promoter region, and a CMV eukaryotic promoter, for the expression of cloned DNA inserts in mammalian cells. pCI-neo also contains the late SV40 polyadenylation sequence

which has been shown to be efficient for polyadenylation and a ampicillin resistance gene for selection during plasmid production (Promega Corporation, 2009).

The VR1020 vector also contains the CMV eukaryotic promoter for expression, but instead contains a Bovine growth hormone (BGH) polyadenylation and the kanamycin resistant gene for selection (Coban et al., 2005). An upstream signal sequence known as the tissue plasminogen activator (TPA) is included in the VR1020 vector and this sequence is able to improve expression in mammalian cells and assist in the secretion of the expressed protein (Lobo et al., 1999).

Characteristics and selection of oppA as vaccine candidate gene

The OppA protein has also been evaluated as antigen in the *Moraxella catarrhalis* and *Yersinia pestis* species as vaccine antigen (Chaudhuri and Ramachandran, 2014; Yang et al., 2011). Agüero et al. (2015) showed that the OppA protein of *M. gallisepticum* is an excellent vaccine candidate as it exhibited immunogenicity.

In *M. hominis* the oligopeptide transport protein was found to contain a substrate binding domain referred to as OppA. The OppA is an extracellular lipoprotein anchored to the cell membrane and in the case of *M. hominis* was found to be involved in host attachment. The OppA protein has several characteristics which makes it a suitable candidate for use in a vaccine. Firstly, being located extracellularly implies that the OppA protein will be exposed to the immune system of the host and can therefore be regarded as a virulence factor (Henrich et al., 1999; Hopfe et al., 2011; Wium et al., 2015). Sedondly, the ABC importers and thus OppA proteins, are not found in eukaryotes but only in prokaryotes, archaea and bacteria (Berntsson et al., 2010; Rice et al., 2014). Therefore, homologous genes should not be present in the ostrich genome. Thirdly, since mycoplasmas need to acquire nutrients from their host the Opp transporter plays an essential role in their survival. Wium et al. (2015) found that with the exception of hemoplasmas, all mycoplasma Opp operons, evaluated in their study, contain an OppA and therefore it most probably has an essential role as a part of the Opp transporter in *Mycoplasma* species. As all three ostrich infecting mycoplasmas also contain an OppA, it can possibly be used as a vaccine candidate for prevention of mycoplasma infections in ostriches.

In previous studies the *oppA* gene of Ms01 and Ms03 was inserted into the DNA vaccine vectors pCI-neo, VR1020 and VR1012 for evaluation in vaccine trials (De Wet, 2015; Van Tonder, 2013; Wium, 2015). In the study done by Wium (2015) the vaccines containing the *oppA* gene of Ms03 were administered in a single dose (100 µg/ml). The trial was however terminated after 3 weeks as a result of an avian influenza outbreak. Results up until week 3 showed that both the pCI-neo and

VR1020 vaccines were able to elicit an immune response. As the trial was not concluded these two vaccines were re-evaluated in this study of which the result are presented in the next chapter.

In the study by Van Tonder (2013) administering the DNA vaccines orally using a bacterial carrier was also evaluated. For this purpose secondary rabbit anti-ostrich IgA antibodies were used which was produced using intact IgA protein. Part of the antibody can, however, be produced recombinantly as an alternative for the production of these antibodies. This would ensure a reproducible source for the production of secondary anti-IgA antibodies. Results on the production of such a protein using a Baculovirus-insect expression system is subsequently presented in Chapter 4.

3. Vaccination Trial

3.1 Introduction

The demand for ostrich products such as meat, leather and feathers has resulted in more intensive rearing strategies which in turn create an environment for the spread of pathogens and thus diseases. Mycoplasma infections are associated with respiratory disease in feedlot ostriches and result in reduced production. To date, three species were found to be involved namely Ms01, Ms02 and Ms03 (Botes et al. 2005). The ostrich industry contributes substantially to the South African economy and to ensure conservation of this industry, disease management is of utmost importance.

In an attempt to address Ms03 infections, a DNA vaccine was developed as part of a previous study. The *oppA* gene of Ms03 was chosen as vaccine antigen and cloned into the VR1020, VR1012 and pCI-neo plasmids (Wium 2015). These vaccines were administrated to ostriches at a single dose (100 µg/ml) to determine if an anti-OppA antibody response could be elicited. As a result of an avian influenza outbreak in the region, the trial was terminated after 3 weeks resulting in only a single vaccination being administered and collection of blood samples at week 0 and 3 only. The results, however, showed that both VR1020_*oppA* and pCI-neo_*oppA* were able to elicit an immune response over the 3 week period, but no response was observed for VR1012_*oppA*. The objective of this study was therefore to re-evaluate the ability of the pCI-neo_*oppA* and VR1020_*oppA* vaccines developed against Ms03 to elicit an anti-OppA antibody response in ostriches. To this end ostriches were vaccinated with the pCI-neo_*oppA* and VR1020_*oppA* DNA vaccines, but using three different doses and also including a booster vaccination after 6 weeks. Serum was collected from trial birds over a period of 9 weeks and analysed for anti-OppA antibodies using an enzyme-linked immunosorbent assay (ELISA). During the course of the trial all ostriches were weighed and monitored using PCR-analysis in order to establish if the ostriches were infected with mycoplasmas.

3.2 Materials and Methods

3.2.1 Ethical clearance

In order to conduct the trial it was necessary to obtain ethical clearance. The Stellenbosch University Animal Ethics Committee (SU-ACUM 13-00019) granted ethical clearance for the trial to be conducted. Ethical permission was also obtained from the Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/1/3) in terms of Section 20 of the Animal Disease Act 1984 (Act No 35 of 1984). Included in the ethical clearance was that the research should be compliant with the

Meat Safety Act (Act No 40 of 2000) for allowance of the animals to enter the human food chain after slaughter.

3.2.2 Preparation of DNA vaccines

The VR1020 and pCI-neo vectors containing the Ms03 *oppA* gene were previously transformed into *E. coli* JM109 competent cells by Wium (2015) and cultures stored at -80ºC. These were used to prepare overnight cultures (O/N) in a Falcon® tube (14 ml) by inoculating 5 ml 2xYT-medium (16 g tryptone, 10 g yeast extract and 5 g NaCl) with 10 µl of freezer stock for VR1020_*oppA* and pCI-neo_*oppA* respectively. The VR1020_*oppA* O/N cultures also contained 10 µl kanamycin (25 mg/ml) whereas the pCI-neo_*oppA* O/N cultures contained 5 µl ampicillin (100 mg/ml). The O/N cultures were grown for 16-18 h at 37°C on an orbital shaker (200 rpm). Starter cultures were prepared for both plasmids in a 50 ml Erlenmeyer flask by adding 20 µl kanamycin (25 mg/ml) for VR1020 and 10 µl ampicillin (100 mg/ml) for pCI-neo, to 10 ml of 2xYT medium and inoculating it with 1 ml of each O/N culture respectively. The starter cultures were incubated at 37°C on an orbital shaker at 200 rpm for 8 h. The starter cultures were then each added to a 2 L Erlenmeyer flask containing 200 ml 2xYT medium with 400 µl kanamycin (25 mg/ml) and 200 µl ampicillin (100 mg/ml) for VR1020 and pCI-neo respectively. The cultures were incubated for 16 h at 37°C on an orbital shaker (150 rpm). This was followed by the isolation of the plasmids using an endotoxin-free plasmid DNA purification kit (NucleoBond® Xtra Midi Plus EF, Macherey-Nagel, Germany) according to manufactures instructions. The yields of the two isolated plasmids were determined using a Nanodrop® ND 1000 spectrophotometer (Novell®, USA). The integrity of both the plasmids were determined by electrophoresis by loading 5 µl of purified plasmid on a 1% (w/v) agarose gel consisting of SeaKem® LE Agarose (Lonza, Switzerland) dissolved in 1x TAE buffer (40 mM Tris-aminomethane; 1 mM EDTA, pH 8; 20 mM glacial acetic acid) containing 0.5 µg/ml ethidium bromide (EtBr) for visualisation under UV-light. The agarose gel was electrophoresed in 1x TAE buffer and depending on the size of the gel, 80 Volts (V) were used for a 3 cm x 10 cm gel, 90 V for a 7 cm x 10 cm gel and 110 V for a 10 cm x 15 cm gel.

For visual confirmation that both plasmids contained the *oppA* gene and were in the supercoiled formation, the plasmids were digested with restriction enzymes. The pCI-neo_*oppA* plasmid was digested with *AccI* (Promega, USA) in a mixture with a total volume of 20 µl containing 16.3 µl autoclaved Milli-Q® water (Milli-Q®), 2 µl 10x buffer G, 0.2 µl bovine serum albumin (BSA) (10 mg/ml), 1 µl pCI-neo_*oppA* (1 µg/µl) and 0.5 µl *AccI*. This mixture was incubated at 37°C for 2 h followed by a deactivation step at 80ºC for 5 min. *AccI* has only one cut site in the plasmid following the insert. The VR1020_*oppA* was digested with *BamHI* Fast-digest (Fermentas, USA) in

a mixture with a final volume of 20 µl which contained 16.5 µl Milli-Q®, 2 µl 10x FastDigest buffer, 0.5 µl VR1020_*oppA* plasmid (1 µg/µl) and 1 µl *BamHI* Fast-digest. The mixture was incubated at 37°C for 5 min followed by a deactivation step at 65°C for 10 min. The *BamHI* has a cut site on the plasmid on either side of the insert. The digested products were then analysed by loading 5 µl of the digested product on a 1% (w/v) agarose gel containing 0.5 µg/µl EtBr in order to visualise the digested products under UV-light. The agarose gel was electrophoresed as previously described.

The two isolated DNA vaccines were diluted to the desired concentration of 100 µg/ml, 600 µg/ml and 1200 µg/ml with sterile PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2) in a class II biological safety cabinet (ESCO, Singapore). The dilutions were made a day before use in 100 ml serum bottles which were sealed with 20 mm rubber silicone stoppers (Sigma-Aldrich) and covered by a 20 mm aluminum seal with a tear away center cap (Sigma-Aldrich).

3.2.3 Animals used for vaccination trial

Ostriches hatch with an immature immune system and as a result rearing of day-old ostrich chicks to an age of 3 to 4 months is a critical phase. During this time high mortalities occur and they are also prone to airway infections (Scholtz, 2014). The trial was initiated in Fraserburg using ± 3 month old ostriches which were reared in Fraserburg (Northern Cape Province) as a precautionary measure to limit pathogen exposure. They were then moved to Oudtshoorn (Western Cape Province) once reaching a weight of 40 kg (± 4 months of age). At the start of the trial each of the ostriches received a tag (Allflex Livestock Identification Tags or Infotag Eartags/Oorplaatjies) under their wing, containing a unique number in order to specifically identify each ostrich in the trial.

The ostriches had access to water ad lib at all times and were kept in open air camps in Fraserburg and Oudtshoorn. Birds were handled by trained and experienced farm personnel at all times. Trial birds were not kept separate in Fraserburg, and in Oudtshoorn and the ostriches forming part of the trial were grouped with other ostriches of the same weight and age that had arrived from Fraserburg.

3.2.4 Vaccination trial design

For the vaccination trial 140 ostriches were divided into a pCI-neo_*oppA* group, a VR1020_ *oppA* group and a control group. As shown in Table 3.1 the pCI-neo_*oppA* and VR1020_ *oppA* groups

each had 60 ostriches whereas the control group contained 20 ostriches. Each of the groups of 60 were further divided into 3 subgroups containing 20 ostriches each that received the pCI-neo_*oppA* and VR1020_ *oppA* vaccines at the respective doses of 100, 600 and 1200 µg/ml whereas the control group did not receive any vaccine. The vaccine doses were administered in a single 1 ml volume by IM injection in the upper thigh of the ostriches at week 0 and a booster administered at week 6 (Table 3.1).

Blood samples (4 ml) were drawn from the jugular vein by using 18Gx1/2" needles (Vacuette®, Austria) and collected in Vacuette® Z serum sep clot activator tubes at week 0, 3, 6 and 9. Centrifugation at low speed for 10 min was used in order to separate the serum before transferring to 1.5 ml Eppendorf tubes and storing at -20°C. The ostriches were kept in Fraserburg from week 0 to 3 and in Oudtshoorn from week 6 to 9.

After arrival of the ostriches in Oudtshoorn one week prior to the week 6 sampling and the administration of a booster vaccine, the birds were kept in quarantine for 29 days and random testing was done for avian influenza. This is standard practice as required by avian influenza control measures. In order to simulate challenge conditions, the birds were then moved to feedlots with other ostriches where they received 2.5 kg of feed per ostrich per day. To further create ideal conditions for possible mycoplasma infections to occur the trial was conducted in late autumn, early winter (March 2015 to June 2015) since mycoplasma infections are known to be a problem during seasonal changes such as autumn and spring.

3.2.5 Weight recording during trial

During the trial period the ostriches were weighed at week 0, 6 and 9 (Table 3.1).This was done to evaluate the influence of the vaccine on the weight gain of the ostriches as well as to monitor the overall well-being of the ostriches. During week 3 there was no scale available and the weight therefore not recorded.

Table 3.1 Vaccination trial groups with administration route, schedule and dose used.

Groups (DNA vaccines)	**Dose * (µg/ml)**	**Week ♦**	**Serum sample collection**	**Swab sample collection**	**Administration of vaccine #**	**Weight Recording**	**Ostriches**
pCI-neo_*oppA*	100	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
	600	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
	1200	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
VR1020_*oppA*	100	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
	600	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
	1200	0	✓	✓	✓	✓	20
		3	✓	✓	-	-	
		6	✓	✓	✓	✓	
		9	✓	✓	-	✓	
Control	-	0	✓	✓	-	✓	20
		3	✓	✓		-	
		6	✓	✓		✓	
		9	✓	✓		✓	
Total							140

* Vaccine dose administered in final volume of 1 ml
# Vaccines administered intramuscularly
♦ Ostriches were in Fraserburg during week 0 to 3 and in Oudtshoorn during week 6 and 9.

3.2.6 Trachea swab sample analysis by PCR

Swab samples were taken during week 0, 3, 6 and 9 from the trachea of the ostriches (Table 3.1). Dry sterile swabs with a plastic applicator and a soft rayon swab tip were used (Copan, Italia). The swabs were inert and non-toxic to micro-organisms as well as the ostriches. Prior to PCR analysis each swab was rinsed in 200 µl sterile PBS buffer, contained in a 1.5 ml Eppendorf tube. These samples were then used for PCR analysis in order to determine the presence of mycoplasma infections. The samples were first tested by using a generic primer pair specific for the *Mycoplasma* genus. The samples that tested positive were further evaluated using species-specific primer pairs for the three ostrich-infecting mycoplasma species namely Ms01, Ms02 and Ms03. The sequences of the primer pairs used are shown in Table 3.2. All primers were designed by Botes (2004) and already available in the laboratory.

Each PCR reaction contained 9.95 µl Milli-Q® water, 2 µl 10x reaction buffer, 2.4 µl $MgCl_2$ (25 mM), 0.8 µl dNTP mix (5 mM) (Kapa Biosystems, RSA), 0.4 µl of each primer depending on the target (20 pmol/µl), 0.05 µl of 5 U/µl Super-Therm polymerase (JMR Holdings, USA) and 4 µl of the trachea sample. A Verti 96 well thermal cycler (Applied Biosystems) was used for amplification with the following conditions: 30 cycles of 94ºC (30 sec), 55ºC (15 sec), 68ºC (1 min) and a single cycle of 6 min at 68ºC before cooling to 15ºC. The reaction mixtures and thermal cycler conditions were the same for all primer sets. The PCR products were analysed on a 1% agarose gel containing 0.5 µg/ml EtBr for visualisation of the PCR products under UV light. Electrophoresis conditions were as previously described.

All samples that tested positive with the generic primer pair but negative with the species-specific primer pair were sequenced. The reaction mixtures contained 5 µl sequencing buffer (400 mM Tris-base (pH 9), 10 mM $MgCl_2 \cdot 6H_2O$), 1 µl of 16R1514 reverse primer or GPO3F forward primer, 1.5 µl of the trachea swab sample and 2 µl BigDye® Terminator mix (v3.1, Applied Biosystems, USA) and 0.5 µl Milli-Q® water to a final volume of 10 µl. The PCR conditions were as follow: 35 cycles of 96ºC (10 sec), 52ºC (30 sec), 60ºC (4 min) and a final step of 60ºC (10 min). The products were analysed by the Central Analytical Facility (CAF) DNA sequencing unit of the University of Stellenbosch by means of an ABI® 3100 Genetic Analyser (Applied Biosystems, USA). The sequences were subsequently analysed further with the Basic Alignment Search Tool (BLAST) by searching for similar sequences in the 16S ribosomal RNA sequence (Bacteria and Archaea) database of the National Center of Biotechnology Information (NCBI).

Table 3.2 Primer pairs and sequences for swab sample analysis

Primer Target		Primer names	Sequence	Product size
Generic primer pair	Genus *Mycoplasma*	GPO3F (F)#	5'-TGGGGAGCAAACAGGATTAGATACC-3'	272
		MGSO (R)*	5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	
Species-specific primer pairs	Ms01	MS012 (F)	5'-AACATTAGTTAATGCCGGATACGC-3'	499
		MS01D (R)	5'-GCCAGTATCCAAAGCGAGCC-3'	
	Ms02	MS02H (F)	5'-AATATAAAAGGAGCGTTTGC-3'	287
		MS02A (R)	5'-AAGGCAATAGCATTTCCTCTACT-3'	
	Ms03	MS03A (F)	5'-AGTGCTAATGCCGGATACTTATAC-3'	521
		MS03C (R)	5'-CGTTAACCTCTATACAATTCTAGCG-3'	
16S rRNA primer		16R1541 (R)	5'-GGTTGGATCACCTCCTT -3'	780

(F) = forward primer
* (R) = reverse primer
(Botes, 2004)

3.2.7 Preparation for analysis of serum samples

To analyse the serum samples of the ostriches taken during the trial, the ELISA technique developed by Wium (2015) was used. This, however, required the preparation of the Ms03 OppA proteins as antigen. Section 3.2.7.2 to section 3.2.7.3 describes the production and isolation of this protein. The ELISA was also re-optimised (section 3.2.7.4) to confirm that the parameters as used by Wium (2015) were optimal.

3.2.7.1 Producing the expression plasmid containing the *oppA* gene

Preparation for expression of the OppA protein

In previous studies the required Ms03 *oppA* gene was cloned into a pGEX-4T-1 vector (GE Healthcare, United Kingdom) (Wium 2015). The pGEX-4T-1 vector allows for expression of proteins with a Glutathione S-transferase (GST) tag which can be isolated by means of affinity chromatography with a Glutathione-Agarose column. The pGEX-4T-1_*oppA* was transformed into *E. coli* BL21(DE3)pLysS cells (Promega, USA) and freezer stocks made by adding 500 µl 80% glycerol to 500 µl of the *E. coli* cells containing the plasmid with the gene of interest and stored at -

80ºC. Freezer stocks were also made of the pGEX-4T-1 vector without any insert that was transformed into *E. coli* BL21(DE3)pLysS cells (Promega, USA). This was used as a control during protein expression studies.

Luria Bertani (LB) (10 g/L Bacto-Tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7) bacteriological agar plates (15 g/L) containing ampicillin (100 mg/ml) was casted and allowed to set. An inoculation loop was used to collect some of the thawed freezer stock of both the pGEX-4T-1_*oppA* as well as the pGEX-4T-1 plasmid containing no gene which was each streaked out on a LB agar plate and incubated at 37ºC for 16-18 h.

Colony PCR

Colony PCR was performed as by Wium (2015) to analyse colonies which formed on the agar plates in reactions containing 1 µl 10x reaction buffer, 0.4 µl dNTP's (5 mM), 0.6 µl $MgCl_2$ (25 mM), 0.5 µl pGEX_F (20 pmol/µl), 0.5 µl T7_R (20 pmol/µl), 0.1 µl Super-Therm polymerase (5 U/µl) and 6.9 µl Milli-Q®. Primer sequences are shown in Table 3.3. The primers used in this reaction were already available in the laboratory for use, therefore it was not necessary to have the primers synthesised. Selected colonies on the agar plates were picked with a sterile tooth pick and mixed into the reaction mixture in 0.2 µl thin wall PCR tubes respectively before the amplification reaction was started. This was done with an initial step of 94ºC (5 min) followed by 25 cycles consisting of a denaturing step at 94ºC (30 sec), annealing step at 55ºC (30 sec) and elongation/extension step at 72ºC (30 sec). The reaction ended with a single 72ºC (7 min) step and cooling to 15ºC. The PCR products were analysed on a 1% agarose gel containing EtBr (0.5 µg/ml) for visualisation under UV light. The agarose gel was electrophoresed as previously described.

O/N cultures were made from the colonies that tested positive by scraping them from the plates using a sterile toothpick which was added to a Falcon® tube containing 5 ml LB medium and 5 µl ampicillin (100 mg/ml) followed by incubation at 37ºC for 16-18 h on an orbital shaker (200 rpm). Freezer stocks were subsequently made by adding 500 µl of the O/N culture to 500 µl 80% glycerol in a 1.5 ml Eppendorf tube and this was then used to start the expression process.

Table 3.3 Primers used for colony PCR

Primer name	Sequence	Comment	Reference
T7_R	5'-GCTGTAATTTGGGCATTTTCTTG-3'	Internal *oppA* primer, binds at position 592-615	Wium (2015)
pGEX_F	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	pGEX-4T-1 vector	pGEX-4T-1 vector (GE Healthcare)

3.2.7.2 Expression of the OppA protein

An O/N culture was made by adding 4.75 ml Terrific-Broth (TB) medium (12 g/L Bacto-Tryptone, 24 g/L Bacto yeast extract, 4 ml/L of 2 M glycerol) supplemented with 100 ml/L phosphate buffer (1 M KH_2PO_4, 1 M K_2HPO_4), to a 14 ml Falcon® tube. To this 250 µl glucose (20% m/v), 5 µl chloramphenicol (34 mg/ml), 5 µl ampicillin (100 mg/ml) and 25 µl freezer stock (pGEX-4T-1_*oppA* in *E. coli* BL 21 cells) was added. The same was done for the control except that only the pGEX-4T-1 plasmid without any insert was used. Both cultures were incubated at 37ºC for 16 h on an orbital shaker (200 rpm).

An expression culture for the pGEX-4T-1_*oppA* was made by adding 95 ml TB-medium, 5 ml glucose (20% (m/v)), 100 µl ampicillin (100 mg/ml), 100 µl chloramphenicol (34 mg/ml) and 2 ml of the O/N culture to a 1 L Erlenmeyer flask. The expression culture for the pGEX-4T-1 control was made by adding 15 ml TB medium, 15 µl ampicillin (100 mg/ml), 15 µl chloramphenicol, 1.5 ml glucose (20% (m/v)) and 200 µl of the O/N culture containing only the pGEX-4T-1 plasmid to a 50 ml Erlenmeyer flask. The expression cultures were incubated at 37ºC on an orbital shaker at 200 rpm until an OD_{600} of 0.6 was reached as determined by using a Spectronic® 20 Genesys™ spectrophotometer. Expression was induced by adding IPTG to a final concentration of 0.4 mM to the culture containing the pGEX-4T-1_*oppA* plasmid as well as the control culture. The expression cultures were incubated at 37ºC for a further 6 h while on an orbital shaker (200 rpm). Samples (1 ml) were taken from both the pGEX-4T-1_*oppA* and pGEX-4T-1 expression cultures at the start of induction and then every 2 h up until 6 h after induction.

After the 6 h incubation period the remaining culture was harvested by centrifugation at 10 000 x g at 4ºC for 10 min (Beckman Coulter Avanti® J-E). The supernatant was discarded and the pellets of the culture containing the pGEX-4T-1_*oppA* as well as the pGEX4T-1 plasmid each resuspended in 1xTen50 buffer (100 µl per 1 ml of culture) (10 mM Tris-HCl, 1 mM EDTA, 50 mM Nacl, 0.1% Triton X-100, 0.2 M dithiothreitol (DTT) and 10% (v/v) glycerol) containing a protease inhibitor (1 tablet per 10 ml extraction solution of cOmplete ULTRA Tablets, Mini, EASYpack tablet, Roche). The samples were subsequently analysed by means of SDS-PAGE.

SDS-PAGE analysis of expressed OppA proteins

An acrylamide gel was caste that consisted of a stacking gel and an 8% resolving gel. The 4.5% stacking gel contained 4.25 ml stacking buffer (0.125 M Tris-base, 0.1% (m/v) SDS, pH 6.8), 0.75 ml stacking monomer (30% T, 2.7% C dissolved in stacking buffer), 15 µl N,N,N',N'-Tetramethylethylenediamine (TEMED) and 30 µl ammonium persulfate (0.2 mg/µl). The resolving

gel contained 9.6 ml resolving buffer (0.375 M Tris-base, 0.1% (m/v) SDS, pH 8.0), 2.4 ml resolving monomer (30% T, 2.7% C dissolved in resolving buffer), 30 µl TEMED and 36 µl ammonium persulfate (0.2 mg/µl).

The expression samples were prepared for electrophoresis by adding 12 µl bromophenol blue (0.1% (m/v) bromophenol blue in 0.1 M NaOH) and 30 µl reducing treatment buffer (0.125 M Tris-base, 4% (m/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, pH 6.8) to 30 µl of the resuspended pellet. The mixture was boiled for 3 min followed by incubation on ice up until the samples were loaded. The SDS-PAGE gel apparatus was set up containing the acrylamide gel and was filled with electrode buffer (25 mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.3). After 25 µl of each sample was loaded on the acrylamide gel it was electrophoresed for 2 h at a constant 25 mA. The resolving gel was subsequently stained for 1 h at 37ºC in Coomasie staining solution (0.125% Coomasie Brilliant Blue R250, 50% (v/v) MetOH and 10% (v/v) HOAc) under agitation in a hybridization oven/shaker (USA). The excess stain was then removed and the gel incubated in destain 1 (50% (v/v) MetOH and 10% HOAc) for 1 h at 37ºC under agitation in the hybridization oven/shaker followed by removal of destain 1 and incubation in destain 2 (5% (v/v) MetOH and 7% HOAc) at RT O/N under agitation at 25 rpm on a GyroTwister (Labnet, USA).

3.2.7.3 Isolation of the expressed OppA protein

The Ms03 OppA-GST protein was isolated from the resuspended *E. coli* cell pellet (Section 3.2.7.3) by means of a Glutathione-Agarose column (Sigma, USA). The lyophilized powder was swelled in Milli-Q® water (200 ml/g) for 30 min at RT and left O/N at 4ºC for 100% swelling of the powder to occur. The column (10.5 mm in height and 2.5 mm in width) was packed with the swelled slurry, avoiding the formation of bubbles, which added up to a volume of 10 ml. The column was equilibrated by washing it with 10 volumes (100 ml) of equilibration buffer (10 mM PBS and 150 mM NaCl, pH 7.4) in order to remove the lactose present in the lyophilized product. The column was then left O/N at 4ºC on 20 ml of the equilibration buffer.

The sample (8 ml) containing the Ms03 OppA-GST protein was prepared by 3 freeze-thawing cycles (20 min at 37ºC followed by 10 min at -80ºC) and 5 cycles of 2 sec sonication followed by 2 min on ice. The samples were then pulled through a 25Gx5/8" needle (Avacare, China) into a 1 ml Injekt-F syringe (BBraun, Germany) 3 times. The same procedure was then followed using a 23Gx1/4" needle (Nipro, Belgium) also for 3 repeats. The sample was then centrifuged (Labnet Prism™ R Refrigerated Microcentrifuge) at 10 000 x g for 10 min (4ºC) in order to obtain a clear supernatant which was loaded onto the column under gravity flow at 4ºC. The clear supernatant was

allowed to run through the column 5 times followed by a 1 h - 2.5 h incubation on the column in an attempt to increase binding of OppA-GST proteins and therefore the yield of the isolated protein.

The clear supernatant was subsequently eluted from the column followed by the first wash step using 5 volumes (50 ml) PBS-T (PBS, pH 7.2 with 1% (v/v) TritonX-100) containing a protease inhibitor (3 tablets per 50 ml) (cOmplete ULTRA Tablets, Mini, EASYpack, Roche). A second wash step was done with 5 volumes (50 ml) of PBS (pH 7.2) also containing a protease inhibitor (3 tablets per 50 ml). The bound Ms03 OppA-GST proteins were then eluted from the column with 3 volumes (30 ml) of elution buffer (10 mM reduced L-Glutathione and 50 mM Tris-HCl, pH 9.5) containing two cOmplete ULTRA Tablets. Twenty samples of 1 ml each were collected in 1.5 ml Eppendorf tubes. The column was subsequently cleaned by 5 volumes (50 ml) of cleansing buffer 1 (0.1 M boric acid and 0.5 M NaCl) followed by 5 volumes (50 ml) of Milli-Q® water. This was followed by 5 volumes of cleansing buffer 2 (0.1 M sodium acetate and 0.5 M NaCl) and finally the column was stored at 4ºC in 2 volumes (20 ml) of storage buffer (2 M NaCl with 0.2% Thimerosal).

The concentration of the isolated protein was determined by means of the Bradford assay. A standard curve was used in order to determine the concentration of the eluted samples. The curve consisted of an increasing protein concentration of 0, 0.25, 0.5, 0.75, 1.00, 1.25 and 1.5 mg/ml of bovine serum albumin (BSA) made up with the elution buffer containing protease inhibitor tablets. The Bradford assay was done in a 96-well polystyrene microtitre plate (Greiner Bio-one, Germany) by loading 5 µl per well of each sample and standard followed by the addition of 250 µl Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) etanol and 8.5% (v/v) phosphoric acid) to the same well (all samples were done in triplicate). The plate was incubated for 2 min at room temperature (RT) and absorbance was measured at 620 nm using a Multiskan Spectrophotometer plate reader (Labsystems, Finland). The samples containing the Ms03 OppA protein as indicated by the Bradford assay were subsequently analysed by SDS-PAGE as previously described.

3.2.7.4 Optimisation of the ELISA for analysis of serum samples collected during the vaccination trial

Biotinylation of secondary rabbit anti-ostrich antibodies for use in ELISA analysis

Rabbit anti-ostrich immunoglobulin (Ig) antibodies were previously produced and provided by Dr. A. Botes, Department of Biochemistry, University of Stellenbosch. These antibodies were isolated from the rabbit serum by mixing 0.5 ml rabbit anti-ostrich Ig serum with 1 ml PBS followed by the addition of 1.5 ml saturated ammonium sulfate ($(NH_4)_2SO_4$; 1 kg/L). The mixture was incubated at

4ºC for 20 min followed by centrifugation at 15 000 x g at 4ºC for 20 min using a Beckman Coulter® Avanti™ J-E centrifuge. The supernatant was discarded and the pellet dissolved in 1 ml PBS (pH 7.2) followed by the addition of 1 ml saturated $(NH_4)_2SO_4$. The mixture was incubated for 20 min at 4ºC and centrifuged at 15 000 x g at 4ºC for 20 min. Again the supernatant was discarded and the pellet redissolved in 0.5 ml PBS. The dissolved pellet was dialysed in a cellulose dialysis tube (average flat width: 10 mm; average diameter: 6 mm) (Sigma-Aldrich, Germany) O/N at 4ºC against an excess of carbonate buffer (0.1 M $NaHCO_3$, pH 8.3), with a single buffer change after 5 hours. After O/N dialysis the concentration of the antibodies in the dialysate was measured by means of the Nanodrop® ND 1000 spectrophotometer (Novell®, USA) at 280 nm followed by dilution of the sample to 5 mg/ml Ig in carbonate buffer (0.1 M $NaHCO_3$, pH 8.3).

The Ig was biotinylated by adding 600 µl of biotinylation reagent (2 mg/ml biotinamidohexanoic acid N-hydroxysuccinimide ester dissolved in dimethylformamide) dropwise to 2.4 ml Ig (5 mg/ml) in a pear-shaped flask and stirring the mixture slowly for 2 h at RT. The mixture was dialysed in a cellulose dialysis tube (average flat width: 25 mm; average diameter: 16 mm) (Sigma-Aldrich, Germany), O/N at 4ºC against an excess of PBS with a single buffer change after 5 h. Following O/N dialysis, glycerol was added to the biotinylated antibodies in a 1:1 ratio and stored at -20ºC.

Determining coating concentration and serum dilution factor

Maxisorp plates (96 well Nunc with certificate, Denmark) were used for ELISA analysis of the serum samples collected during the vaccine trial. For this purpose the optimal coating concentration of the wells with antigen (Ms03 OppA-GST protein) needed to be determined. Six rows were coated with different concentrations of Ms03 OppA-GST protein diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6). Row A and B was coated with 1 µg/ml, C and D with 5 µg/ml and rows E and F with 10 µg/ml (Figure 3.1). The coated plate was incubated for 18 h at 4ºC to allow for binding of the antigen to the plate surface.

The coating antigen was decanted followed by the addition of 200 µl casein buffer (10 mM Tris-HCl, 154 mM NaCl, 0.5% casein and 0.02% Thimerosal, pH 7.6) to all wells to block non-specific binding. The plate was incubated at 37ºC for only 1 h instead of 2 h as done by Wium (2015). The casein buffer was then decanted and the plate washed 5x with PBS-Tween (PBS buffer, pH 7.2 containing 0.1% (v/v) Tween® 20) and 3x with Milli-Q® water, compared to the 8x PBS-Tween wash used by Wium (2015).

A week 0 and week 3 serum sample from ostrich 207 (pCI-neo_*oppA* 1200 µg/ml group) and from 266 (VR1020_*oppA* 1200 µg/ml group), was used for optimising the serum dilution factor to be

used during ELISA analysis. These sera were diluted (1:20) in casein-Tween (casein buffer, pH 7.6 containing 0.1% (v/v) Tween®20) and 200 µl of these dilutions added to the wells in column 2 and 8 respectively (Figure 3.1). To the rest of the columns 100 µl/well of casein-Tween was added. A serial dilution was made by transferring 100 µl of the serum sample from column 2 into column 3, mixing, and repeating the transfer up until column 6 ending with a dilution factor of 1:320. The same serial dilution was repeated from column 8-12. The final 100 µl taken from column 6 and 12 was discarded. Column 1 and 8 acted as blank and therefore only received 100 µl/well casein-Tween. The plate was incubated at 37ºC for 1 h, the serum dilutions decanted and the plate washed 5x in PBS-Tween and 3x using Milli-Q® water.

After selecting a coating concentration the serial dilution was repeated as before except that a single coating concentration was used for all wells and the serial dilution started at 1:40 and ended at 1:640. A week 0 and week 9 serum sample from ostrich 261 (VR1020_*oppA* 1200 µg/ml subgroup) was used for the serial dilution. This was done to determine the highest dilution factor where a difference is still visible between two samples in order to not over- or underestimate results observed. Increasing the washing steps to 10x with PBS-Tween followed by a 3x wash with Milli-Q® water was also evaluated.

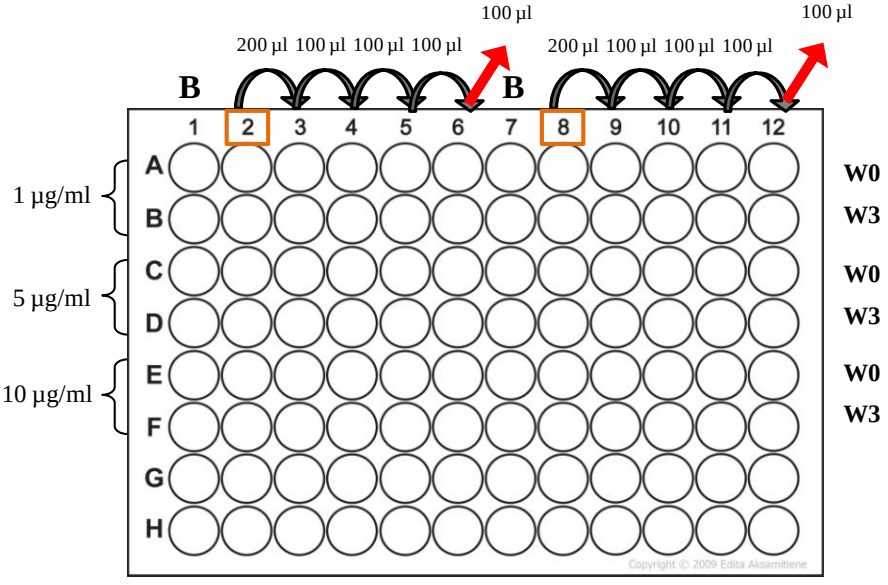

Figure 3.1 Plate layout used for optimising the ELISA coating concentration and serum dilutions. The 1 µg/ml, 5 µg/ml and 10 µg/ml refer to the coating concentrations. Indicated in the orange blocks are the rows which received 200 µl of the 1:20 diluted serum. The black arrows indicate the serial dilution with the transfer of 100 µl from the first column to the next. The red arrows indicate the discarding of the final 100 µl serum sample at the end of the serial dilution. W0 = Week 0 and W3 = Week 3.

Biotinylated antibody (rabbit anti-ostrich Ig) was diluted 1:100 in casein-Tween and 100 µl of this dilution added to each well followed by 1 h incubation at 37 ºC. The plate was washed 5 or 10 times with PBS-Tween as well as 3 times with Milli-Q®. The streptavidin horseradish peroxidase (HRP)

conjugate (Invitrogen) (2 ml streptavidin HRP, 3 ml 0.5% casein buffer, 40 ml glycerol) was diluted (1:100) in casein-Tween and 100 µl of this dilution added to each well followed by a 1 h incubation at 37ºC. A wash step was performed again as before followed by the addition of 100 µl substrate (0.5 mg/ml 2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 0.5 µl/ml H_2O_2 in 0.1 M citrate buffer, pH 5) to each well. The plate was subsequently incubated for 30 min at 37ºC and the absorbance of the wells measured at 405 nm by using a Multikan Spectrophotometer plate reader (Labsystems, Finland). The coating concentration was plotted graphically against the serial dilution to analyse the results.

3.2.8 ELISA analysis of ostrich serum samples

All serum samples collected from the ostriches during the vaccination trial from week 0-9 were analysed in triplicate following the procedure described in section 3.2.7.4 except that all wells were coated with 10 µg/ml OppA-GST protein and two serum dilutions used for analysis namely 1:100 and 1:500. Plates were washed 5 times with PBS-Tween and 3 times with Milli-Q® between all incubation steps. Absorbance values measured during ELISA analysis represent antibody levels and are referred to as titre values.

All of the plates contained the same plate control sample (ostrich number 217) from the pCI-neo_*oppA* 1200 µg/ml subgroup, which was randomly selected, in order to monitor plate to plate variation. In order to evaluate the specificity of the ELISA a no-coat column (column 12) was included in one of the ELISA plates using a sample that gave a high titre value using the described ELISA. The no-coat column was coated with 100 µl/well carbonate buffer (50 mM $NaHCO_3$, pH 9.6) but contained no protein. Except for the column containing no protein, all the steps following coating were the same as described before and serum samples applied to the no coat column were diluted 1:100.

3.2.1 Statistical analysis of trial data

Statistical analysis for the ELISA results obtained by diluting the serum 1:100 and 1:500 as well as weight data recorded during the trial was done by using the General Linear Model (GLM) procedure by means of the Agrobase Generation II® (Agronomix Software Inc.) software. The analysis of variance (ANOVA) and least significant difference (LSD) was calculated and a significance level of $P<0.05$ was chosen for analysis. The only ostriches that were not included in the ELISA analysis and subsequent statistical analysis were those who lost a tag or those with more than two data sampling points (week 0, 3, 6 and 9) missing due to serum samples not being collected. The data used for the statistical analysis are given in Addendum A.

3.3 Results

3.3.1 Preparation of DNA vaccines

Both the pCI-neo_*oppA* as well as the VR1020_*oppA* plasmids were isolated successfully with final concentrations of 89300.32 µg (11 preps) and 89300.68 µg (10 preps) respectively which was sufficient for vaccination at week 0 and week 6. On a 1% agarose gel (Figure 3.2) the majority of both the plasmids were identified as supercoiled as indicated by a band at 7000 bp for the undigested pCI-neo_*oppA* isolated plasmid (Figure 3.2 A) and a band at 8000 bp for the undigested VR1020_*oppA* isolated plasmid (Figure 3.2 B). A small percentage of the undigested pCI-neo_*oppA* and VR1020_*oppA* plasmids were nicked. The restriction enzyme digestion also confirmed the presence of the Ms03 *oppA* gene in both plasmids. The pCI-neo_*oppA* digestion with *AccI* resulted in a single band between 8000 bp and 10 000 bp on the agarose gel representing the pCI-neo plasmid (5472 bp) together with the *oppA* gene (3867 bp) in a linear form (Figure 3.2. A). Digestion of the VR1020_*oppA* with *BamHI* resulted in two bands, the first band representing the linear plasmid (5044 bp) and the second band, representing the Ms03 *oppA* gene (3867 bp) (Figure 3.2 C).

3.3.2 Vaccination trial

3.3.3 Weight recording during trial

The average weight of the different groups as recorded during the vaccination trial is shown in Table 3.4. The weight gain/loss of the different groups was all in the same range from one time point to the next as there was no statistically significant difference between the groups over the 9 week period (pCI-neo_*oppA* P = 0.9564; VR1020_*oppA* P = 0.5290) . The only exception was the VR1020_*oppA* 600 µg/ml subgroup which, based on the LSD (1.8101) value for the weight change, had a statistically significant lower average weight at week 9 compared to the control group (Addendum A). The control group and the vaccinated groups gained weight from week 0 to week 6 but from week 6 up until week 9 there was an average weight loss of 0.05 kg amongst all groups. Similar to the period of week 0 to week 6, weight gains was expected from week 6 to week 9 as the ostriches were in a seemingly good condition at week 6, had no mycoplasma infections and were on a standard feeding schedule formulated to favour weight gain. Problems were however experienced with the availability of the normal pelleted feed between the week 6 and 9 time points and birds were therefore given feed in a mashed form. Due to the change in feed the ostriches stopped eating and needed to be pastured after spending only 1 week in the feedlot. They were pastured for the remainder of the trial receiving only 1 kg of extra feed per bird per day.

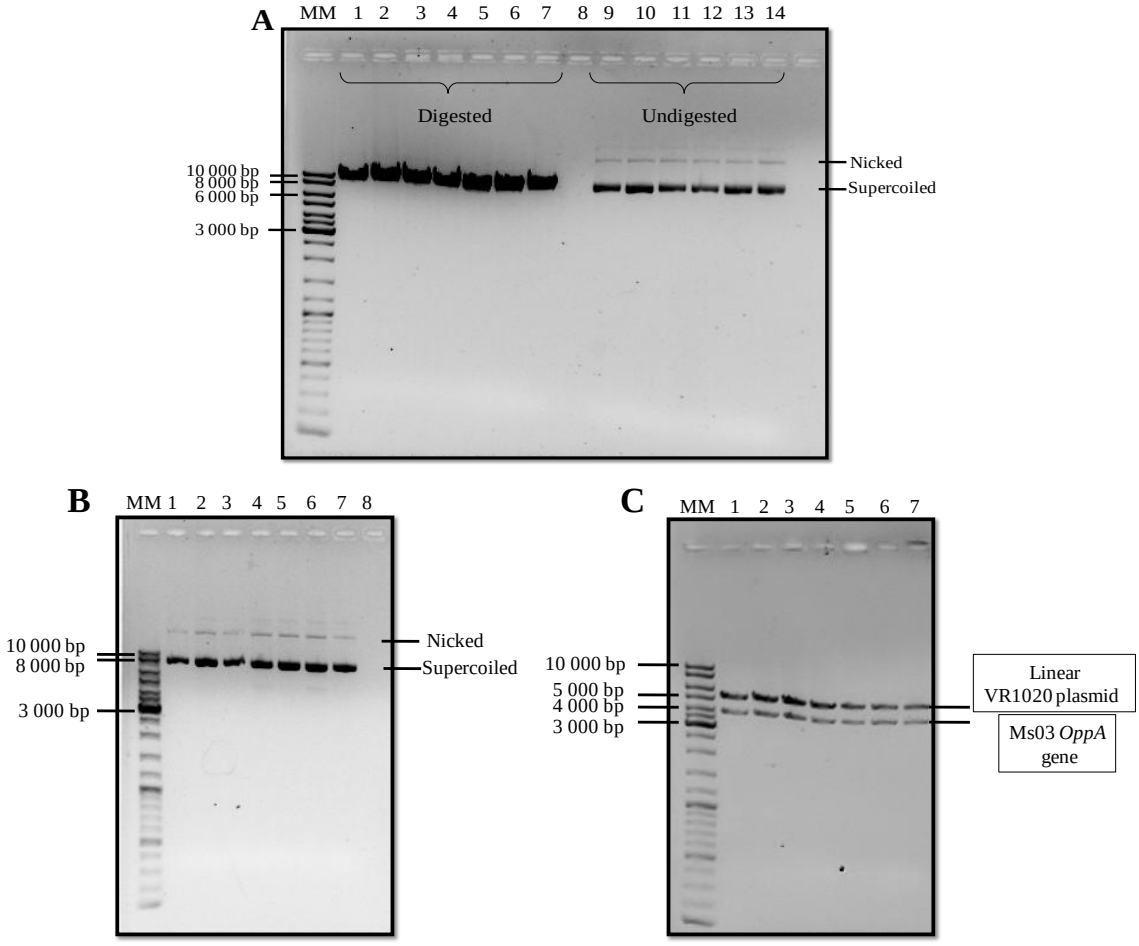

Figure 3.2 A 1% agarose gel showing the integrity of the DNA vaccine plasmids, pCI-neo_*oppA* **(A)** and VR1020_*oppA* **(B and C)** after plasmid isolation.
(A): MM: GeneRuler™ (Fermentas); Lane 1-7: Isolated pCI-neo_*oppA* plasmid digested with the *AccI* restriction enzyme; Lane 9-14: Isolated pCI-neo_*oppA* plasmids undigested.
(B): MM: GeneRuler™ (Fermentas); Lane 1-7: Isolated VR1020_*oppA* plasmids undigested digested.
(C): MM: GeneRuler™ (Fermentas); Lane 1-7: Isolated VR1020_*oppA* digested with the *BamHI* restriction enzyme.

Table 3.4 The average weight of each trial group as recorded during the vaccination trial.

	Average Weight (kg)			
	Week 0	**Week 3**	**Week 6**	**Week 9**
Control	27.2	No weight recorded	44.21	43.84
pCI-neo				
100 µg/ml	26.53		43.23	44.87
600 µg/ml	25.95		42.72	43.74
1200 µg/ml	26.75		45.8	45.39
VR1020				
100 µg/ml	27.53		47.88	47.24
600 µg/ml	26.23		42.56	41.08
1200 µg/ml	25.95		44.5	42.85

3.3.4 Trachea swab sample analysis by PCR

The results of the PCR analysis of the swab samples are shown in Table 3.5 A for the pCI-neo_*oppA* vaccine groups and Table 3.5 B for the VR1020_*oppA* vaccine groups. The control group shown in Table 3.5 A and B refer to the same group. No mycoplasma infections could be detected by PCR for any of the groups in week 0 and week 3 during which time the ostriches were in Fraserburg. After moving the ostriches to Oudtshoorn also no infections could be detected by PCR at week 6, but at this point the ostriches have only been in Oudtshoorn for one week and still in quarantine. At week 9, however, there was an increase in PCR detected infections. At week 9 the ostriches were already out of quarantine for 8 days and were therefore exposed to the rest of the farm environment and therefore an increase in infections could be expected.

For the pCI-neo_*oppA* vaccine groups the highest percentage of infections was due to Ms02 at 7.27% of the total infections followed by Ms03 with 3.63% of the total infection and Ms01 with total infections of 1.81% (Table 3.5 A). As for the VR1020_*oppA* vaccine groups the highest percentage of infections was again due to Ms02 infections with 10.52% of the total infections followed by Ms03 infections which were 5.26% and lastly Ms01 with the lowest infection rate of 3.50% (Table 3.5 B). Overall the pCI-neo_*oppA* vaccination groups had fewer infections due to the ostrich-infecting mycoplasma species than the VR1020_*oppA* vaccination groups, with only the VR1020_*oppA* 1200 µg/ml subgroup and the control group having no infections. Multiple infections were present in some birds. The pCI-neo_*oppA* 1200 µg/ml subgroup contained one ostrich and VR1020_*oppA* 600 µg/ml subgroup contained four ostriches infected by more than one of the ostrich-infecting mycoplasmas.

Not all infections detected at week 9 were as a result of mycoplasma infections as determined by BLAST analysis (Table 3.6 and Table 3.7). Some of the trachea samples tested positive for mycoplasma when using the genus specific primers but when testing for the ostrich-infecting mycoplasmas, results were negative. Therefore these samples were sequenced and BLAST analysis performed to confirm if the infections were indeed due to mycoplasmas (Table 3.6). Results revealed no mycoplasma infections but instead Gram-negative bacteria of the family Entrobacteriaceae were found, which included *Cronobacter zurichensis*, *Pantoea agglomerans* and *Citrobacter murliniae* (Table 3.7). Some of the members of this family are present in the gut of many animals without causing harmful effects (Linton and Hinton, 1988). The only exception was that of the Gram-positive plant associated bacterium that can also be found in soil namely *Curtobacterium plantarum* which is a member of the Microbacteriaceae family (Table 3.7). It is therefore not impossible that these bacteria could be found in some samples. Ostriches could have

encountered both of these by either pecking the soil or other birds faeces. It should be noted that these bacteria would by no means represent all the bacteria that the trial ostriches were exposed to but only the ones that were present and had cross reactivity with the *Mycoplasma* genus specific primers.

Table 3.5 Ms01, Ms02 and Ms03 infections as detected by PCR analysis during the course of the vaccination trial for the **(A)** pCI-neo_*oppA* and **(B)** VR1020_*oppA* vaccinated groups.

A

Experimental groups			Percentage infections at each sample point			
		µg/ml*	Week			
			0	3	6	9
Ms01	pCI-neo_*oppA*	100	-	-	-	1/19 # (5.26%)
		600	-	-	-	-
		1200	-	-	-	-
	Control		-	-	-	-
Ms02	pCI-neo_*oppA*	100	-	-	-	2/19 (10.53%)
		600	-	-	-	1/17 (5.88%)
		1200	-	-	-	1/19 (5.26%)
	Control		-	-	-	-
Ms03	pCI-neo_*oppA*	100	-	-	-	1/19 (5.26%)
		600	-	-	-	-
		1200	-	-	-	1/19 (5.26%)
	Control		-	-	-	-

B

Experimental groups			Percentage infections at each sample point			
		µg/ml*	Week			
			0	3	6	9
Ms01	VR1020_*oppA*	100	-	-	-	-
		600	-	-	-	2/18 # (11.11%)
		1200	-	-	-	-
	Control		-	-	-	-
Ms02	VR1020_*oppA*	100	-	-	-	2/19 (10.53%)
		600	-	-	-	4/18 (22.22%)
		1200	-	-	-	-
	Control		-	-	-	-
Ms03	VR1020_*oppA*	100	-	-	-	1/19 (5.26%)
		600	-	-	-	2/18 (11.11%)
		1200	-	-	-	-
	Control		-	-	-	-

* Vaccine concentration administered

\# Total number of birds in group at end of trial

Table 3.6 Bacteria other than the three ostrich-infecting mycoplasmas detected when using mycoplasma genus primers.

Experimental groups		Total infections at each sample point not due to ostrich infecting mycoplasmas			
Vaccine	Dose	Week 0	Week 3	Week 6	Week 9
pCI-neo_*oppA*	100 µg/ml	-	-	-	2/19 (10.53%)
	600 µg/ml	-	-	-	1/17 (5.88%)
	1200 µg/ml	-	-	-	-
VR1020_*oppA*	100 µg/ml	-	-	-	-
	600 µg/ml	-	-	-	1/18 (5.56%)
	1200 µg/ml	-	-	-	3/20 (15.00%)
Control	-	1/20 (5.00%)	-	-	1/17 (5.88%)

Table 3.7 Identity of bacteria other than the ostrich-infecting mycoplasmas as detected by sequencing and BLAST analysis.

Organism	Max Score (bit-score)	Query cover	E-value	Identity	Accession number
Citrobacter murliniae	961	98%	0	97%	NR028688.1
Cronobacter zurichensis	1230	93%	0	97%	NR104924.1
Curtobacterium plantarum	1230	97%	0	97%	NR104943.1
Pantoea agglomerans	1206	91%	0	99%	NR114111.1
Pantoea agglomerans	612	99%	$4e^{-}175$	95%	NR114735.1

3.3.5 Preparation for analysis of serum samples

3.3.5.1 Expression and Isolation of OppA proteins

Producing the expression plasmid containing the oppA gene

A number of colonies resulting from the transformation of pGEX-4T-1_*oppA* into *E. coli* BL21(DE3)pLysS cells were selected for analysis by colony PCR and all contained the pGEX-4T-1_*oppA* plasmid as shown in Figure 3.3 (lane 3-11) with a band at about 600 bp. The pGEX_F primer together with the T7_R primer results in a PCR product of 592 bp as the T7_R primer binds at position 592-615 inside the *oppA* gene whereas the pGEX_F binds to the pGEX-4T-1 vector.

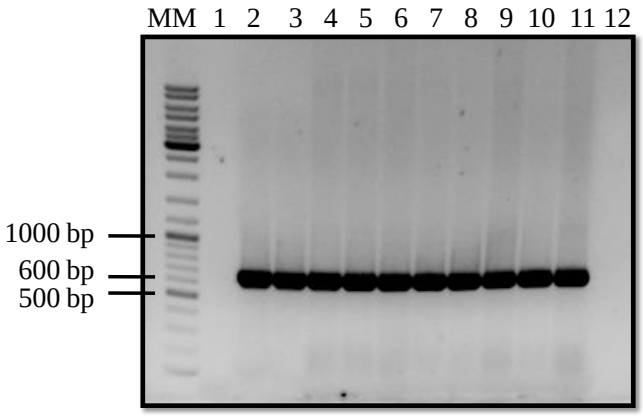

Figure 3.3 Colony PCR results of colonies containing pGEX-4T-1_*oppA* on a 1% agarose gel. MM: GeneRuler™ (Fermentas), Lane 1: Negative control, PCR containing no DNA only Milli-Q®, Lane 2: Positive control containing isolated pGEX-4T-1_*oppA* plasmid, Lane 3-11: PCR products of colonies tested.

Expression of the OppA protein and SDS-PAGE analysis

Ms03 OppA proteins were successfully expressed as confirmed by SDS-PAGE analysis (Figure 3.4). The expressed fusion protein consisting of an OppA protein (145 kDa) and a GST tag (26 kDa) resulted in a band of 171 kDa as indicated by the red block in Figure 3.4. No expression was observed in the control (lane 1 and 4) at 0 h and 6 h due to the fact that the control contained a plasmid without the *oppA* gene. The samples containing the pGEX-4T-1_*OppA* plasmid also showed no expression at 0 h (lane 2-3) but the Ms03 OppA-GST protein was expressed at 6 h (lane 5-6).

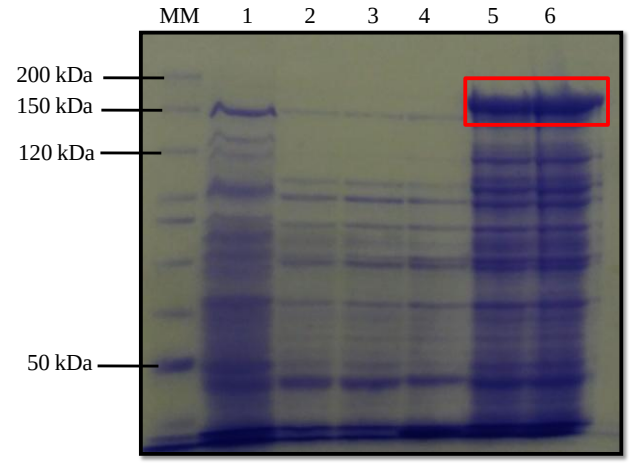

Figure 3.4 8% SDS-PAGE analysis of the OppA-GST protein expressed in *E. coli* BL21(DE3)pLysS cells. MM: PageRuler™ Unstained protein ladder (Thermo Scientific). Lane 1: Expression of control at 0 h. Lane 2-3: Expression of Ms03 OppA-GST at 0 h. Lane 4: Expression of control at 6 h. Lane 5-6: Expression of Ms03 OppA-GST at 6 h.

3.3.5.2 Isolation of the expressed OppA protein

The Ms03 OppA-GST protein was successfully isolated by means of a Glutathione-Agarose column as confirmed by the Bradford and SDS-PAGE analysis. Figure 3.5 shows the elution profile as

detected by Bradford analysis of the 20 samples collected during the isolation process. The purified Ms03 OppA-GST protein starts eluting at sample 5 and the highest concentration of purified eluted Ms03 OppA fusion protein is reached at sample 9, 10 and 11 with concentrations of 0.673 mg/ml, 0.650 mg/ml and 0.708 mg/ml respectively. The highest concentration that was reached based on Bradford analysis could be increased from 0.554 mg/ml to 0.947 mg/ml protein by increasing the incubation time of the sample on the column from 1h to 2 h and 30 min.

After initial isolation of the Ms03 OppA-GST protein, SDS-PAGE analysis showed that the GST tag was cleaved from the Ms03 OppA protein during isolation. To prevent this from happening and ensuring efficient isolation of the protein with the Glutathione-agarose column, a protease inhibitor was added to the lysis buffer and all the buffers used during the isolation process to ensure that the protein remained in its fusion state. No protease inhibitor was, however, added to the equilibrium buffer as almost all of the equilibrium buffer was eluted before the addition of the sample containing the OppA-GST protein.

SDS-PAGE analysis confirmed that the protein that was isolated with the Glutathione-Agarose column was indeed the Ms03 OppA-GST protein as indicated by the protein bands at 170 kDa in lanes 6-9 (Figure 3.6, red block). In Figure 3.6, Lane 2 shows that the supernatant which was loaded on the column contained the Ms03 OppA-GST protein as well as other proteins. Lane 3 indicated that most of the Ms03 OppA-GST proteins were able to bind to the column and in lane 4 and 5 it is shown that most of the proteins not containing a GST tag were eluted by the wash steps. There were still some unwanted proteins after isolation (lanes 6-9), although the largest amount of protein isolated was the Ms03 OppA-GST protein as this is the most prominent band in these lanes.

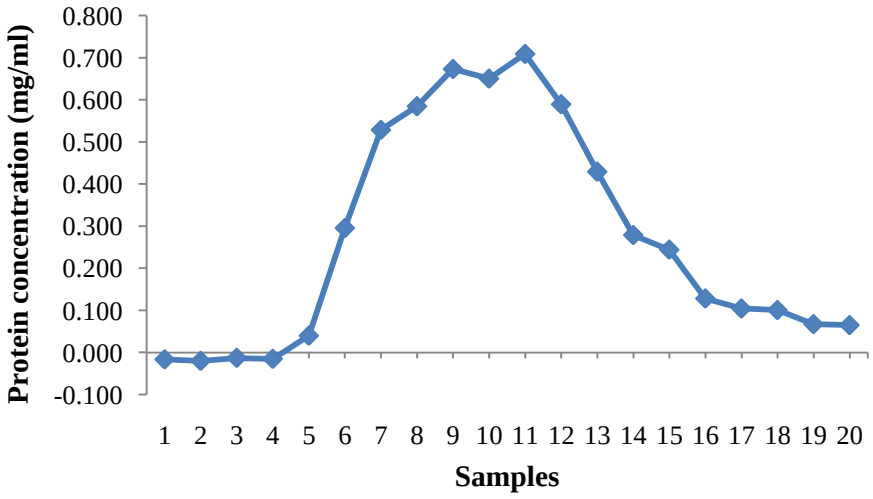

Figure 3.5 Elution profile illustrating the concentration of protein (mg/ml) per sample eluted during isolation of the Ms03 OppA-GST protein, as determined by Bradford analysis. The elution profile indicates that sample 9, 10 and 11 contains the highest concentration of purified protein.

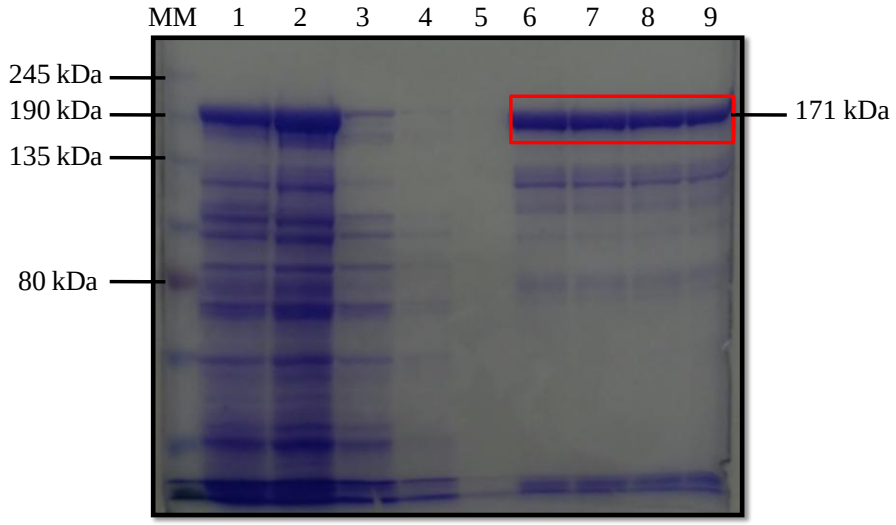

Figure 3.6 8% SDS-PAGE gel analysis of the isolated OppA-GST proteins isolated using a glutathione-agarose column. MM: Color Prestained Protein Standard, Broad Range (BioLabs® Inc., New England). Lane 1: Cells harvested after 6 h of expression. Lane 2: Supernatant before loading on column. Lane 3: Flow through after incubation period on column. Lane 4: After wash 1. Lane 5: After wash 2. Lane 6-9: Eluted OppA-GST proteins as indicated in red block (171 kDa).

3.3.5.3 Optimisation of ELISA for analysis of serum samples collected during the vaccination trial

Figure 3.7 A and B shows the absorbance values obtained for the VR1020_*oppA* and pCI-neo_*oppA* serum samples respectively when serial dilutions ranging from 1:20 to 1:320 of different coating concentrations were used. For both A and B of Figure 3.7 the 1 and 10 µg/ml coating concentrations showed a difference in absorbance values between the week 0 and 3 serum samples. A serum dilution between 1:80 and 1:160 in combination with both the 1 and 10 µg/ml coating concentration resulted in a low absorbance value for the week 0 serum, but still allowed a high signal to background ratio to be achieved for the corresponding week 3 sera. Although both the 1 and 10 µg/ml coating concentrations allowed differences in absorbance values, the 10 µg/ml coating concentration was chosen for further analysis as this was also the coating concentration used in the previous study by Wium (2015). For simplicity, a dilution factor of 1:100 (indicated at the black vertical line) was chosen for further ELISA analysis.

Serum samples that are diluted with a too low dilution factor can cause overestimation of binding as the antibodies will bind non-specifically or with low binding efficiency, whereas a too high dilution factor can cause an underestimation of the binding as there is not enough antibody (due to the dilution) to result in an effective signal (Tate and Ward, 2004). The serial dilution was therefore repeated using a range from 1:40 to 1:640 and results shown in Figure 3.8 A. For simplicity the 1:500 dilution (indicated at the black vertical line) was chosen for further ELISA analysis as a difference between the week 0 and 9 serum could still be observed at this dilution factor. All the

serum samples collected during the trial from week 0 to week 9 were subsequently analysed using a 1:100 and 1:500 dilution factor.

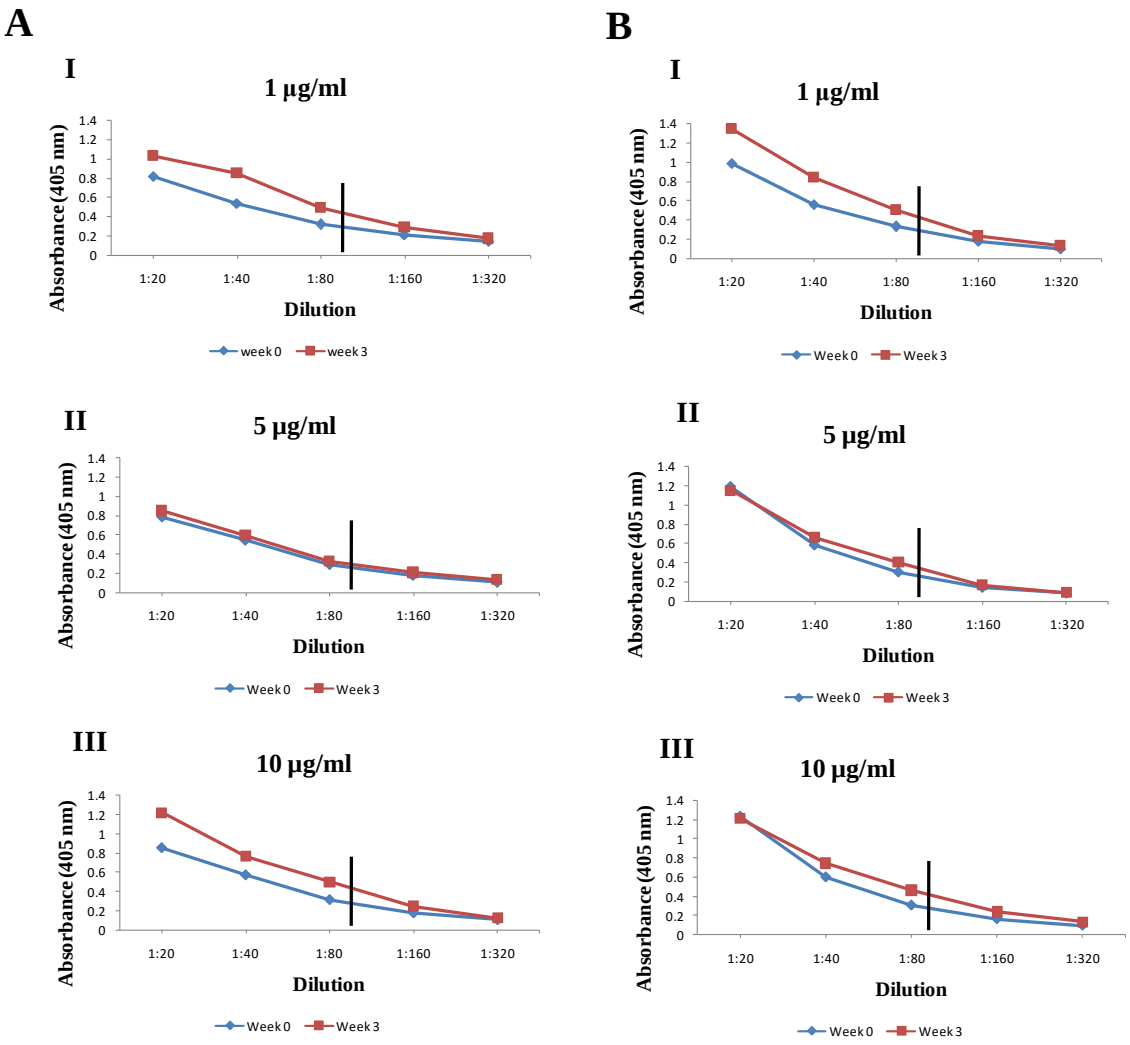

Figure 3.7 Plots of absorbance values of ELISA determinations of different serum dilutions and different coating concentrations. **(A)** Optimisation of the ELISA technique with different coating concentrations of the OppA-GST protein. Week 0 and week 3 serum of the bird 266 in the VR1020_*oppA* 1200 µg/ml subgroup was used in order to perform the optimisation of the ELISA technique (I): Coating concentration of 1 µg/ml. (II): Coating concentration of 5 µg/ml. (III): Coating concentration of 10 µg/ml. The 1:100 dilution is indicated by the vertical black line. **(B)** Optimisation of the ELISA technique with different coating concentrations of the OppA-GST protein. Week 0 and week 3 serum of the bird 207 in the pCI-neo_*oppA* 1200 µg/ml subgroup was used in order to perform the optimisation of the ELISA technique (I): Coating concentration of 1 µg/ml. (II): Coating concentration of 5 µg/ml. (III): Coating concentration of 10 µg/ml. The 1:100 dilution is indicated by the vertical black line.

Compared to the results obtained by Wium (2015) there was no appreciable difference observed in background due to the decrease from 2 h to 1 h at 37ºC, for the block step.

Residual Tween can interfere with the enzymatic activity of peroxidase conjugates (Gibbs, 2001) and therefore plates were washed 5x with PBS-Tween followed by a 3x Milli-Q® wash in order to determine if the background could be reduced as compared to washing the plate 8x with only PBS-Tween as done by Wium (2015). There was however no difference observed in background values

of the week 0 and week 3 serum used in combination with a serial dilution of 1:20 to 1:320 with this modified washing technique.

Using a week 0 and week 9 serum in combination with a 1:40 to 1:640 serial dilution the 5x PBS-Tween wash followed by the 3x Milli-Q® water wash was compared to a 10x PBS-Tween wash followed by the 3x Milli-Q® water wash (Figure 3.8 A and B)

Increasing the wash steps to 10x PBS-Tween resulted a in higher ELISA absorbance titre value for the week 9 serum at almost all of the dilution factors compared to the 5x PBS-Tween wash. For both wash steps the titre values were in the same range for the week 0 serum. The increase in PBS-Tween wash steps, however, increased the blank column values and thus background form 0.102-0.116 to 0.113-0.131. The 5x PBS-Tween wash step followed by the 3x Milli-Q® wash was subsequently used for further ELISA analysis.

3.3.5.4 Biotinylation of rabbit anti-ostrich antibodies

The rabbit anti-ostrich antibody was successfully biotinylated since it allowed for interaction of biotin with the streptavidin HRP with a resulting colour reaction seen after addition of the substrate to each of the wells (Figure 3.9). This procedure has also been used in numerous ELISA approaches in this laboratory and performed very similarly.

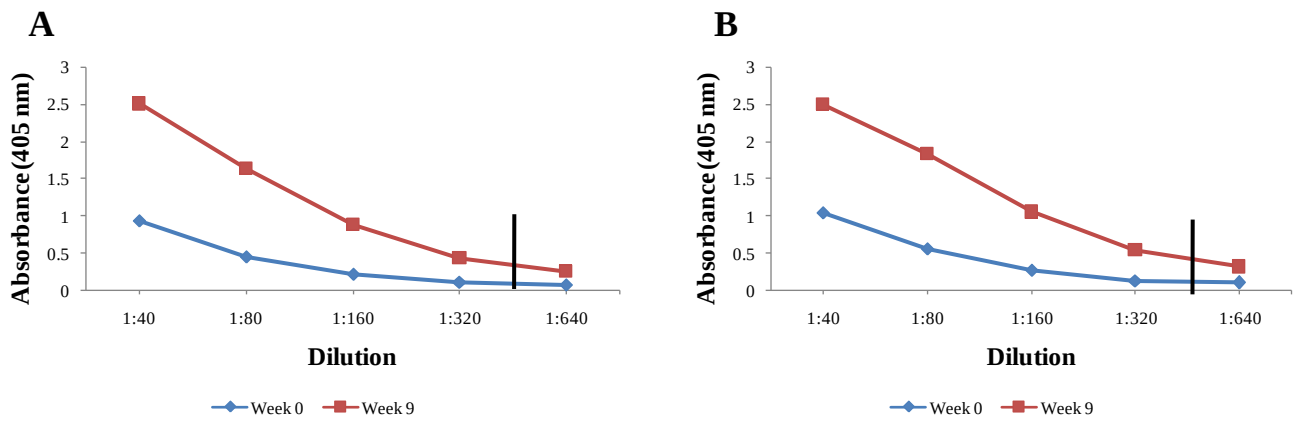

Figure 3.8 Graphical representation of the serial dilution from 1:40 to 1:640 of the week 0 and 9 serum of ostrich 261 in the VR1020_*oppA* 1200 µg/ml subgroup by means of the ELISA technique with a single coating concentration of 10 µg/ml. **(A)** plate washed 5x with PBS-Tween followed by 3x water wash. **(B)** Plate washed 10x with PBS-Tween followed by 3x water wash. The vertical black line indicates the 1:500 dilution that was chosen for ELISA analysis.

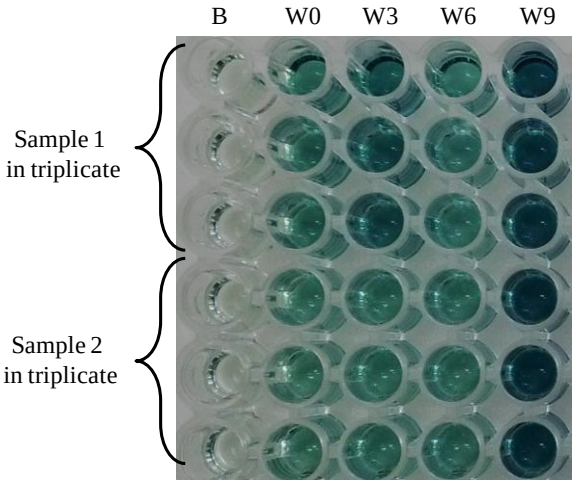

Figure 3.9 96 well Maxisorb plate showing the colour reaction of two serum samples from the VR1020 1200 µg/ml subgroup (sample 1: ostrich 265 and sample 2: 266) with the use of the biotinylated antibody in the ELISA technique. B: Blank row receiving no serum, W0: Serum sample taken at week 0, W3: Serum sample taken at week 3, W6: Serum sample taken at week 6, W9: Serum sample taken at week 9. The plate was coated with 10 µg/ml Ms03 OppA protein and all serum samples were diluted 1:100 in casein-Tween.

3.3.6 ELISA analysis of ostrich serum samples

The range of the titre values for the plate control samples for both serum dilutions are shown in Table 3.8. The plate control sample (pCI-neo_*oppA* 1200 µg/ml subgroup, ostrich 217) was chosen randomly and was one of the samples with a high titre value at week 0 compared to other samples in the same subgroup. The titre values obtained for the plate control sample were consistently within the same range. Although aliquots were made of the plate control sample they were still exposed to freeze/thaw cycles which might have had an influence on the variation of the range. As a result of plate control variation form plate to plate, some variation would be expected between all plates analysed. The no-coat column (1:100 serum dilution) also indicated that there was no non-specific binding as the titre values were in the same range as the blank (0.130-0.238).

Table 3.8 Range of titre values for both serum dilutions for week 0, 3, 6 and 9 of the plate control sample.

Serum dilution	Range			
	Week 0	Week 3	Week 6	Week 9
1:100	0.754-1.251	0.603-0.983	0.512-1.194	1.140-1.830
1:500	0.131-0.245	0.071-0.158	0.159-0.233	0.209-0.333

Four ostriches were excluded from the trial and subsequent ELISA analysis. Three of these were from the control group where one was accidently injected with the pCI-neo_*oppA* (1200 µg/ml) vaccine, and the other two together with the fourth ostrich from the VR1020_*oppA* 600 µg/ml subgroup that lost their tags during the trial. Figure 3.10 and Figure 3.11 represents the average anti-OppA antibody response as produced in response to the pCI-neo_*oppA* and the VR1020_*oppA* vaccines respectively as determined by the ELISA when using a dilution factor of 1:100 for the

serum whereas Figure 3.12 and Figure 3.13 represents the average anti-OppA antibody response when using a dilution factor of 1:500 for the serum.

As shown by the results in Figure 3.10, Figure 3.11, Figure 3.12 and Figure 3.13 the control group, which received no vaccine, had an overall average titre in the same range from week 0-6. At week 9 however, there is a slight increase in the average titre. The serum samples from the control group for the 1:100 dilution and 1:500 dilution were not all analysed on the same plate and also not with the same batch of isolated OppA proteins. Instead the serum samples from week 0-9 of each ostrich were analysed together on the same plate and therefore at the same time point and using the same batch of coating antigen. The increase in absorbance values from week 6 to week 9 for the control group can therefore not be ascribed to handling error alone.

ANOVA analysis of the pCI-neo_*oppA* vaccination results (1:100 serum dilution, Figure 3.10) indicated a significant treatment x time interaction for the data as a whole with $P = 0.0462$. By using the calculated LSD value (0.2235) a pair wise comparison was done between the immune responses of each of the vaccinated subgroups and the control group to determine if there was a statistical significant difference between doses. This revealed that there was no statistical significant difference between the control group and any of the vaccinated subgroups at week 0, week 3 or week 6. At week 9, however, the antibody response produced by the 100 and 600 µg/ml doses differed significantly from the control. There was no statistical significant difference between the control and the 1200 µg/ml dose at any of the time points and at week 9 there was no statistical difference between the different doses when using a 1:100 serum dilution.

ANOVA analysis of the VR1020_*oppA* vaccination results (1:100 serum dilution, Figure 3.11) indicated a significant treatment x time interaction for the data as a whole with a $P < 0.05$. The LSD value (0.2796) calculated was also used for pair wise comparison between the immune responses of each of the vaccinated subgroups and the control group to determine if there was any statistical significant differences between doses. No statistical significant difference was observed between the control group and any of the vaccinated subgroups at week 0, week 3 or week 6. Compared to the pCI-neo_*oppA* vaccine the antibody response produced by all of the subgroups differed significantly from the control group, however, the significant difference again could only be observed at week 9. A significant difference was also observed between the 100 and 600 µg/ml, 600 and 1200 µg/ml as well as the 100 and 1200 µg/ml doses at week 9.

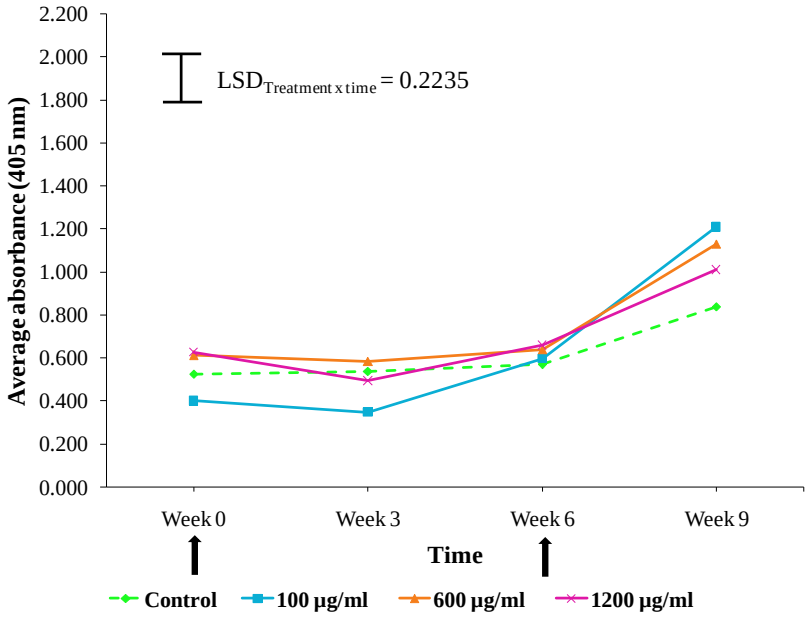

Figure 3.10 Average titre values as determined by means of the ELISA technique illustrating the anti-oppA antibody response against the pCI-neo_*oppA* vaccine injected into ostriches during the field trial. The results were obtained by with a 1:100 dilution of the serum samples. The black arrows indicate the time points at which the ostriches were vaccinated.

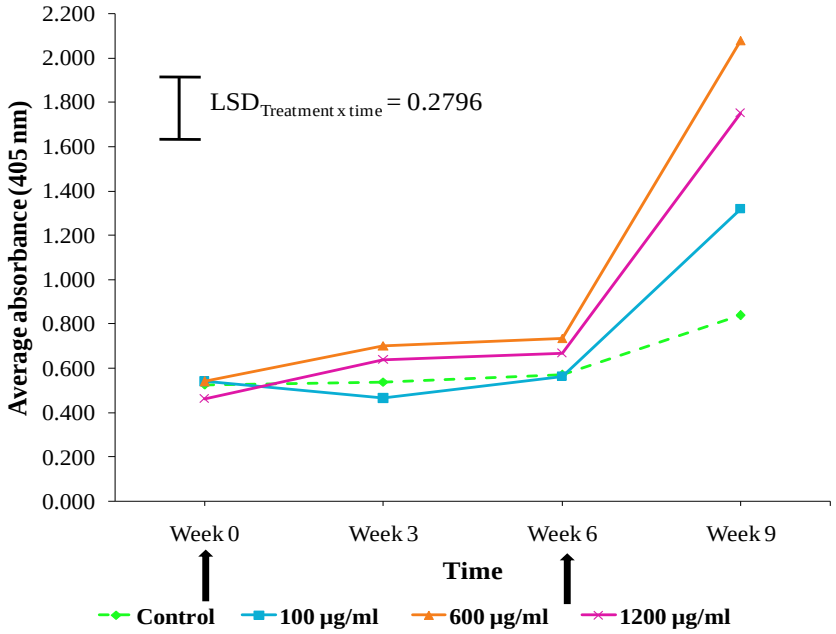

Figure 3.11 Average titre values determined by means of the ELISA technique illustrating the anti-OppA antibody response against the VR1020_*oppA* vaccine injected into ostriches during the field trial. The results were obtained with a 1:100 dilution of the serum samples. The black arrows indicate the time points at which the ostriches were vaccinated.

The statistical significance of the ELISA results for the pCI-neo_*oppA* vaccination group (1:500 serum dilution, Figure 3.12) was determined as before and indicated there was no significant treatment x time interaction for the data as a whole with P = 0.2774. These results differ from that obtained with the 1:100 serum dilution where a significant treatment x time interaction was obtained. The LSD value (0.0944) indicated no statistical significant difference between the control

group and any of the vaccinated subgroups at week 0, week 3 or week 6. When using a 1:500 dilution only the pCI-neo_*oppA* 100 and 1200 µg/ml doses differed significantly from the control, however, the significant difference could only be observed at week 9. These results once again differed from the 1:100 dilution results where the 100 µg/ml and 600 µg/ml doses produced a significant difference when compared to the control at week 9.

Similar to the 1:100 results an ANOVA analysis of the 1:500 serum dilution VR1020_*oppA* vaccination (Figure 3.13) results indicated a significant treatment x time interaction for the data as a whole with $P < 0.05$. As before, the LSD value (0.2120) indicated no statistical significant difference between the control group and vaccinated subgroups at week 0, week 3 and week 6. Once again, similar to the 1:100 dilution factor the antibody response produced by the VR1020_*oppA* 100, 600 and 1200 µg/ml doses differed significantly from the control and only at week 9. Different from the 1:100 dilution factor there was no significant difference between the 600 and 1200 µg/ml doses but as for the 1:100, there was a significant difference between both the 100 and 600 µg/ml as well as the 100 and 1200 µg/ml doses.

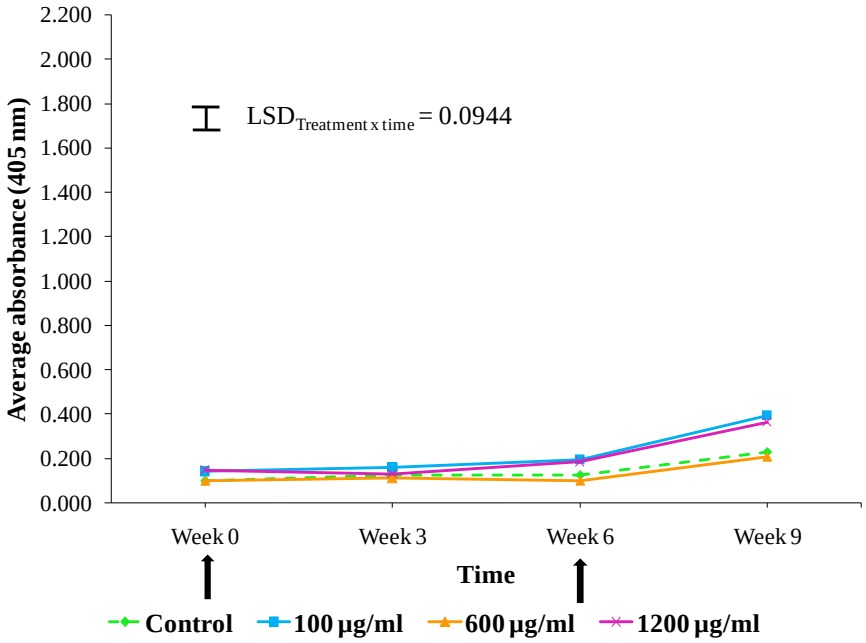

Figure 3.12 Average titre values determined by means of the ELISA technique illustrating the anti-oppA antibody response against the pCI-neo_*oppA* vaccine injected into ostriches during the field trial. The results were obtained with a 1:500 dilution of the serum samples. The black arrows indicate the time points at which the ostriches were vaccinated.

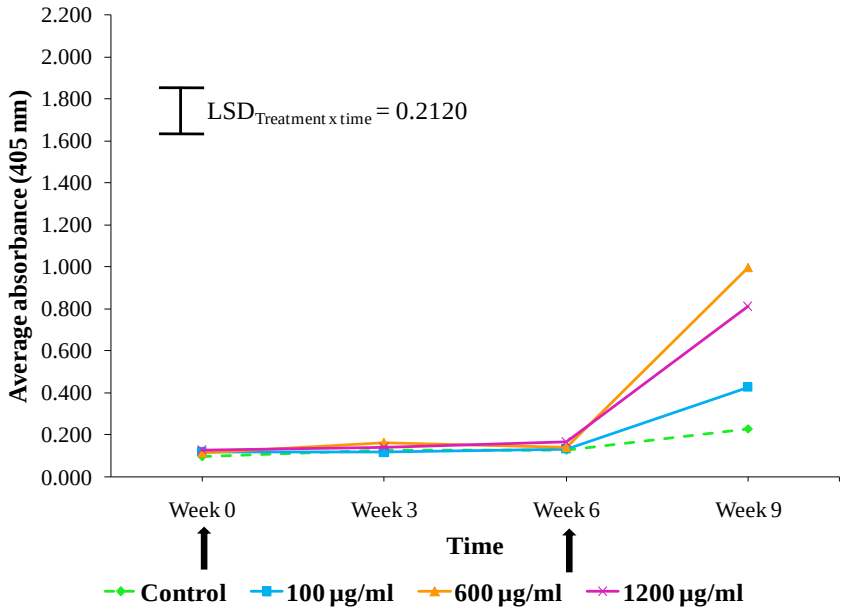

Figure 3.13 Average titre values determined by means of the ELISA technique illustrating the anti-oppA antibody response against the VR1020_*oppA* vaccine injected into ostriches during the field trial. The results were obtained with a 1:500 dilution of the serum samples. The black arrows indicate the time points at which the ostriches were vaccinated.

3.4 Discussion

Ostriches hatch with an immature immune system making them more susceptible to especially respiratory infections such as those caused by mycoplasmas (Scholtz, 2014). The mortality of chicks up to the age of three months can be as high as 50% (Cloete et al., 2001). Due to the large number of ostrich farms in the Oudtshoorn district, infections are common on these farms and as a result day old ostrich chicks are moved to distant areas such as Fraserburg until they reach an age of about 3-4 months. As there are fewer ostrich farms in Fraserburg compared to Oudtshoorn, spread of infections from farm to farm is limited. The vaccination trial described here was therefore started in Fraserburg with 3 month old ostriches at the end of autumn. Ostriches were kept at Fraserburg during the week 0 and week 3 sampling points and only moved to Oudtshoorn one week prior to the week 6 sampling point. During the first sampling point (week 0) ostriches that formed part of the trial needed to be tagged for identification purposes. In a previous study by De Wet (2015) the ostriches were tagged in the neck which attracted the attention of other ostriches. They subsequently pecked at each other's tags resulting in the loss of tags and because these birds were untraceable the number of trial birds was decreased over time. In this study, the ostriches were therefore tagged underneath their wings which resulted in fewer tags being lost.

In the vaccination trial, the pCI-neo_*oppA* and VR1020_*oppA* vaccines were administered to the ostriches in three different doses at week 0 and a booster at week 6. All of the vaccine plasmids were administered in supercoiled form as it has been shown that this conformation results in higher transfection rates into mammalian cells and is less susceptible to intracellular degradation than other plasmid conformations (Maucksch et al., 2009; Ghanem, Healey, & Adly, 2013).

The anti-OppA antibody responses against both the pCI-neo_*oppA* and VR1020_*oppA* vaccines were evaluated by means of an ELISA. The dilution factor used for sera can cause either over- or underestimation of results due to a too low or too high dilution. Two different dilution factors were therefore used namely 1:100 and 1:500 and the results statistically analysed. Overall the outcome of the ELISA results were not different when using the 1:100 or 1:500 dilution factor except for the pCI-neo_*oppA* 600 (1:100 dilution) and 1200 µg/ml (1:500 dilution) doses which showed a significant difference from the control group at the different serum dilutions. Although both serum dilutions resulted in a high coefficient of variance, this is expected as none of the animals will have the same reaction to the vaccine and this reaction can also not be controlled. The coefficient of variance, however, increased dramatically from analyses with the 1:100 dilution (pCI-neo: 51.74%; VR1020: 53.82%) to analyses with the 1:500 dilution (pCI-neo: 86.11%; VR1020: 133.10%) and

therefore the statistical significance is decreased. Hence, the 1:100 dilution factor will preferably be used in future studies and only these results will be discussed.

The 1:100 dilution indicated that the 100 and 600 µg/ml doses of the pCI-neo_*oppA* vaccine was able to produce an anti-OppA immune response (P = 0.0462) but only after administering a booster vaccination. All of the VR1020_*oppA* doses that were administered to the ostriches resulted in significantly higher average titre values than that of the control but similar to the pCI-neo vaccine only after a booster vaccine was administered. This indicates the necessity of a booster vaccination. The 600 µg/ml dose produced a higher average titre than the 1200 µg/ml dose and the difference between these two doses was statistically significant. This implies that 600 µg/ml is the preferred dose for the specific age and weight of the ostriches that were vaccinated. Sample collection, however, was only done up until week 9 and a different result may have been obtained with more sample points beyond week 9. Although the 100 µg/ml dose resulted in the lowest average titre values it was also able to elicit a statistically significant anti-OppA immune response at week 9. Therefore, given more time, the 100 µg/ml dose might be sufficient for eliciting an anti-OppA antibody response.

After IM injection of a DNA vaccine the plasmids are taken up by the muscle cells near the injection site. Although the mechanism of plasmid uptake into the muscle cells is not yet clear it is speculated that this process is facilitated by T tubules which are only found in skeletal and cardiac muscle cells (Dupuis et al., 2000; Kutzler and Weiner, 2008; Saade and Petrovsky, 2012). Following the uptake of the DNA vaccine by the muscle cells the plasmid enters the cell nucleus by means of the host cellular machinery and is expressed (Kutzler and Weiner, 2008). Expressed antigen will trigger the activation of a primary immune response which results in antibody production against the antigen, and is mediated by both B- and T-cells (Janeway et al., 2001). When comparing the antibody response elicited by the pCI-neo_*oppA* vaccine to that of the VR1020_*oppA* vaccine, the VR1020_*oppA* vaccine had higher average titre values indicating a greater antibody response. This might be due to the fact that the VR1020 plasmid contains a tPa signal sequence upstream from the *oppA* gene. This sequence assists with expression, export of the protein and immunogenicity as shown by Malin et al. (2000). As the pCI-neo plasmid does not contain a tPa signal like the VR1020 plasmid, it is possible that synthesised proteins were not transported out of the cell and therefore not presented well enough for an immune response to be elicited during that time. Another possibility is that it the transport of the protein out of the cell takes longer and therefore needs more time to be sufficiently exposed to the immune system. Therefore the choice of plasmid in DNA vaccines can have an influence on the immune response as

seen with the VR1020_*oppA* vaccine when compared to the pCI-neo_*oppA* vaccine's ability to elicit an anti-OppA immune response.

There were no significant differences between antibody levels of the vaccinated subgroups and the control group from week 0 to week 6. Hence, no primary antibody response was elicited by any of the vaccine doses. A general trend was, observed for the VR1020_*oppA* 600 and 1200 µg/ml doses where average titre values increased from week 0 to week 3 followed by a decrease from week 3 to week 6. If a first vaccination is able to elicit a primary immune response with the formation of memory B cells the production of a secondary immune response (after administration of a booster) can generally be observed. This is due to the fact that the host will be able to recognize the same antigen faster and with greater magnitude than the primary immune response (Reynolds et al., 2015). In a study done by Blignaut et al. (2000) an immune response to an inactivated vaccine in ostriches was only seen by day 14 with a maximum response at about 21 days. As in the case of both the DNA vaccines no increase in immune response was seen at week 3 for any of the vaccinated groups. It might be that not enough time was allowed for the production of the OppA protein before the administration of a booster. The inactivated vaccine as in the case of the study by Blignaut et al. (2000) can immediately start to activate the immune system as the antigen is already present. In the case of a DNA vaccine it possibly takes the antigen longer to reach the immune system as it still has to be expressed or it might have been expressed at levels that are too low to elicit an anti-OppA response during that time. As there is possibly a delay in the time that it takes for the antigen to be presented to the immune system it might explain why only a secondary immune response is observed and together with the booster results in an anti-OppA immune response for both the vaccines.

As ostriches are susceptible to mycoplasma infections during seasonal changes, the health of the ostriches was monitored during the course of the trial (late autumn, early winter) which included weight measurement at week 0, 6 and 9 as well as swab sample collection and analysis of those by means of PCR at week 0, 3, 6 and 9. Comparing the average weight change of the vaccinated groups and control group over the course of the trial revealed that the birds gained weight up until week 6 but their weight gain decreased from week 6 to week 9 with some birds even losing weight. Given that this trend was also observed for the control group ostriches, the weight loss cannot be ascribed to either of the vaccines. After the ostriches were moved into the feedlot system problems were experienced in the supply of pelleted feed and they were given feed in a mashed form. Ostriches in feedlot systems tend not to consume feeds that they are not used to (Brand and Gous, 2006) and the sudden introduction of the other feed resulted in them not eating with a subsequent lack of weight gain. It is also unlikely that the weight loss was due to the transport of the ostriches

since the weight loss only started to occur after two weeks in Oudtshoorn and coincided with feed problems.

The PCR results revealed that none of the trial birds had infections from week 0 to 6 which might be ascribed to the fact that the ostriches were in Fraserburg from week 0 to week 3 where less infections were expected and they were still in quarantine at the week 6 sampling point in Oudtshoorn. The ostriches also only arrived in Oudtshoorn one week prior to the week 6 sampling point and therefore it is possible that the birds were not yet exposed to mycoplasmas. After the 29 day quarantine the ostriches were moved out into a feedlot system for one week and pastured for the remainder of the trial. Exposure of ostriches to the rest of the farming environment resulted in low levels of mycoplasma infections which were detected by PCR analysis at week 9 with Ms03 and Ms01 infections being lower in comparison with Ms02. Mycoplasma infections are common on Oudtshoorn ostrich farms and especially prominent during seasonal changes. The trial was conducted during late autumn, early winter and therefore an increased exposure to mycoplasma infections was expected. Having feed problems in addition to this and birds not eating, further increased their susceptibility to mycoplasma infections. As their immune system was already under pressure as a result of the vaccination, the challenge conditions created by the feedlot system, weather and the feed problem possibly all had an effect on the number of infections seen during week 9.

During the course of the trial some of the samples that tested positive were not actually due to mycoplasmas. This indicated that the general primers have some cross reactivity and that the genus specific primers need to be re-designed.

In order to determine the ability of the vaccines to protect the ostriches against Ms03 infections they should be challenged with the pathogen. Enough time should, however, be allowed for the vaccines to elicit a primary and secondary anti-OppA antibody response before they are challenged with the pathogen. Collop (2011) showed that anti-Ms03 OppA antibodies have a low level of cross reactivity with the OppA protein of Ms01 but none with the OppA protein of Ms02. Therefore the vaccines used for this study might be able to provide limited protection against Ms01 infections but no protection against Ms02 infections will be provided.

4. Production of IgA_H proteins by means of the Baculovirus-insect expression system

4.1 Introduction

Mycoplasma infections in ostriches could be controlled by using a vaccine but currently there is no commercially available vaccine against ostrich infecting mycoplasmas. Two DNA vaccines against ostrich-infecting mycoplasmas were developed by Wium (2015) and in the previous chapter we were able to induce an immune response using these vaccines. The DNA vaccines were however administered intramuscularly and as Botes et al. (2005) found that mycoplasmas target the mucosal surfaces in ostriches, the possibility of administering the same DNA vaccine mucosally should be investigated.

A mucosal vaccine is administered to a mucosal surface mucosally (oral, eyes, nose) and should result in the production of IgA antibodies (Hobson et al., 2003). The ELISA technique can be used to measure an immune response elicited by a mucosal vaccine but requires secondary anti-IgA antibodies. Currently secondary antibodies against ostrich IgA is not commercially available and the production of such antibodies would require the isolation of ostrich IgA. Isolation of IgA from ostrich nasal samples is problematic and results in low yields (Botes, 2004). The process of IgA isolation from the ostrich can be avoided by producing the protein *in vitro*. For the purpose of producing secretory antibodies the entire protein is, however, not required but only the IgA heavy chain constant region (IgA_H) of the ostrich.

Immunoglobulins are known as glycoproteins containing glycosylated sites which can influence their function and plays a role in protection against proteolytic degradation. These modifications can have a structural and functional effect on the protein and have an influence on its immunogenicity. Ostrich IgA_H contains three N-linked glycosylation sites and it is therefore important that an *in vitro* expression system can provide post-translational modifications such as glycosylation for applications in which the native protein is required (Huang et al., 2012; Schroeder and Cavacini, 2010; Zhong and Wright, 2013). The most popular expression system used to produce such post-translational modified proteins is the baculovirus-insect cell expression system. The yield produced by this expression system is larger than those of mammalian cell lines and this expression system is also capable of post translational modifications similar to those of higher eukaryotes. Therefore the baculovirus-insect cell expression system holds an advantage over bacterial expression systems as these systems do not have post translational modification capabilities (Verma et al., 1998).

The Sf9 insect cell line is commonly used for production of the recombinant baculovirus as it allows both small and large scale protein production, and can be used for virus propagation prior to transfection. Several baculovirus vectors are commercially available depending on the protein to be expressed. The ProFold™-ER1 baculovirus is specifically recommended for glycoproteins as well as secreted proteins since it encodes for the molecular chaperones calreticulin and protein disulfide isomerase (PDI). Both of these chaperones ultimately facilitate folding of the protein of interest in the endoplasmic reticulum (ER). Calreticulin is a lectin chaperone able to interact with glycoproteins whereas PDI participates in disulfide bond formation and can also fold proteins independently of disulfide bonds. Overexpession of PDI is also able to improve the longevity of the infected insect cells by about 24 h compared to cells infected with control viruses that do not contain PDI, as well as improve yields of secreted proteins. (Dalton and Barton, 2014; Taylor et al., 2004; Turano et al., 2002). The ProFold™-ER1 baculovirus vector also contains the gene encoding *Aequorea victoria* green-fluorescent protein (GFP) which allows for visualising cells infected with the recombinant baculovirus. All of these genes are expressed during the very late stage of infection at about the same time as the target protein and are situated away from the polyhedrin site to avoid interference of strong promoters. Therefore expression of the gene of interest is not compromised by the expression of the PDI, Calreticulin or GFP (AB Vector, 2014).

For insertion of the *IgA$_H$* gene into a baculovirus vector a transfer plasmid is used and several different plasmids are commercially available. The pAB-6xHis™ transfer plasmid used in this study is compatible with the ProFold™-ER1 baculovirus vector and allows expression of proteins with an N-terminal 6xHis tag. It also contains a thrombin site for the removal of the tag after isolation of the protein of interest if desired. Also present in the transfer plasmid is a gene encoding ampicillin resistance, a bacterial origin of replication and the MCS with unique RE sites which is flanked by a strong polyhedrin promoter as well as the essential baculovirus *orf 1629* gene which allows insertion of the gene to be expressed into the baculovirus vector by homologous recombination.

The objective for this study was to evaluate the use of a baculovirus-insect expression system for the production of the ostrich IgA$_H$ protein. To this end the *IgA$_H$* gene was sub-cloned into the pAB-6xHis™ transfer plasmid and used to co-transfect Sf9 insect cells with the linearised viral ProFold™-ER1 baculovirus vector for the generation of ProFold™-ER1_*IgA$_H$* recombinant baculovirus. Sf9 cells were transfected with the ProFold™-ER1_*IgA$_H$* baculovirus for the production of the IgA$_H$ protein. The aim was then to verify the produced IgA$_H$ protein using the SDS-PAGE and western blot techniques.

4.2 Materials and Methods

4.2.1 Sub-cloning of the *IgA$_H$* gene into the pAB-6xHis™ baculovirus transfer vector

4.2.1.1 Primer Design

Primers were designed by using the computer program Primer Designer (version 1.0.1, Scientific and Educational Software) and synthesized by Integrated DNA Technologies (IDT). The primers were designed according to the ostrich *IgA$_H$* DNA sequence obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database (Accession number: JN709445.1). The forward primer (Figure 4.1) was designed to bind from base pair (bp) 1 to 18 (position 1 = start of sequence) and the reverse primer from bp 1277 to 1298 (end of sequence). For subsequent cloning into the transfer plasmid suitable restriction endonuclease (RE) sites were added to each of the primers. The forward primer contained the *EcoRI* RE recognition site whereas the reverse primer contained the *NotI* RE recognition site. Five random base pairs were added to the 5' end of each primer to allow for efficient cutting of the RE.

Forward Primer (IgA_F(EcoRI)):

5'-TATTTGAATTCGCCACGGCCAAAGCGCCC-3'

A **B** **C**

Reverse Primer (IgA_R(NotI)):

5'-ATAATGCGGCCGCCAGTAGCAGGTGATGTCGGCGT-3'

A **B** **C**

Figure 4.1 The primers designed for the amplification of the *IgA$_H$* gene. **Forward Primer (IgA F(EcoRI))**: **A**- Five added bases, **B**- *EcoRI* RE recognition site, **C**- Primer. **Reverse Primer (IgA R(NotI))**: **A**- Five added bases, **B**- *NotI* recognition site, **C**- Primer.

4.2.1.2 Amplification and isolation of the *IgA$_H$* gene

The *IgA$_H$* gene cloned into a pGEX-4T-1 expression vector was obtained from co-workers at Onderstepoort (Pretoria). The gene represented the mRNA sequence of the IgA$_H$ which consists of four domains including the secretory coding segment (Addendum C). The vector containing the *IgA$_H$* gene was multiplied by making an O/N culture as described in section 3.2.2, with the use of LB media, and incubating the culture at 37ºC for 16-18 h on an orbital shaker (200 rpm). The pGEX-4T-1_*IgA$_H$* was isolated from the *E. coli* culture by means of the Invisorb® Spin Plasmid Mini Two kit (Invitek, Berlin). The plasmid was isolated according to the manufacturer's instructions with the exception of eluting the plasmid in 30 µl autoclaved Milli-Q® water (Milli-Q®)

pre-heated to 65ºC. The concentration of the isolated pGEX-4T-1_*IgA*$_H$ plasmid was determined by means of the Nanodrop® ND 1000 spectrophotometer (Novell®, USA).

The gene was PCR amplified from the vector with the use of the designed primers in order to obtain the gene containing the RE recognition sites. For amplification, the optimal annealing temperature of the designed primers was firstly determined by using different annealing temperatures of 66ºC, 64ºC, 62ºC, 60ºC, 58ºC and 56ºC. The *IgA*$_H$ gene was then PCR amplified form the pGEX-4T-1 plasmid using the following reaction conditions: 5 µl 5x Fidelity buffer, 0.75 µl dNTP's (10 mM), 0.75 µl IgA_F(EcoRI) forward primer (10 µM), 0.75 µl IgA_R(NotI) reverse primer (10 µM), 1 µl plasmid DNA (130.9 ng/µl diluted 1:20 in Milli-Q® water), 0.5 µl KAPAHifi DNA polymerase (1 U/µl) (Roche, RSA) and Milli-Q® water to a final volume of 25 µl. The parameters of the Veriti™ 96 Well Thermal Cycler (Applied Biosystems) used were as follow, an initial step of 5 min (95°C), followed by 25 cycles of 20 sec (98°C), 15 sec (66°C), 40 sec (72°C) with a final step (single cycle) for 5 min (72°C) followed by cooling to 15°C. The PCR products were analysed on a 1% agarose gel containing EtBr (0.5 µg/ml) for visualisation of the products under UV light. Depending on the size of the agarose gel used, PCR products were electrophoresed in 1x TAE buffer at 80 Volts (V) for a 3 cm x 10 cm gel, 90 V for a 7 cm x 10 cm gel and110 V for a 10 cm x 15 cm gel.

In order to produce a larger yield of the *IgA*$_H$ gene for RE-digestion and subsequent cloning the PCR reaction was scaled up to a final volume of 100 µl using the same PCR parameters as before. Due to non-specific binding of the primers the PCR product had to be excised and cleaned from the agarose gel. The total volume of PCR product was therefore loaded on to a 0.5% agarose gel containing EtBr (0.5 µg/ml) and electrophoresed (120 V). The *IgA*$_H$ gene with a size of approximately 1300 bp was excised from the gel and purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Sweden) as per manufacturer's instructions except for the elution step where Milli-Q® was used to elute the product. The concentration of the purified product was determined using a Nanodrop® ND 1000 spectrophotometer (Novell®, USA) and the product analysed on a 1% agarose gel containing 0.5 µg/ml EtBr for visualisation in order to determine if the purification step was able to remove all non-specific PCR products. The agarose gel was electrophoresed as previously described.

4.2.1.3 Restriction enzyme digestion of the pAB-6xHis™ transfer plasmid and *IgA*$_H$ gene

The pAB-6xHis™ transfer plasmid (AB vector, USA) was kindly provided by Prof. Pieter Swart (Department of Biochemistry, Stellenbosch University) in *E. coli* JM109 cells (Promega, USA). An O/N culture was made as in section 3.2.2 of the *E. coli* JM109 cells containing the pAB-6xHis™ transfer plasmid in order to increase the plasmid concentration. The plasmid was isolated and the

concentration determined by means of the Nanodrop® ND 1000 spectrophotometer (Novell®, USA). This was followed by a double digestion of the isolated pAB-6xHis™ transfer plasmid using the *EcoRI* and *NotI* RE's. The reaction contained 2 µl 10x FastDigest buffer (Fermentas), 11.6 µl plasmid DNA (pDNA) (86.4 ng/µl), 1 µl *NotI* , 1 µl *EcoRI* and 4.4 µl Milli-Q® water adding up to a total volume of 20 µl. The reaction mixture was incubated at 37ºC for 13 min followed by a 5 min heat inactivating step at 80ºC. Purification of the reaction mixture was done by means of the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) according to the manufactures instructions. This was followed by dephosphorylation of the digested plasmid product by means of shrimp alkaline phosphatase (SAP, Promega) in a reaction containing 3 µl 10x Reaction buffer for SAP, 1 µl SAP (1 U/µl), 5.7 µl digested plasmid (after cleanup) and 20.3 µl Milli-Q® water. The reaction mixture was incubated for 15 min at 37ºC.

The isolated PCR product of the IgA_H gene was also double digested with the *BamHI* and *NotI* RE in a reaction mixture containing 4 µl 10x FastDigest buffer (Fermentas), 3.4 µl IgA_H gel purified PCR product (145.2 ng/µl), 1 µl *BamHI*, 1 µl *NotI* and 30.6 µl Milli-Q® water. The reaction mixture was incubated at 37 ºC for 13 min and heat inactivated at 80ºC for 5 min followed by purification with the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) as per manufactures instructions.

4.2.1.4 Ligation of the IgA_H gene and the pAB-6xHis™ transfer plasmid and transformation into *E. coli* JM109 cells

The IgA_H gene was ligated into the pAB-6xHis™ transfer plasmid using a vector to insert ratio of 1:1 and 1:3. The 1:1 reaction contained 4.1 µl pDNA (24.6 ng/µl), 0.3 µl insert DNA (42 ng/µl), 1 µl 10x Ligase buffer, 1 µl T4 DNA ligase (Promega) and 3.6 µl Milli-Q® water. The 1:3 reaction contained 4.4 µl pDNA (22.6 ng/µl), 0.9 µl insert DNA (42 ng/µl), 1 µl 10x Ligase buffer, 1 µl T4 DNA ligase and 2.7 µl Milli-Q® water. The reactions were incubated at 4ºC O/N.

Each of the ligated plasmid products were transformed into *E. coli* JM109 (Promega, USA) competent cells as follows: the JM109 cells were removed from the -80ºC freezer and allowed to thaw on ice. The cells were mixed and 50 µl pipetted into chilled 14 ml Falcon® tubes followed by the addition of 5 µl of either the 1:1 or 1:3 ligation products. The contents of the tubes were gently mixed by flicking the tube and incubated on ice for 20 min. Subsequently, a 42ºC water bath was used in order to heat shock the cells for 45 sec followed by 2 min incubation on ice. LB medium (950 µl; 10 g/L Bacto-Tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH7) was added to the Falcon® tubes containing the transformation product and incubated at 37ºC for 1 h 30 min on a orbital shaker (200 rpm). The incubated cells were plated on LB plates containing agarose (15 g/L) as well as 1

µl/ml ampicillin (100 mg/ml), by spreading 100 µl of the transformation product on a plate for each of the 1:1 and 1:3 reactions respectively. The rest of the transformation product was concentrated by means of centrifugation at 10 000 x g for 10 min and 600 µl of the supernatant discarded. The pellets were resuspended in the remaining supernatant and 50 µl and 100 µl of concentrated samples spread on a plate respectively. This was repeated for the 1:1 and 1:3 transformation products. All the agar plates were incubated for 16-18 h at 37ºC. Colonies which formed on the agar plates were analysed by means of colony PCR as previously described with the only exception being the pAB-6xHis™ primers pHF and mR (Table 4.1) which was used instead of the pGEX_F and T7_R primers.

O/N cultures were made from the colonies that contained the plasmid with insert as confirmed by the colony PCR. This was done by scraping the colony from the plate by means of a sterile toothpick and adding it to 5 ml of LB medium in a Falcon® tube containing 5 µl ampicillin (100 mg/ml) followed by incubation for 16-18 h at 37ºC on an orbital shaker (200 rpm). The pAB-6xHis™_IgA_H was isolated from the O/N culture as previously described. The isolated pAB-6xHis™_IgA_H was digested with *NotI* and *EcoRI* RE's to confirm that the plasmid did indeed contain the IgA_H insert. The reaction mixture contained 1 µl 10x FastDigest buffer, 0.5 µl *NotI* RE, 0.5 µl *EcoRI* RE, 3 µl pDNA (130.6 µg/µl) and 5 µl Milli-Q® water. The reaction was incubated for 13 min at 37 ºC followed by a heat inactivation step for 5 min at 80 ºC. The product was purified by means of the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) and analysed on a 1% agarose gel containing EtBr (0.5 µg/ml) for visualisation.

Isolated pAB-6xHis™_IgA_H insert was also sequenced to confirm that the IgA_H insert had the correct orientation and sequence. The sequencing reactions contained 1 µl BigDye® Terminator mix (v3.1, Applied Biosystems, USA), 3 µl Half-Dye Mix (Bioline, UK), 3 µl pDNA (300 ng) and 3 µl pHF forward primer (3.3 pmol/µl). For the reverse sequence reaction the same components at the same concentrations were used with the exception that only the mR reverse primer was used. The parameters of the Veriti™ 96 Well Thermal Cycler (Applied Biosystems) was set to 35 cycles of 96ºC (10 sec), 52ºC (30 sec), 60ºC (4 min) and a final step of 60ºC (10 min) followed by a cooling step to 15ºC. The samples were then sent to the Central Analytical Facility (CAF) DNA sequencing unit of the University of Stellenbosch, South Africa for analyses using an ABI® 3100 Genetic Analyser (Applied Biosystems, USA). The resulting sequences were analysed by aligning the obtained sequence to the IgA_H sequence from NCBI (Accession number: JN709445.1) by means of the BioEdit sequence alignment editor (version 7.2.5).

Table 4.1 Primer sequences for the pAB-6xHis™ transfer plasmid

Primer	Sequence	Plasmid	Binding position on	Comment
pHF	5'-AGACGCACAAACTAATATCACAAACTGGA-	pAB-6xHis™	3945-3973 bp	(AB Vector, 2014)
mR	5'- CGTGTCGGGTTTAACATTACGGATT-3'	pAB-6xHis™	4284-4308 bp	(AB Vector, 2014)

Primers provided by Prof Pieter Swart (Department of Biochemistry, Stellenbosch University)
* According to the pAB-6xHis™ transfer plasmid map as supplied by AB vector.

4.2.2 Culturing insect host cell line

4.2.2.1 Monolayer cultures

An aliquot of *Spodoptera frugipedra* (Sf9) cells stored in liquid nitrogen was kindly supplied by Prof. Pieter Swart (Department of Biochemistry, University of Stellenbosch). Cell densities were first determined by counting the cells with a haemocytometer (Neubauer) and cell viability was assessed by the Tryptan Blue exclusion method (Johnson et al., 2013). In order to count the cells the aliquot was thawed in a 28ºC water bath and 20 µl of cells were mixed with 20 µl 0.4% Trypan Blue solution (Life Technologies,USA) and 20 µl of this mixture pipetted into a haemocytometer (Neubauer). The cells were counted by means of a microscope (Olympus CKX 41) at a magnification of 10x and subsequently plated at seeding densities of 3 x 10^4 viable cells/cm^2 in complete TNM-FH medium (named after Dr. W.F. Hink who modified Grace's medium for the growth of *Tricoplusia ni* cells and is therefore known as TNM-FH medium). The cells were plated in 75 cm^2 Cell Star® tissue culture flasks (Greiner bio-one) and grown as monolayers in a non-humidified, ambient air-regulated incubator at 27ºC. Passages of the monolayers were performed at 90-95% confluency by dislodging the cells, with a method known as sloughing, from the surface of the 75 cm^2 tissue culture flask and counting the cells as well as assessing the cell viability of the cells as described previously. The cells were subsequently seeded at densities of 3 x 10^4 viable cells/cm^2 (dilutions made with complete TNM-FH media) in a new 75 cm^2 tissue culture flask and incubated as before.

The complete TNM-FH medium consisted of Grace's insect cell culture medium containing L-glutamine (Gibco®, Life Technologies) which was supplemented with 10% fetal bovine serum (FBS) (Biochrom: FBS superior, standardized (cat no. S0615, Merck)), 3.303 g/L lactalbumin hydrolysate (Sigma), 3.330 g/L yeastolate (BD Biosciences), 0.35 g/L sodium bicarbonate (Sigma), 100 U/ml Penicillin-Streptomycin (PenStrep) (Sigma), 10 µg/ml gentamicin (Sigma) and 0.25 µgl/ml amphotericin B (Gibco®, Life Technologies). The media was prepared by adding Grace's insect cell culture powder (Gibco®, Life Technologies) to a 1 L Schott bottle containing 700 ml

sterilized Milli-Q® water. This was followed by the addition of 3.303 g of lactalbumin hydrolysate, 3.330 g yeastolate and 0.35 g sodium bicarbonate. The pH was set to 6 and sterile Milli-Q® water was added to a final volume of 1 L. The medium was passed through a glass fiber prefilter (Merc) followed by a Sterivex 0.22 µm bell filter by means of a Millipore easy-load® Masterflex® L/S® pump (Millipore). A sample was taken from the media and incubated at 37ºC to ensure that it was sterile. Subsequently complete TNM-FH media was prepared by adding FBS, gentamicin, amphotericin B and PenStrep to the prepared media and stored at 4ºC.

4.2.2.2 Suspension cultures

After about three passages in which regular exponential growth with viability of greater than 90% was obtained with the monolayer cultures, they were transferred to an Erlenmeyer flask to form a suspension culture. A minimum density of 0.5 x 10^6 cells/ml is required to start a suspension culture from an adherent culture and therefore 0.5 x 10^6 cells/ml were suspended in 70 ml complete TNM-FH medium in a sterile 150 ml Erlenmeyer flask containing 0.1% Pluronic® F-68 (Gibco, Life technologies). The cultures where agitated continuously at 120 rpm at 27ºC (Ecotron, Infors HT) and passaged to cell densities of 3 x 10^5 cells/ml until they reached a density of 2 x 10^6 to 4 x 10^6 cells/ml after 3-4 days. Passages were done by using the haemocytometer and Trypan Blue exclusion method as described previously to determine the amount of cells and their viability. They were subsequently again seeded at the recommended cell densities in complete TNM-FH media in a 150 ml Erlenmeyer flask.

4.2.3 Production of the recombinant virus by means of co-transfection of Sf9 cells

4.2.3.1 Preparation of Sf9 monolayers

Sf9 monolayers were prepared from suspension cultures by seeding a 9.6 cm^2 6-well cell culture plate (Greiner bio-one, Austria) with 2.0 ml complete TNM-FH medium containing 9.6 x 10^4 viable cells/cm^2 per well to obtain 60% confluency for efficient baculovirus replication. The cells were incubated at 27ºC in a non-humidified ambient air-regulated incubator for 1 h in order for the cells to attach to the surface of each well. Following the incubation period the TNM-FH medium was removed and each well washed twice with 1 ml 1 x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2) for the removal of residual FBS and antibiotics. Incomplete TNM-FH media (2 ml) containing L-glutamine and sodium bicarbonate but no FBS or antibiotics, were added to each well.

4.2.3.2 P0 virus production by homologous recombination and co-transfection

A DNA-profectin mixture was prepared in a 1.5 ml Eppendorf tube by adding 5 µl ProFold™-ER1 linearised baculovirus DNA (AB vector) to 100 ng pAB-6xHis™_IgA_H plasmid and adding sterilized Milli-Q® water to a final volume of 50 µl. The ProFectin™ transfection reagent (AB vector) was prepared by diluting it 1:10 in Milli-Q® water to a final volume of 50 µl. This was then added dropwise to the mixture of ProFold™-ER1 linearised baculovirus DNA and pAB-6xHis™_IgA_H plasmid resulting in a final volume of 100 µl. The DNA-ProFectin™emulsion was added dropwise to the monolayer of insect cells in the 6-well plate (100 µl per well). The controls included a No control (NC) and Green control (GC) (AB vector). NC virus is a negative control which is identical to the wild type *Autographa californica* nuclearpolyhedrosis virus with the only exception being that it does not express polyhedrin or any other protein (AB vector). GC virus which is positive control, expresses *Aequorea victoria* green-fluorescent protein (GFP) which can be used for detection of virus propagation (AB vector). Of each control, 10 µl was added to two wells of the 6-well plate respectively. Three of the wells did not receive any virus and was referred to as the untransfected (UT) wells. This was followed by incubation at 27ºC for 24 h before the addition of 1 ml complete TNM-FH media containing 10% FBS and antibiotics. The 6-well plate was incubated for a further 60 h at 27ºC. After 60 h the monolayer was gently resuspended by pipetting the media over the cells and 1.5 ml of the suspension transferred to a 25 cm^2 Cell Star® tissue culture flasks (Greiner bio-one) containing 5 ml complete TNM-FH medium for each sample. These flasks were incubated for 4 days at 27ºC. Following the 4 day incubation the virus containing medium was transferred to a 15 ml Falcon® tube and centrifuged (Eppendorf Centrifuge 5702) at 376 x g for 15 min. The supernatant containing the P0 virus was transferred to a sterile 15 ml Falcon® tube and stored at 4ºC protected from light.

4.2.4 Production of higher titer P1 virus

As initial P0 virus stocks are of a titer which is too low for protein expression studies they have to be amplified. Therefore a T75 flask was seeded with Sf9 cells from a monolayer culture in complete TNM-FH medium at 4 x 10^4 cells/cm^2 to obtain a confluency of 25%. P0 virus stock (500 µl) was added to the monolayer and incubated for 4 days at 27ºC. After 4 days the media of these cultures which contained the P1 virus stock could be harvested. The media containing the virus was transferred to a 15 ml Falcon® tube and centrifuged (Eppendorf centrifuge 5702) at 1500 rpm for 15 min. The supernatant (P1 virus stock) was transferred to a sterile 15 ml Falcon® tube and stored at 4ºC protected from light.

4.2.5 Production of protein on small scale

Sf9 suspension culture cells that reached a cell density of between 2 x 10^6- 4 x 10^6 was used to seed a 6-well plate with 1.5x10^5 cells/cm^2 (95% confluency) in 2 ml complete TNM-FH medium per well and incubated for 1 h at 27ºC. Three wells were infected with 200 µl P1 virus. Two of the wells were infected with 20 µl GC and NC respectively. The last well was not infected with any virus and served as an UT control. The plate was incubated at 27ºC and the ProFold-ER1™_IgA_H transfected samples were collected at 48 h, 60 h and 72 h respectively. The GC, NC and UT samples were collected at 60 h.

Samples were collected by firstly removing the medium from each well and transferring it to a 2 ml Eppendorf tube followed by centrifugation for 3 min at 376 x g. The supernatant was then removed and represents the media which were also kept for analysis. The pellet represents the cells that were already dislodged from plate where the recombinant protein is expected. Secondly, each well was washed with 1 ml PBS buffer which was transferred from the well to the Eppendorf tube containing the pellet and used to resuspend the pellet. The resuspended pellet was again centrifuged at 376 x g for 3 min followed by discarding of the supernatant. To each of the wells 100 µl lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X 100) was added which was used to dislodge the monolayers. The dislodged monolayer was transferred again to the Eppendorf tube containing the pellet followed by resuspension of the pellet (this was done for each of the wells). The samples were stored at -20ºC until further analysed.

4.2.6 Verification of the expressed IgA_H proteins by means of western blot

4.2.6.1 Western Blot with anti-HisProbe-HRP antibodies

All of the samples containing the expressed protein were analyzed on a SDS-PAGE gel as in section 3.2.7.2. The only exception was that 30 µl of the 60 h and 72 h samples were centrifuged for 15 min at 17 000 x g. The supernatant was transferred to a 1.5 ml Eppendorf tube containing 30 µl treatment buffer and12 µl loading dye. The pellet was resuspended in 30 µl treatment buffer and 12 µl loading dye was added. The GC, NC, UT and 48 h samples were handled as in section 3.2.7.2 but all samples were boiled for 13 min instead of 3 min. The samples were loaded (15 µl of each sample) onto a SDS-PAGE gel and the gel was electrophoresed as in section 3.2.7.2.

Proteins were transferred from a 12% SDS-PAGE gel (as described in section 3.2.7.2) to a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, Sigma) by electrophoresis (up to 1 A; 25 V for 30 min) using the Trans-Blot® Turbo™ Blotting System (Bio-rad). The nitrocellulose membrane was

placed in blocking buffer (25 mM Tris-base, 0.15 M NaCl, pH7.2 containing 10% BSA) to block non-specific binding, for 1 h at RT with agitation (Gyro Twister, Labnet). The membrane was washed twice with Tris-buffered saline containing Tween (TBST) (25 mM Tris-base, 0.15 M NaCl and 0.05% Tween® 20, pH 7.2) for 10 min each at 37ºC with agitation (hybridization oven/shaker, USA) This was followed by the addition of the HisProbe-HRP (Thermo Scientific) working solution (HisProbe diluted 1:5000 in blocking buffer) and incubation for 1 h at 37ºC with agitation. The HisProbe-HRP working solution was discarded and the membrane was washed four times in TBST buffer for 10 min each at 37ºC with agitation. The membrane was subsequently developed for 2 sec to 30 min in a solution containing 0.05% (w/v) 4-chloro-1-naphthol, 16% (v/v) methanol, 30 ml 1xPBS and 0.025% (v/v) H_2O_2.

4.2.6.2 Western Blot with rabbit anti-IgA antibodies

Proteins were transferred from a SDS-PAGE gel as in section 4.2.6.1 and the membrane was washed in PBS-Tween (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2 containing 0.1% (v/v) Tween® 20) for 10 min at 37ºC with agitation (hybridization oven/shaker, USA). The PBS-Tween was removed and non-specific binding was blocked by incubating the blot in casein buffer (10 mM Tris-HCl, 154 mM NaCl, 0.5% fat-free milk powder and 0.02% Thimerosal, pH 7.6) for 1 h at 37ºC with agitation (hybridization oven/shaker, USA). Following the blocking step the membrane was washed three times with PBS-Tween for 5 min each at 37ºC with agitation. Rabbit anti-ostrich IgA diluted 1:500 in casein-Tween (casein buffer, pH 7.6 containing 0.1% (v/v) Tween®20), was added to the membrane and incubated O/N at 4ºC with agitation on a Gyro Twister (Labnet). The anti-ostrich IgA antibodies were provided by Dr. A. Botes, Department of Biochemistry, Stellenbosch University and were previously developed against IgA isolated from ostrich nasal secretions.

Following incubation with the primary antibody the membrane was washed three times with PBS-Tween for 5 min each at 37ºC with agitation. Subsequently goat anti-rabbit IgG (Sigma) diluted 1:500 in casein-Tween was added to the membrane and incubated for 1 h at 37ºC with agitation. The membrane was washed three times with PBS-Tween for 5 min at 37ºC with agitation. This was followed by the addition of peroxidase anti-peroxidase (PAP) soluble complex antibody produced in rabbit (diluted 1:500 in casein-Tween) which was incubated for 1 h at 37ºC with agitation. The PAP was removed after the hour incubation and the membrane washed three times with PBS-Tween for 5 min each at 37ºC with agitation. This was followed by the development of the membrane as described in section 4.2.6.1.

4.2.7 PCR amplification of insert obtained in the P1 recombinant virus

The gene inserted into the ProFold™-ER1 baculovirus vector was PCR amplified from the recombinant ProFold™-ER1_*IgA*$_H$ P1 stocks, with the use of the pHF and mR primers. Four microliter of the P1 virus stocks were added to 25 µl GES buffer (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% (v/v) Triton X-100) respectively and boiled at 95ºC for 10 min. This virus sample mixture was used in the following reaction conditions: 1 µl 10x reaction buffer, 0.4 µl dNTP's (5 mM), 0.6 µl $MgCl_2$ (25 mM), 0.5 µl pHF (20 pmol/µl), 0.5 µl mR (20 pmol/µl), 0.1 µl Super-Therm polymerase (5 U/µl), 1.5 µl virus sample mixture and 5.4 µl Milli-Q® water. The parameters for the Veriti™ 96 Well Thermal Cycler (Applied Biosystems) used were as follow, an initial step of 5 min (95ºC), followed by 25 cycles of 20 sec (98ºC), 15 sec (58ºC), 40 sec (72ºC) with a final step (single cycle) of 5 min (72ºC) followed by cooling to 15ºC. The PCR products were analysed on a 1% agarose gel containing EtBr (0.5 µg/ml) for visualisation of the products under UV light and electrophoresed as in section 4.2.1.2.

4.3 Results

4.3.1 Sub-cloning of the *IgA*$_H$ gene into the pAB-6xHis™ baculovirus transfer vector

4.3.1.1 Amplification and isolation of the *IgA*$_H$ gene

The *IgA*$_H$ gene could be amplified successfully with all the annealing temperatures as indicated by the red block in Figure 4.2 A. The PCR product amplified with an annealing temperature of 66ºC (lane 1) yielded the brightest band at a size of 1300 bp representing the *IgA*$_H$ gene, indicated in the red block, which indicates a higher concentration, with the least non-specific binding as the surrounding bands are lighter. The annealing temperature of 66ºC was therefore used to amplify the *IgA*$_H$ gene and the gene was successfully isolated from the 0.5% agarose gel as a single band could be seen in Figure 4.2 B with a size of 1300 bp after purification.

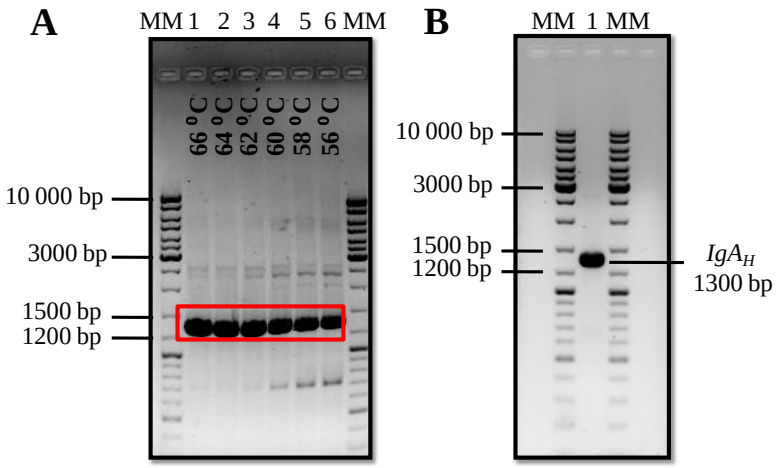

Figure 4.2 1% Agarose gel showing the results of the PCR amplification of the *IgA*$_H$ gene using the pGEX_*IgA*$_H$ plasmid as template. **(A)** Optimization of annealing temperatures. MM: GeneRuler™ (Fermentas), Lane 1: Annealing temperature 66ºC, Lane 2: Annealing temperature 64ºC, Lane 3: Annealing temperature 62ºC, Lane 4: Annealing temperature 60ºC, Lane 5: Annealing temperature 58ºC, Lane 6: Annealing temperature 56ºC. **(B)** PCR product excised and purified from the agarose gel. MM: GeneRuler™ (Fermentas), Lane 1: Isolated IgA$_H$ with size of 1300 bp.

4.3.1.2 Restriction enzyme digestion of the pAB-6xHis™ transfer plasmid and *IgA*$_H$ gene

The pAB-6xHis™ transfer plasmid (9698 bp) was successfully increased and digested by the selected enzymes as shown in Figure 4.3 in lane 4-7 as the digested plasmid produced a band at about 10 000 bp on a agarose gel. The digestion of the *IgA*$_H$ gene (1298 bp) as in lane 1-3 of Figure 4.3 did not result in more than one band. This indicates that the gene only contained one digestion site for each of the enzymes as incorporated during the amplification of the gene by means of the designed primers and the gene of interest was not cut at unwanted sites.

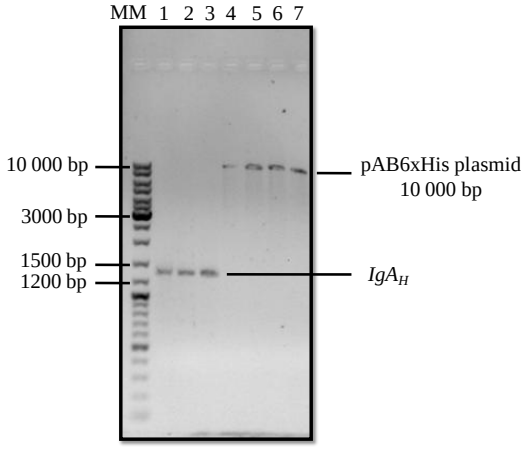

Figure 4.3 Products produced after digestion and cleanup of the *IgA*$_H$ gene and pAB-6xHis™ plasmid as analysed on a 1% agarose gel. MM: GeneRuler™ (Fermentas), Lane 1-3: Digested *IgA*$_H$, Lane 4-7: Digested pAB-6xHis™ plasmids after SAP and cleanup.

4.3.1.3 Ligation of the *IgA$_H$* gene and the pAB-6xHis™ plasmid

The colony PCR confirmed the presence of the *IgA$_H$* gene in most of the plasmids of the tested colonies as shown in Figure 4.4. Representing the *IgA$_H$* gene a band of 1600 bp was obtained for all of the tested colonies except the ones in lanes 4 and 11. Therefore cloning of the *IgA$_H$* gene into the pAB-6xHis™ plasmid by means of restriction enzymes was successful. The positive control used, which is represented in lane 2, was only the pAB-6xHis™ plasmid without any insert as confirmed by the band of 400 bp. The negative control in lane 1 did not result in any band. Sequencing results also confirmed that the pAB-6xHis™ transfer plasmid contained the correct sequence representing the *IgA$_H$* gene.

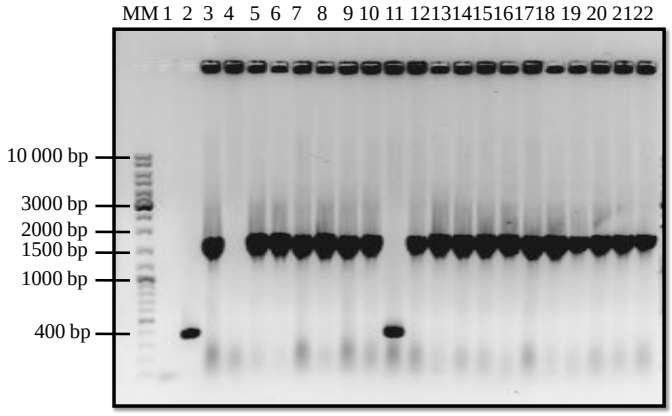

Figure 4.4 Colony PCR products on a 1% agarose gel. MM: GeneRuler™ (Fermentas), Lane 1: Negative Control, Lane 2: Positive control, Lane 3-22: PCR products of colonies tested where only Lane 4 and 11 tested negative for pAB-6xHis™_*IgA$_H$*.

The pAB-6xHis™_*IgA$_H$* was also digested with *EcoRI* and *NotI* to ensure that the insert was indeed the *IgA$_H$* gene with a size of 1300 bp. As indicated in Figure 4.5 only two bands were seen on the gel namely the 10 000 bp representing the pAB-6xHis™ plasmid and a band at 1300 bp representing the *IgA$_H$* gene.

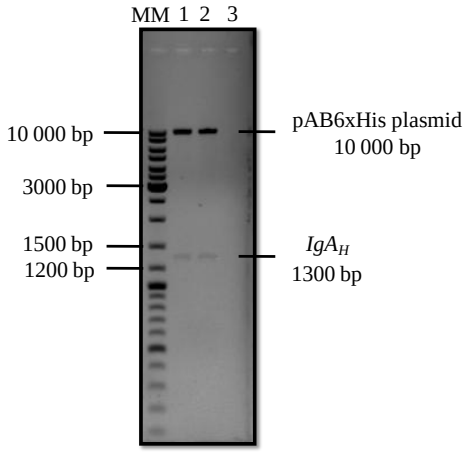

Figure 4.5 1% agarose gel of the RE digested pAB-6xHis™_*IgA$_H$* plasmid.MM: GeneRuler™ (Fermentas), Lane 1-2: pAB-6xHis™_*IgA$_H$* plasmid digested with *NotI* and *EcoRI* resulting in two bands.

4.3.2 Production of the recombinant virus by means of co-transfection of Sf9 cell

Cells were considered as infected when they became enlarged and non-adherent as compared to the UT control with smaller cells that still adhered to the surface of the plate. As the virus needs to be in a circular form to be able to infect cells it was assumed that the double homologous cross over event took place and that the ProFold™-ER1 baculovirus vector contained the *IgA$_H$* gene. Without the double homologous cross over event taking place the virus would still be linear and not able to infect the cells. Therefore enlargement of the cells would be highly unlikely. It was also visible that the cells were infected as indicated in Figure 4.6 A and B as they appeared black under UV due to the expression of GFP, as compared to the NC in Figure 4.6 C which had no black cells.

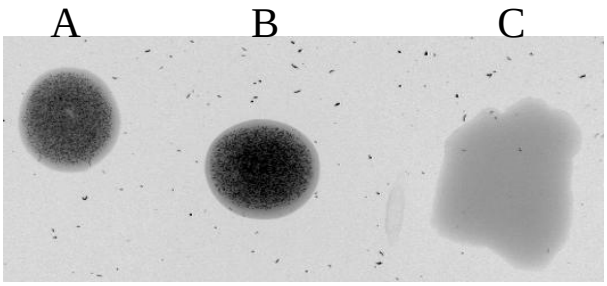

Figure 4.6 Cells infected with (A) the ProFold™-ER1_*IgA$_H$* virus, (B) GC and (C) NC virus**.** The cells that were infected by the ProFold™-ER1_*IgA$_H$* and GC viruses appear black under UV due to the expression of GFP by the viruses.

4.3.3 Production of protein on small scale

The IgA$_H$ protein (46.5 kDa) was expected to be 51.6 kDa including the 6xHis-tag and thrombin site. After 48 h, 60 h and 72 h a protein product was observed between 46-58 kDa on the SDS-PAGE gel (indicated by the arrow for lanes 4-8 in Figure 4.7) which was not present in the control samples (Lane 1-3).

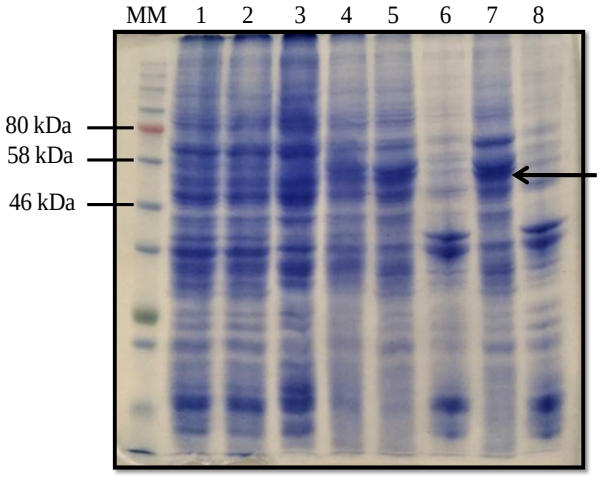

Figure 4.7 12% SDS-PAGE analysis of the IgA$_H$ protein expressed by means of the baculovirus-insect expression system. MM: Color Prestained Protein Standard, Broad Range (BioLabs® Inc., New England). Lane 1: GC after 60 h. Lane 2: NC after 60 h. Lane 3: UT after 60 h. Lane 4: 48 h expression of IgA$_H$. Lane 5: 60 h expression of IgA$_H$, supernatant after centrifugation. Lane 6: 60 h expression of IgA$_H$, pellet after centrifugation. Lane 7: 72 h expression of IgA$_H$, supernatant after centrifugation. Lane 8: 72 h expression of IgA$_H$, pellet after centrifugation.

4.3.4 Verification of the expressed IgA_H proteins by means of western blot

4.3.4.1 Western Blot with anti-HisProbe-HRP antibodies

The same band as in Figure 4.7, indicated by the arrow (between 38 kDa and 52 kDa), was also visible in lanes 2-4 in Figure 4.8 as detected by the HisProbe-HRP antibodies during western blot analysis.

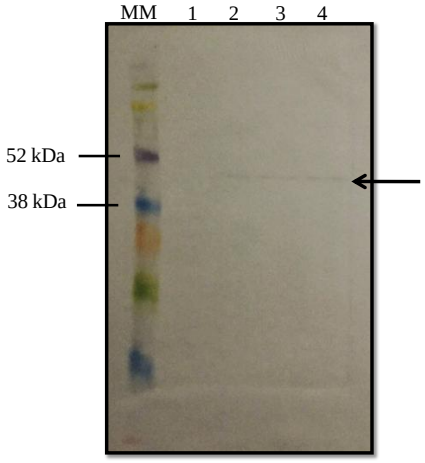

Figure 4.8 Verification of the expression of the IgA_H protein by means of Western Blot analysis. MM: Full-range Rainbow™ marker (Amersham™). Lane 1: NC after 60h. Lane 2: 48 h expression of IgA_H. Lane 3: 60 h expression of IgA_H. Lane 4: 72 h expression of IgA_H.

4.3.4.2 Western Blot with rabbit anti-IgA antibodies

Western blot analysis using the anti-ostrich IgA antibody resulted in a product of the same size as the western blot analysis with the HisProbe-HRP antibody as shown in Figure 4.9 between 38 kDa and 52 kDa, lanes 2-4 indicated by the arrow. Other bands seen in Figure 4.9 can be ascribed to non-specific binding as these bands are also visible in lane 1 which is a control sample.

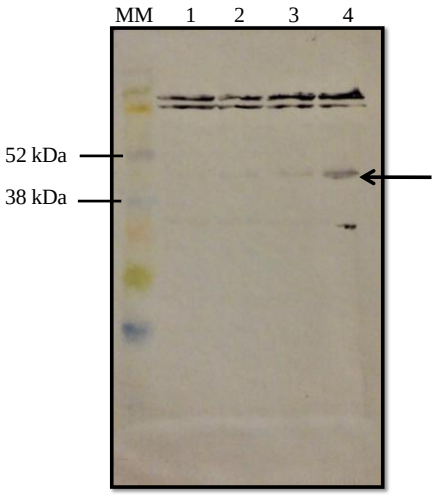

Figure 4.9 Verification of the expression of the IgA_H protein by means of Western Blot analysis. MM: Full-range Rainbow™ marker (Amersham™). Lane 1: NC after 60h. Lane 2: 48 h expression of IgA_H. Lane 3: 60 h expression of IgA_H. Lane 4: 72 h expression of IgA_H.

4.3.5 PCR amplification of insert in the P1 recombinant virus

The PCR product amplified from the ProFold™-ER1_*IgA*$_H$ recombinant virus was 1600 bp as shown in Figure 4.10 lanes 4-9. The 1600 bps band correspond with the size of the *IgA*$_H$ gene, thus homologous recombination was successful.

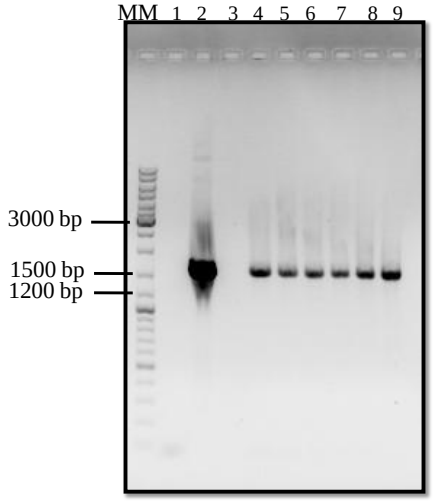

Figure 4.10 1% agarose gel of PCR products amplified form the ProFold™-ER1_*IgA*$_H$ recombinant virus. MM: Generuler™(Fermentas), Lane 1: negative control. Lane 2: Positive control (pAB-6xHis™_*IgA*$_H$). Lane 3: open. Lane 4-9: PCR product obtained from P1 ProFold™-ER1_*IgA*$_H$ recombinant virus.

4.4 Discussion

Administering a vaccine mucosally may result in an IgA immune response which can be measured by means of ELISA. Secondary antibodies are, however, necessary for this technique and as the focus of this study is ostriches, IgA needs to be isolated form ostrich samples for the production of such secondary antibodies. However, since the class of antibody is determined by the heavy chain of the antibody it is not necessary to use the entire antibody for the production of these secondary antibodies.

In this study the gene representing the heavy chain constant region of the IgA antibody (IgA$_H$) was inserted into the ProFold™-ER1 virus. The recombinant ProFold™-ER1_*IgA*$_H$ virus was used to infect Sf9 cells and the protein of interest was produced. It was however not clear from the SDS-PAGE gel (Figure 4.7) that the IgA$_H$ protein was produced, which might have been due to a low yield. A PCR was done to amplify the *IgA*$_H$ gene from the ProFold™-ER1 virus to confirm that the gene of interest was present in the ProFold™-ER1 virus. However, the P0 stock had a virus titer that was too low for detection and the PCR was done with the P1 virus stock. The GES buffer was used in the PCR protocol as it improved viral template release (La Notte et al., 1997), and a band was amplified form the ProFold™-ER1 virus which corresponded with the band size obtained from colony PCR. This confirmed that homologous recombination occurred successfully and that the

gene of interest was successfully cloned into the ProFold™-ER1 virus. Furthermore GFP expression was also observed which indicated that the virus was producing the protein of interest. Western blot analysis confirmed expression of the IgA_H protein yielding a band between 38 kDa and 52 kDa as detected by HisProbe-HRP and rabbit anti-ostrich IgA antibodies.

The size observed on the western blot with both the HisProbe-HRP and rabbit anti-ostrich IgA antibodies deviated slightly from the expected size of the IgA_H protein. As homologous recombination was successful and the *IgA_H* gene was present in the ProFold™-ER1 virus the size difference observed might be ascribed to glycosylation or other post-translational modifications. Glycoproteins behave aberrantly on SDS-PAGE gels (Roy and Kumar, 2014) and it was found in a study by Barnett et al. (1994) that the electrophorectic ability of the protein can vary due to glycosylation. It is therefore concluded that the IgA_H protein was successfully expressed. In order to isolate the IgA_H protein in sufficient quantities the virus should be amplified and more cells should be infected for an increased production of the IgA_H protein.

As IgA_H protein production was successful and anti-ostrich IgA antibodies were able to detect the protein, it is confirmed that the protein representing the IgA_H can still be detected by anti-ostrich IgA antibodies. Conversely therefore, using the IgA_H protein for the production of secondary antibodies should be successful and will ensure a reproducible source to use for the production of secondary antibodies.

5. Concluding remarks and future perspectives

The first objective of this study was to re-evaluate two previously developed DNA vaccines against *Mycoplasma nasistruthionis* sp. nov. str. Ms03 in a vaccination trial to determine the ability of the vaccines to elicit an immune response in ostriches. No statistically significant primary immune responses were elicited by either of the vaccines whereas a secondary immune response was elicited by both vaccines at all of the doses except for the pCI-neo_*oppA* 1200 µg/ml dose. This is an indication that the booster administration is important in eliciting adequate immune responses against the vaccines. Adequate time should be given for the production of both primary and secondary immune responses against the vaccines to ensure the development of immune memory. The ability of the vaccines to protect ostriches against Ms03 infections should however be established by challenging the ostriches with the pathogen.

DNA vaccine responses were evaluated by means of the ELISA technique which required isolation of recombinant OppA protein for coating of the ELISA plate. The recombinant OppA protein was isolated by means of affinity chromatography, but more than one batch had to be isolated since the yield of isolated OppA was not high enough for evaluation of all serum samples. Although an increase in incubation time on the column during the binding step resulted in a higher yield of OppA proteins, increasing the incubation time even further should be considered in future isolation attempts. This might result in a high enough yield of isolated OppA to allow for all ELISA analysis using a single batch of isolated protein. This will ensure additional consistency and accuracy.

Since mycoplasmas infect the mucosal surfaces of the ostrich respiratory tract, mucosal administration of the DNA vaccines should also be considered. The immune response elicited will be a mucosal immune response, and therefore IgA needs to be measured by ELISA. The second objective of this study was therefore to produce IgA_H antibodies in the baculovirus-insect expression system. Sub-cloning of the *IgA*$_H$ gene was successful and following homologous recombination between the virus and transfer plasmid containing the *IgA*$_H$ gene, the recombinant virus containing the *IgA*$_H$ gene was produced.

The IgA_H protein was successfully expressed by means of the baculovirus-insect system as verified by western blot analysis but expression levels were low. However, large amounts of IgA_H are not required for vaccination of rabbits and may therefore be adequate. To increase expression levels the ProFold™-ER1_*IgA*$_H$ virus should be titrated in order to infect Sf9 insect cells at the correct multiplicity of infection for effective amplification of the virus as well as to increase expression of the IgA_H protein for subsequent isolation by means of immobilised metal affinity chromatography

(IMAC). As the previously produced rabbit anti-ostrich IgA antibodies were able to detect the IgA_H protein during western blot analysis, secondary antibodies produced in the rabbit against the IgA_H protein should not influence the ability of the ELISA to detect the IgA antibodies elicited against a mucosal vaccine. The IgA_H protein produced in insect cells can therefore be administered to the rabbit for production of secondary antibodies for future use in the ELISA technique to assess mucosal IgA immune responses in mucosally vaccinated ostriches.

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7. Addenda

7.1 Addendum A

This addendum contains the results of the statistical analysis as obtained using Agrobase Generation II® for the ostrich vaccination trial data.

The humoral anti-OppA response elicited by the ostriches vaccinated by either pCI-neo_*oppA* or VR1020_*oppA* vaccines were analysed by means of ELISA with a 1:100 and 1:500 serum dilution factor. The ELISA results obtained for both the dilution factors was used for an analysis of variance (ANOVA) calculation and subsequent least significant difference (LSD) calculations. An ANOVA and LSD calculation was also done for the weight data.

ANALYSIS OF VARIANCE

pCI-neo 1:100, 2015

Variable: Response to DNA vaccine pCI-neo_*oppA* (1:100)

Source	df	SS	MS	F-value	Pr>F
Total	298	50.943			
FAC_A	3	0.548	0.183	1.52	0.2104
FAC_B	3	14.188	4.729	39.25	0.00000
FAC_A by FAC_B	9	2.106	0.234	1.94	0.0462
Residual	283	34.101	0.12		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 0.671	R-squared = 0.3306	C.V. = 51.74%

C.V = coefficient of variance

LSD for FAC_A = 0.1118	S.E.D = 0.0571	r = 74.0
T(2-sided a=0.050, 283 df) = 1.9684		MSE = 0.12050

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
2	0.73	60.6	1pCIneo 600
3	0.7	51.7	2pCIneo 1200
1	0.64	80.8	3 pCIneo 100
4	0.62	51.3	4 Control

LSD for FAC_B = 0.1118	S.E.D = 0.0571	r = 74.0
T(2-sided a=0.050, 283 df) = 1.9684		MSE = 0.12050

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	1.05	47.2	1 Week 9
6	0.61	51.9	2 Week 6
0	0.54	56.5	3
3	0.49	52.3	4 Week 3

LSD for FAC_A*FAC_B = 0.2235	S.E.D = 0.1136	r = 18.7
T(2-sided a=0.050, 283 df) = 1.9684		MSE = 0.12050

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.348	0.000	0.000	0.595	0.000	0.000	1.207
2	0.000	0.000	0.581	0.000	0.000	0.580	0.000	0.000	1.129
3	0.000	0.000	0.494	0.000	0.000	0.679	0.000	0.000	1.011
4	0.000	0.000	0.538	0.000	0.000	0.568	0.000	0.000	0.838

ANALYSIS OF VARIANCE

pCI-neo 1:100, 2015

Variable: Weight

Source	df	SS	MS	F-value	Pr>F
Total	223	20685.410			
FAC_A	3	71.845	23.984	0.96	0.4111
FAC_B	2	15302.793	7651.397	307.65	0.0000
FAC_A by FAC_B	6	38.214	6.369	0.26	0.9564
Residual	212	5272.558	24.871		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 38.490	R-squared = 0.7451	C.V. = 12.96%

C.V = coefficient of variance

LSD for FAC_A = 1.8578	S.E.D = 0.9425	r = 56.0
T(2-sided a=0.050, 212 df) = 1.9712		MSE = 24.87055

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error.

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
4	39.13	24.9	1 Control
3	38.97	24.5	2 pCIneo 1200
1	38.21	25.0	3 pCIneo 100
2	37.65	27.9	4 pCIneo 600

LSD for FAC_B = 0.1118	S.E.D = 0.0571	r = 74.0
T(2-sided a=0.050, 283 df) = 1.9684		MSE = 0.12050

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	44.60	11.9	1 Week 9
6	44.10	12.9	2 Week 6
0	26.77	13.4	3

LSD for FAC_A*FAC_B = 3.2178	S.E.D = 1.6324	r = 18.7
T(2-sided a=0.050, 212 df) = 1.9712		MSE = 24.87055

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.000	0.000	0.000	43.255	0.000	0.000	44.868
2	0.000	0.000	0.000	0.000	0.000	43.273	0.000	0.000	43.735
3	0.000	0.000	0.000	0.000	0.000	44.778	0.000	0.000	45.395
4	0.000	0.000	0.000	0.000	0.000	45.118	0.000	0.000	44.412

Data used for statistical analysis of the pCI-neo_*oppA* vaccine results using a 1:100 serum dilution factor. The data is arranged in columns, each containing the ostrich number, treatment received (trt), time (in weeks), response obtained from ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right.

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	TIME	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
161	0	pCIneo 100	0.304	22.5	166	0	pCIneo 100	0.662	28.5	171	0	pCIneo 100	0.117	26
162	0	pCIneo 100	0.245	27	167	0	pCIneo 100	0.152	26.5	172	0	pCIneo 100	1.006	23
163	0	pCIneo 100	0.755	34	168	0	pCIneo 100	0.157	21.5	173	0	pCIneo 100	0.404	26
164	0	pCIneo 100	0.545	27	169	0	pCIneo 100	0.506	28.5	174	0	pCIneo 100	0.383	31.5
165	0	pCIneo 100	0.336	30	170	0	pCIneo 100	0.342	21	175	0	pCIneo 100	0.252	29.5

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	TIME	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
176	0	pCIneo 100	0.205	22.5	216	0	pCIneo 1200	0.17	24.5	179	3	pCIneo 100	0.534	-9
177	0	pCIneo 100	0.359	22.5	217	0	pCIneo 1200	0.98	22.5	180	3	pCIneo 100	0.383	-9
178	0	pCIneo 100	0.556	29	218	0	pCIneo 1200	0.255	29.5	181	3	pCIneo 600	0.382	-9
179	0	pCIneo 100	0.356	30.5	219	0	pCIneo 1200	0.954	25.5	182	3	pCIneo 600	0.684	-9
180	0	pCIneo 100	0.365	23.5	220	0	pCIneo 1200	0.556	29	183	3	pCIneo 600	1.113	-9.00
181	0	pCIneo 600	0.396	24.5	141	0	control	0.829	29.5	184	3	pCIneo 600	0.682	-9.00
182	0	pCIneo 600	0.481	28.5	142	0	control	0.448	32.5	185	3	pCIneo 600	0.380	-9.00
183	0	pCIneo 600	0.598	27.5	143	0	control	0.759	24.5	186	3	pCIneo 600	0.331	-9.00
184	0	pCIneo 600	0.825	33.5	144	0	control	0.967	30	187	3	pCIneo 600	0.790	-9.00
185	0	pCIneo 600	0.421	22.5	146	0	control	0.251	31	188	3	pCIneo 600	0.191	-9.00
186	0	pCIneo 600	0.322	21.5	147	0	control	0.496	29.5	189	3	pCIneo 600	0.417	-9.00
187	0	pCIneo 600	0.556	26	149	0	control	0.98	29.5	190	3	pCIneo 600	0.247	-9.00
188	0	pCIneo 600	0.281	21	150	0	control	0.196	26	191	3	pCIneo 600	0.396	-9.00
189	0	pCIneo 600	0.328	26.5	151	0	control	0.328	26	192	3	pCIneo 600	0.662	-9.00
190	0	pCIneo 600	0.372	26	152	0	control	0.157	21	193	3	pCIneo 600	0.326	-9.00
191	0	pCIneo 600	0.428	31.5	153	0	control	0.249	27.5	194	3	pCIneo 600	1.108	-9.00
192	0	pCIneo 600	0.43	24	154	0	control	0.725	26	195	3	pCIneo 600	0.635	-9.00
193	0	pCIneo 600	0.457	32.5	155	0	control	0.461	33.5	196	3	pCIneo 600	0.400	-9.00
194	0	pCIneo 600	1.534	29	156	0	control	0.266	29.5	197	3	pCIneo 600	0.494	-9.00
195	0	pCIneo 600	0.982	22	157	0	control	0.919	22.5	198	3	pCIneo 600	0.552	-9.00
196	0	pCIneo 600	0.429	23.5	158	0	control	0.184	25	199	3	pCIneo 600	1.291	-9.00
197	0	pCIneo 600	0.905	19.5	160	0	control	0.731	30	200	3	pCIneo 600	0.537	-9.00
198	0	pCIneo 600	0.611	26	161	3	pCIneo 100	0.206	-9	201	3	pCIneo 1200	0.508	-9.00
199	0	pCIneo 600	1.153	25.5	162	3	pCIneo 100	0.196	-9	202	3	pCIneo 1200	0.336	-9.00
200	0	pCIneo 600	0.721	28	163	3	pCIneo 100	0.509	-9	203	3	pCIneo 1200	0.427	-9.00
201	0	pCIneo 1200	0.968	27.5	164	3	pCIneo 100	0.299	-9	204	3	pCIneo 1200	0.197	-9.00
202	0	pCIneo 1200	0.381	30.5	165	3	pCIneo 100	0.291	-9	205	3	pCIneo 1200	0.545	-9.00
203	0	pCIneo 1200	0.325	30	166	3	pCIneo 100	0.449	-9	206	3	pCIneo 1200	0.465	-9.00
204	0	pCIneo 1200	0.273	24.5	167	3	pCIneo 100	0.107	-9	207	3	pCIneo 1200	0.340	-9.00
205	0	pCIneo 1200	0.607	28.5	168	3	pCIneo 100	0.173	-9	208	3	pCIneo 1200	0.454	-9.00
206	0	pCIneo 1200	1.095	26	169	3	pCIneo 100	0.395	-9	209	3	pCIneo 1200	0.410	-9.00
207	0	pCIneo 1200	0.286	19.5	170	3	pCIneo 100	0.422	-9	210	3	pCIneo 1200	0.313	-9.00
208	0	pCIneo 1200	0.371	32.5	171	3	pCIneo 100	0.206	-9	211	3	pCIneo 1200	0.929	-9.00
209	0	pCIneo 1200	0.694	29	172	3	pCIneo 100	0.76	-9	212	3	pCIneo 1200	0.660	-9.00
210	0	pCIneo 1200	0.402	31	173	3	pCIneo 100	0.3	-9	213	3	pCIneo 1200	0.635	-9.00
211	0	pCIneo 1200	1.313	28	174	3	pCIneo 100	0.406	-9	214	3	pCIneo 1200	0.485	-9.00
212	0	pCIneo 1200	1.075	25.5	175	3	pCIneo 100	0.175	-9	215	3	pCIneo 1200	0.388	-9.00
213	0	pCIneo 1200	0.823	25	176	3	pCIneo 100	0.158	-9	216	3	pCIneo 1200	0.213	-9.00
214	0	pCIneo 1200	0.518	25.5	177	3	pCIneo 100	0.546	-9	217	3	pCIneo 1200	0.678	-9.00
215	0	pCIneo 1200	0.465	21	178	3	pCIneo 100	0.406	-9	218	3	pCIneo 1200	0.678	-9.00

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
219	3	pCIneo 1200	0.704	-9.00	184	6	pCIneo 600	0.735	49.50	147	6	control	-9.000	49.00
220	3	pCIneo 1200	0.500	-9.00	185	6	pCIneo 600	0.396	35.00	149	6	control	0.762	46.00
141	3	control	0.796	-9.00	186	6	pCIneo 600	-9.000	-9.00	150	6	control	0.515	44.50
142	3	control	0.155	-9.00	187	6	pCIneo 600	1.187	42.00	151	6	control	0.722	34.50
143	3	control	0.411	-9.00	188	6	pCIneo 600	0.438	39.00	152	6	control	0.500	46.00
144	3	control	0.760	-9.00	189	6	pCIneo 600	0.227	46.50	153	6	control	0.381	52.50
146	3	control	0.529	-9.00	190	6	pCIneo 600	0.307	42.50	154	6	control	0.758	46.50
147	3	control	0.627	-9.00	191	6	pCIneo 600	0.446	56.50	155	6	control	0.483	54.00
149	3	control	0.527	-9.00	192	6	pCIneo 600	0.472	34.00	156	6	control	0.220	46.50
150	3	control	0.489	-9.00	193	6	pCIneo 600	0.569	50.00	157	6	control	1.314	37.50
151	3	control	1.284	-9.00	194	6	pCIneo 600	0.968	50.50	158	6	control	0.501	40.50
152	3	control	0.397	-9.00	195	6	pCIneo 600	0.570	38.50	160	6	control	0.385	53.00
153	3	control	0.348	-9.00	196	6	pCIneo 600	0.516	33.50	161	9	pCIneo 100	1.209	41.00
154	3	control	0.616	-9.00	197	6	pCIneo 600	0.601	38.50	162	9	pCIneo 100	0.574	49.50
155	3	control	0.489	-9.00	198	6	pCIneo 600	0.851	41.50	163	9	pCIneo 100	1.042	54.00
156	3	control	0.234	-9.00	199	6	pCIneo 600	-9.000	-9.00	164	9	pCIneo 100	2.257	46.00
157	3	control	1.050	-9.00	200	6	pCIneo 600	1.068	40.50	165	9	pCIneo 100	1.296	48.00
158	3	control	0.156	-9.00	201	6	pCIneo 1200	0.552	51.50	166	9	pCIneo 100	0.642	45.50
160	3	control	0.263	-9.00	202	6	pCIneo 1200	0.446	49.00	167	9	pCIneo 100	1.696	52.00
161	6	pCIneo 100	0.373	42.50	203	6	pCIneo 1200	0.471	51.50	168	9	pCIneo 100	-9.000	-9.00
162	6	pCIneo 100	0.266	47.00	204	6	pCIneo 1200	0.258	36.50	169	9	pCIneo 100	0.529	44.00
163	6	pCIneo 100	0.922	50.00	205	6	pCIneo 1200	0.902	40.50	170	9	pCIneo 100	2.195	34.50
164	6	pCIneo 100	1.470	44.00	206	6	pCIneo 1200	0.723	46.00	171	9	pCIneo 100	0.451	47.00
165	6	pCIneo 100	1.484	48.00	207	6	pCIneo 1200	1.366	33.00	172	9	pCIneo 100	2.299	37.50
166	6	pCIneo 100	0.328	45.50	208	6	pCIneo 1200	0.605	50.00	173	9	pCIneo 100	0.731	43.00
167	6	pCIneo 100	0.134	51.50	209	6	pCIneo 1200	0.550	46.00	174	9	pCIneo 100	1.378	45.00
168	6	pCIneo 100	0.313	38.00	210	6	pCIneo 1200	0.289	51.00	175	9	pCIneo 100	0.534	48.00
169	6	pCIneo 100	0.622	45	211	6	pCIneo 1200	0.771	45.00	176	9	pCIneo 100	0.881	39.50
170	6	pCIneo 100	0.653	34.00	212	6	pCIneo 1200	1.426	47.00	177	9	pCIneo 100	0.844	37.50
171	6	pCIneo 100	0.338	44.00	213	6	pCIneo 1200	0.570	44.50	178	9	pCIneo 100	1.772	44.50
172	6	pCIneo 100	0.831	33.50	214	6	pCIneo 1200	0.383	47.00	179	9	pCIneo 100	0.992	47.00
173	6	pCIneo 100	0.254	47.50	215	6	pCIneo 1200	0.456	44.00	180	9	pCIneo 100	1.631	49.00
174	6	pCIneo 100	0.922	43.00	216	6	pCIneo 1200	0.356	44.50	181	9	pCIneo 600	0.371	41.50
175	6	pCIneo 100	0.237	47.00	217	6	pCIneo 1200	0.910	44.50	182	9	pCIneo 600	0.600	49.00
176	6	pCIneo 100	0.385	37.50	218	6	pCIneo 1200	0.839	52.50	183	9	pCIneo 600	2.261	43.50
177	6	pCIneo 100	0.626	38.50	219	6	pCIneo 1200	0.936	43.00	184	9	pCIneo 600	0.966	46.00
178	6	pCIneo 100	0.386	44.50	220	6	pCIneo 1200	0.366	49.00	185	9	pCIneo 600	0.772	34.00
179	6	pCIneo 100	-9.000	42.00	141	6	control	0.892	49.00	186	9	pCIneo 600	-9.000	-9.00
180	6	pCIneo 100	0.773	41.50	142	6	control	0.222	47.00	187	9	pCIneo 600	1.220	44.00
181	6	pCIneo 600	0.421	38.50	143	6	control	0.434	30.00	188	9	pCIneo 600	0.615	38.00
182	6	pCIneo 600	0.617	48.00	144	6	control	0.568	46.50	189	9	pCIneo 600	0.965	48.00
183	6	pCIneo 600	1.113	44.50	146	6	control	0.450	44.00	190	9	pCIneo 600	0.587	44.00

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
191	9	pCIneo 600	1.314	52.00	207	9	pCIneo 1200	0.592	33.50	155	9	control	0.883	53.50
192	9	pCIneo 600	1.952	33.50	208	9	pCIneo 1200	0.886	49.50	156	9	control	0.416	40.50
193	9	pCIneo 600	0.446	48.50	209	9	pCIneo 1200	1.347	45.50	157	9	control	1.187	40.00
194	9	pCIneo 600	1.642	49.00	210	9	pCIneo 1200	0.686	50.50	158	9	control	1.380	43.50
195	9	pCIneo 600	1.131	43.50	141	9	control	1.145	43.50	160	9	control	0.450	53.50
196	9	pCIneo 600	-9.000	-9.00	142	9	control	0.304	46.00	211	9	pCIneo 1200	1.511	46.50
197	9	pCIneo 600	-9.000	-9.00	143	9	control	0.648	34.00	212	9	pCIneo 1200	-9.000	-9.00
198	9	pCIneo 600	1.594	38.50	144	9	control	0.796	40.00	213	9	pCIneo 1200	1.343	42.00
199	9	pCIneo 600	1.450	43.50	146	9	control	1.390	45.00	214	9	pCIneo 1200	0.884	46.00
200	9	pCIneo 600	1.318	47.00	147	9	control	0.834	47.50	215	9	pCIneo 1200	0.496	44.00
201	9	pCIneo 1200	1.427	48.00	149	9	control	0.913	46.50	216	9	pCIneo 1200	0.325	41.50
202	9	pCIneo 1200	0.701	49.50	150	9	control	0.715	42.00	217	9	pCIneo 1200	1.062	44.50
203	9	pCIneo 1200	1.365	53.50	151	9	control	0.763	31.50	218	9	pCIneo 1200	0.787	50.50
204	9	pCIneo 1200	0.679	37.00	152	9	control	0.859	50.50	219	9	pCIneo 1200	1.657	47.00
205	9	pCIneo 1200	1.543	44.50	153	9	control	0.772	54.00	220	9	pCIneo 1200	0.571	45.50
206	9	pCIneo 1200	1.316	43.50	154	9	control	0.787	43.50					

*Ostrich-number of ostrich; TRT- Treatment received; Time- Time in weeks; RESP- Titre response; Weight- weight of specific ostrich

ANALYSIS OF VARIANCE

VR1020 1:100, 2015

Variable: Response to DNA vaccine VR1020_*oppA* (1:100)

Source	Df	SS	MS	F-value	Pr>F
Total	299	117.577			
FAC_A	3	6.545	2.182	11.54	0.0000
FAC_B	3	47.351	15.784	83.45	0.0000
FAC_A by FAC_B	9	9.966	1.107	5.85	0.0000
Residual	284	53.715	0.189		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 0.808	R-squared = 0.5432	C.V. = 53.82%

C.V = coefficient of variance

LSD for FAC_A = 0.1398	S.E.D = 0.0710	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.18914

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
2	1.01	79.8	1 VR1020 600
3	0.88	76.1	2 VR1020 1200
1	0.72	77.6	3 VR1020 100
4	0.62	51.3	4 Control

LSD for FAC_B = 0.1398	S.E.D = 0.0710	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.18914

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	1.05	52.1	1 Week 9
6	0.63	45.8	2 Week 6
0	0.58	60.8	3 Week 3
3	0.52	67.9	4

LSD for FAC_A*FAC_B = 0.2796	S.E.D = 0.1420	r = 18.8
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.18914

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.464	0.000	0.000	0.564	0.000	0.000	1.319
2	0.000	0.000	0.700	0.000	0.000	0.737	0.000	0.000	2.078
3	0.000	0.000	0.638	0.000	0.000	0.666	0.000	0.000	1.751
4	0.000	0.000	0.538	0.000	0.000	0.568	0.000	0.000	0.838

ANALYSIS OF VARIANCE

VR1020 1:100, 2015

Variable: Weight

Source	df	SS	MS	F-value	Pr>F
Total	224	21669.723			
FAC_A	3	420.716	140.239	5.91	0.0007
FAC_B	2	16075.611	8037.806	338.90	0.0000
FAC_A by FAC_B	6	121.659	20.277	0.85	0.5290
Residual	213	5051.736	23.717		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 38.814	R-squared = 0.7669	C.V. = 12.55%

C.V = coefficient of variance

LSD for FAC_A = 1.8101	S.E.D = 0.9203	r = 56.0
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
1	40.88	27.0	1 VR1020 100
4	39.13	24.9	2 Control
3	37.81	25.0	3 VR1020 1200
2	37.44	24.2	4 VR1020 600

LSD for FAC_B = 1.5676	S.E.D = 0.7953	r = 75.0
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
6	45.04	11.7	1 Week 6
9	44.52	13.3	2 Week 9
0	26.88	13.4	3

LSD for FAC_A*FAC_B = 3.1352	S.E.D = 1.5905	r = 18.8
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.000	0.000	0.000	47.875	0.000	0.000	47.237
2	0.000	0.000	0.000	0.000	0.000	42.556	0.000	0.000	43.583
3	0.000	0.000	0.000	0.000	0.000	44.625	0.000	0.000	42.850
4	0.000	0.000	0.000	0.000	0.000	45.118	0.000	0.000	44.412

Data used for statistical analysis of the VR1020_*oppA* vaccine results using a 1:100 serum dilution factor. The data is arranged in columns, each containing the ostrich number, treatment received (trt), time (in weeks), response obtained from ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right.

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
221	0	VR1020 100	0.293	31.50	244	0	VR1020 600	0.485	28.00	268	0	VR1020 1200	0.424	27.50
222	0	VR1020 100	0.644	29.00	245	0	VR1020 600	0.311	32.00	269	0	VR1020 1200	0.524	29.00
223	0	VR1020 100	0.571	27.50	246	0	VR1020 600	0.200	24.50	270	0	VR1020 1200	0.272	23.50
224	0	VR1020 100	1.008	25.00	247	0	VR1020 600	1.203	24.50	271	0	VR1020 1200	0.959	28.50
225	0	VR1020 100	0.424	33.00	248	0	VR1020 600	0.324	28.00	272	0	VR1020 1200	0.401	26.00
226	0	VR1020 100	0.419	27.50	249	0	VR1020 600	0.725	24.00	273	0	VR1020 1200	0.310	23.00
227	0	VR1020 100	0.787	22.00	250	0	VR1020 600	0.436	15.00	274	0	VR1020 1200	0.490	26.50
228	0	VR1020 100	0.256	29.50	251	0	VR1020 600	0.503	28.00	275	0	VR1020 1200	1.754	19.50
229	0	VR1020 100	0.372	18.50	252	0	VR1020 600	0.709	26.50	276	0	VR1020 1200	0.363	23.50
230	0	VR1020 100	0.288	26.00	253	0	VR1020 600	1.020	32.00	277	0	VR1020 1200	0.098	32.00
231	0	VR1020 100	0.244	24.00	254	0	VR1020 600	0.320	26.00	278	0	VR1020 1200	0.174	26.50
232	0	VR1020 100	1.994	25.00	255	0	VR1020 600	1.230	24.00	279	0	VR1020 1200	0.275	23.50
233	0	VR1020 100	0.175	25.00	256	0	VR1020 600	0.285	25.50	280	0	VR1020 1200	0.203	21.00
234	0	VR1020 100	0.230	31.00	257	0	VR1020 600	0.190	24.50	141	0	control	0.829	29.50
235	0	VR1020 100	0.416	29.00	258	0	VR1020 600	0.676	25.50	142	0	control	0.448	32.50
236	0	VR1020 100	0.140	28.50	260	0	VR1020 600	0.256	27.50	143	0	control	0.759	24.50
237	0	VR1020 100	0.700	28.00	261	0	VR1020 1200	0.499	28.00	144	0	control	0.967	30.00
238	0	VR1020 100	0.493	32.00	262	0	VR1020 1200	0.876	33.00	146	0	control	0.251	31.00
239	0	VR1020 100	0.731	28.50	263	0	VR1020 1200	0.284	25.50	147	0	control	0.496	29.50
240	0	VR1020 100	0.598	30.00	264	0	VR1020 1200	0.135	24.50	149	0	control	0.980	29.50
241	0	VR1020 600	0.621	27.50	265	0	VR1020 1200	0.556	27.00	150	0	control	0.196	26.00
242	0	VR1020 600	0.250	30.50	266	0	VR1020 1200	0.407	29.50	151	0	control	0.328	26.00
243	0	VR1020 600	0.502	24.00	267	0	VR1020 1200	0.261	21.50	152	0	control	0.157	21.00

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
153	0	control	0.249	27.50	256	3	VR1020 600	0.248	-9.00	222	6	VR1020 100	0.510	48.00
154	0	control	0.725	26.00	257	3	VR1020 600	0.303	-9.00	223	6	VR1020 100	1.191	44.00
155	0	control	0.461	33.50	258	3	VR1020 600	0.698	-9.00	224	6	VR1020 100	1.483	43.50
156	0	control	0.266	29.50	260	3	VR1020 600	0.770	-9.00	225	6	VR1020 100	0.460	55.50
157	0	control	0.919	22.50	261	3	VR1020 1200	1.956	-9.00	226	6	VR1020 100	0.490	47.00
158	0	control	0.184	25.00	262	3	VR1020 1200	0.645	-9.00	227	6	VR1020 100	0.607	41.00
160	0	control	0.731	30.00	263	3	VR1020 1200	0.275	-9.00	228	6	VR1020 100	0.250	53.50
221	3	VR1020 100	0.332	-9.00	264	3	VR1020 1200	0.404	-9.00	229	6	VR1020 100	0.511	35.00
222	3	VR1020 100	0.777	-9.00	265	3	VR1020 1200	0.785	-9.00	230	6	VR1020 100	0.210	45.00
223	3	VR1020 100	0.974	-9.00	266	3	VR1020 1200	0.548	-9.00	231	6	VR1020 100	0.485	44.50
224	3	VR1020 100	0.499	-9.00	267	3	VR1020 1200	0.210	-9.00	232	6	VR1020 100	0.625	44.50
225	3	VR1020 100	0.346	-9.00	268	3	VR1020 1200	0.398	-9.00	233	6	VR1020 100	0.215	45.50
226	3	VR1020 100	0.385	-9.00	269	3	VR1020 1200	0.676	-9.00	234	6	VR1020 100	0.286	53.00
227	3	VR1020 100	0.578	-9.00	270	3	VR1020 1200	0.309	-9.00	235	6	VR1020 100	0.765	49.00
228	3	VR1020 100	0.181	-9.00	271	3	VR1020 1200	0.797	-9.00	236	6	VR1020 100	0.538	51.50
229	3	VR1020 100	0.484	-9.00	272	3	VR1020 1200	1.183	-9.00	237	6	VR1020 100	0.396	53.50
230	3	VR1020 100	0.254	-9.00	273	3	VR1020 1200	0.533	-9.00	238	6	VR1020 100	0.709	47.00
231	3	VR1020 100	0.284	-9.00	274	3	VR1020 1200	0.812	-9.00	239	6	VR1020 100	0.649	52.00
232	3	VR1020 100	0.921	-9.00	275	3	VR1020 1200	1.167	-9.00	240	6	VR1020 100	0.482	50.00
233	3	VR1020 100	0.219	-9.00	276	3	VR1020 1200	0.634	-9.00	241	6	VR1020 600	0.702	44.00
234	3	VR1020 100	0.580	-9.00	277	3	VR1020 1200	0.351	-9.00	242	6	VR1020 600	0.965	44.50
235	3	VR1020 100	0.595	-9.00	278	3	VR1020 1200	0.177	-9.00	243	6	VR1020 600	0.447	40.00
236	3	VR1020 100	0.155	-9.00	279	3	VR1020 1200	0.520	-9.00	244	6	VR1020 600	0.620	45.00
237	3	VR1020 100	0.460	-9.00	280	3	VR1020 1200	0.374	-9.00	245	6	VR1020 600	0.516	45.50
238	3	VR1020 100	0.498	-9.00	141	3	control	0.796	-9.00	246	6	VR1020 600	0.547	35.50
239	3	VR1020 100	0.402	-9.00	142	3	control	0.155	-9.00	247	6	VR1020 600	0.824	41.50
240	3	VR1020 100	0.349	-9.00	143	3	control	0.411	-9.00	248	6	VR1020 600	0.637	47.00
241	3	VR1020 600	0.480	-9.00	144	3	control	0.760	-9.00	249	6	VR1020 600	0.702	40.00
242	3	VR1020 600	0.961	-9.00	146	3	control	0.529	-9.00	250	6	VR1020 600	-9.000	-9.00
243	3	VR1020 600	0.348	-9.00	147	3	control	0.627	-9.00	251	6	VR1020 600	0.680	40.50
244	3	VR1020 600	0.568	-9.00	149	3	control	0.527	-9.00	252	6	VR1020 600	0.767	50.00
245	3	VR1020 600	0.709	-9.00	150	3	control	0.489	-9.00	253	6	VR1020 600	1.227	41.00
246	3	VR1020 600	0.740	-9.00	151	3	control	1.284	-9.00	254	6	VR1020 600	0.770	38.50
247	3	VR1020 600	1.695	-9.00	152	3	control	0.397	-9.00	255	6	VR1020 600	1.007	41.50
248	3	VR1020 600	0.341	-9.00	153	3	control	0.348	-9.00	256	6	VR1020 600	0.276	44.00
249	3	VR1020 600	0.740	-9.00	154	3	control	0.616	-9.00	257	6	VR1020 600	0.526	41.00
250	3	VR1020 600	1.043	-9.00	155	3	control	0.489	-9.00	258	6	VR1020 600	1.336	41.00
251	3	VR1020 600	0.439	-9.00	156	3	control	0.234	-9.00	260	6	VR1020 600	0.688	45.50
252	3	VR1020 600	0.631	-9.00	157	3	control	1.050	-9.00	261	6	VR1020 1200	0.553	44.50
253	3	VR1020 600	0.552	-9.00	158	3	control	0.156	-9.00	262	6	VR1020 1200	0.700	51.00
254	3	VR1020 600	0.350	-9.00	160	3	control	0.263	-9.00	263	6	VR1020 1200	0.500	42.50
255	3	VR1020 600	1.687	-9.00	221	6	VR1020 100	0.396	54.50	264	6	VR1020 1200	0.986	48.00

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
265	6	VR1020 1200	0.394	44.00	225	9	VR1020 100	-9.000	-9.00	263	9	VR1020 1200	1.332	44.00
266	6	VR1020 1200	0.622	50.00	226	9	VR1020 100	1.566	45.50	264	9	VR1020 1200	2.554	43.50
267	6	VR1020 1200	0.367	42.00	227	9	VR1020 100	1.108	42.50	265	9	VR1020 1200	1.867	43.50
268	6	VR1020 1200	0.514	43.00	228	9	VR1020 100	0.821	54.00	266	9	VR1020 1200	2.525	45.00
269	6	VR1020 1200	0.797	45.00	229	9	VR1020 100	2.520	34.50	267	9	VR1020 1200	1.090	45.00
270	6	VR1020 1200	0.674	43.50	230	9	VR1020 100	0.528	43.00	268	9	VR1020 1200	1.376	38.00
271	6	VR1020 1200	0.626	49.00	231	9	VR1020 100	0.884	41.00	269	9	VR1020 1200	1.433	46.50
272	6	VR1020 1200	1.587	46.00	232	9	VR1020 100	1.501	47.50	270	9	VR1020 1200	1.614	44.50
273	6	VR1020 1200	0.680	41.50	233	9	VR1020 100	0.480	40.00	271	9	VR1020 1200	2.186	44.50
274	6	VR1020 1200	0.431	49.50	234	9	VR1020 100	1.046	52.50	272	9	VR1020 1200	2.844	42.00
275	6	VR1020 1200	1.056	38.50	235	9	VR1020 100	0.908	54.50	273	9	VR1020 1200	1.503	43.00
276	6	VR1020 1200	0.811	38.00	236	9	VR1020 100	2.414	46.00	274	9	VR1020 1200	1.545	46.00
277	6	VR1020 1200	0.551	54.50	237	9	VR1020 100	2.370	51.00	275	9	VR1020 1200	1.507	38.50
278	6	VR1020 1200	0.264	43.50	238	9	VR1020 100	1.256	43.50	276	9	VR1020 1200	2.400	37.50
279	6	VR1020 1200	0.464	42.00	239	9	VR1020 100	1.020	56.00	277	9	VR1020 1200	1.147	45.00
280	6	VR1020 1200	0.754	36.50	240	9	VR1020 100	1.088	54.50	278	9	VR1020 1200	0.680	39.00
141	6	control	0.892	49.00	241	9	VR1020 600	2.996	42.00	279	9	VR1020 1200	1.847	34.50
142	6	control	0.222	47.00	242	9	VR1020 600	2.940	37.00	280	9	VR1020 1200	2.620	39.00
143	6	control	0.434	30.00	243	9	VR1020 600	0.812	42.50	141	9	control	1.145	43.50
144	6	control	0.568	46.50	244	9	VR1020 600	2.314	42.50	142	9	control	0.304	46.00
146	6	control	0.450	44.00	245	9	VR1020 600	2.934	49.00	143	9	control	0.648	34.00
147	6	control	-9.000	49.00	246	9	VR1020 600	2.975	34.00	144	9	control	0.796	40.00
149	6	control	0.762	46.00	247	9	VR1020 600	2.379	48.00	146	9	control	1.390	45.00
150	6	control	0.515	44.50	248	9	VR1020 600	0.950	45.00	147	9	control	0.834	47.50
151	6	control	0.722	34.50	249	9	VR1020 600	2.503	41.00	149	9	control	0.913	46.50
152	6	control	0.500	46.00	250	9	VR1020 600	-9.000	-9.00	150	9	control	0.715	42.00
153	6	control	0.381	52.50	251	9	VR1020 600	1.375	37.50	151	9	control	0.763	31.50
154	6	control	0.758	46.50	252	9	VR1020 600	1.692	54.00	152	9	control	0.859	50.50
155	6	control	0.483	54.00	253	9	VR1020 600	2.922	47.00	153	9	control	0.772	54.00
156	6	control	0.220	46.50	254	9	VR1020 600	1.217	42.50	154	9	control	0.787	43.50
157	6	control	1.314	37.50	255	9	VR1020 600	1.980	44.00	155	9	control	0.883	53.50
158	6	control	0.501	40.50	256	9	VR1020 600	1.024	45.50	156	9	control	0.416	40.50
160	6	control	0.385	53.00	257	9	VR1020 600	0.692	46.50	157	9	control	1.187	40.00
221	9	VR1020 100	0.498	63.00	258	9	VR1020 600	3.066	41.00	158	9	control	1.380	43.50
222	9	VR1020 100	1.008	48.00	260	9	VR1020 600	2.639	45.50	160	9	control	0.450	53.50
223	9	VR1020 100	2.669	44.00	261	9	VR1020 1200	0.977	42.50					
224	9	VR1020 100	1.364	36.50	262	9	VR1020 1200	1.962	55.50					

*Ostrich-number of ostrich; TRT- Treatment received; Time- Time in weeks; RESP- Titre response; Weight- weight of specific ostrich

pCI-neo 1:500, 2015

Variable: Response to DNA vaccine pCI-neo_*oppA* (1:500)

Source	df	SS	MS	F-value	Pr>F
Total	299	8.285			
FAC_A	3	0.488	0.163	7.56	0.0001
FAC_B	3	1.440	0.480	22.28	0.0000
FAC_A by FAC_B	9	0.238	0.026	1.23	0.2774
Residual	284	6.119	0.022		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 0.170	R-squared = 0.2615	C.V. = 86.11%

C.V = coefficient of variance

LSD for FAC_A = 0.0472	S.E.D = 0.0240	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.02154

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
1	0.22	98.6	1 pCIneo 100
3	0.20	94.2	2 pCIneo 1200
2	0.13	83.5	3 pCIneo 60
4	0.13	72.3	4 Control

LSD for FAC_B = 0.0472	S.E.D = 0.0240	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.02154

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	0.29	87.2	1 Week 9
6	0.14	83.5	2 Week 6
3	0.13	55.0	3 Week 3
0	0.12	72.1	4

LSD for FAC_A*FAC_B = 0.0944	S.E.D = 0.0479	r = 18.8
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.02154

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.159	0.000	0.000	0.197	0.000	0.000	0.394
2	0.000	0.000	0.111	0.000	0.000	0.114	0.000	0.000	0.206
3	0.000	0.000	0.129	0.000	0.000	0.155	0.000	0.000	0.363
4	0.000	0.000	0.114	0.000	0.000	0.105	0.000	0.000	0.203

ANALYSIS OF VARIANCE

pCIneo 1:500, 2015

Variable: Weight

Source	df	SS	MS	F-value	Pr>F
Total	223	20685.410			
FAC_A	3	71.845	23.948	0.96	0.4111
FAC_B	2	15302.793	7651.397	307.65	0.0000
FAC_A by FAC_B	6	38.214	6.369	0.26	0.9564
Residual	212	5272.558	24.871		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 38.490	R-squared = 0.7451	C.V. = 12.96%

C.V = coefficient of variance

LSD for FAC_A = 1.8578	S.E.D = 0.9425	r = 56.0
T(2-sided a=0.050, 212 df) = 1.9712		MSE = 24.87055

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
4	39.13	24.9	1 Control
3	38.97	24.5	2 pCIneo 1200
1	38.21	25.0	3 pCIneo 100
2	37.65	27.9	4 pCIneo 600

LSD for FAC_B = 1.6089	S.E.D = 0.8199	r = 74.0
T(2-sided a=0.050, 212 df) = 1.9712		MSE = 24.87055

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	44.60	11.9	1 Week 9
6	44.10	12.9	2 Week 6
0	26.77	13.4	3

LSD for FAC_A*FAC_B = 3.2178	S.E.D = 1.6324	r = 18.7
T(2-sided a=0.050, 212 df) = 1.9712		MSE = 24.87055

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.000	0.000	0.000	43.225	0.000	0.000	44.868
2	0.000	0.000	0.000	0.000	0.000	43.273	0.000	0.000	43.735
3	0.000	0.000	0.000	0.000	0.000	44.778	0.000	0.000	45.395
4	0.000	0.000	0.000	0.000	0.000	45.118	0.000	0.000	44.412

Data used for statistical analysis of the pCI-neo_*oppA* vaccine results using a 1:500 serum dilution factor. The data is arranged in columns, each containing the ostrich number, treatment received (trt), time (in weeks), response obtained from ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
161	0	pCIneo 100	0.225	22.5	175	0	pCIneo 100	0.084	29.5	197	0	pCIneo 600	0.082	19.5
162	0	pCIneo 100	0.172	27	176	0	pCIneo 100	0.056	22.5	198	0	pCIneo 600	0.339	26
163	0	pCIneo 100	0.39	34	177	0	pCIneo 100	0.05	22.5	199	0	pCIneo 600	0.17	25.5
164	0	pCIneo 100	0.179	27	178	0	pCIneo 100	0.058	29	200	0	pCIneo 600	0.138	28
165	0	pCIneo 100	0.147	30	179	0	pCIneo 100	0.124	30.5	201	0	pCIneo 1200	0.153	27.5
166	0	pCIneo 100	0.138	28.5	180	0	pCIneo 100	0.097	23.5	202	0	pCIneo 1200	0.023	30.5
167	0	pCIneo 100	0.148	26.5	181	0	pCIneo 600	0.08	24.5	211	0	pCIneo 1200	0.376	28
168	0	pCIneo 100	0.178	21.5	190	0	pCIneo 600	0.032	26	212	0	pCIneo 1200	0.319	25.5
169	0	pCIneo 100	0.296	28.5	191	0	pCIneo 600	0.055	31.5	213	0	pCIneo 1200	0.149	25
170	0	pCIneo 100	0.012	21	192	0	pCIneo 600	0.055	24	214	0	pCIneo 1200	0.145	25.5
171	0	pCIneo 100	0.092	26	193	0	pCIneo 600	0.075	32.5	215	0	pCIneo 1200	0.049	21
172	0	pCIneo 100	0.114	23	194	0	pCIneo 600	0.192	29	216	0	pCIneo 1200	0.043	24.5
173	0	pCIneo 100	0.115	26	195	0	pCIneo 600	0.075	22	217	0	pCIneo 1200	0.098	22.5
174	0	pCIneo 100	0.149	31.5	196	0	pCIneo 600	0.019	23.5	218	0	pCIneo 1200	0.054	29.5

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
219	0	pClneo 1200	0.013	25.5	184	3	pClneo 600	0.125	-9.00	147	3	control	0.097	-9.00
220	0	pClneo 1200	0.195	29	185	3	pClneo 600	0.084	-9.00	149	3	control	0.092	-9.00
141	0	control	0.135	29.5	186	3	pClneo 600	0.080	-9.00	150	3	control	0.076	-9.00
142	0	control	0.122	32.5	187	3	pClneo 600	0.219	-9.00	151	3	control	0.288	-9.00
143	0	control	0.161	24.5	188	3	pClneo 600	0.046	-9.00	152	3	control	0.158	-9.00
144	0	control	0.265	30.00	189	3	pClneo 600	0.135	-9.00	153	3	control	0.061	-9.00
146	0	control	0.042	31.00	190	3	pClneo 600	0.073	-9.00	154	3	control	0.089	-9.00
147	0	control	0.054	29.50	191	3	pClneo 600	0.069	-9.00	155	3	control	0.073	-9.00
149	0	control	0.141	29.50	192	3	pClneo 600	0.073	-9.00	156	3	control	0.056	-9.00
150	0	control	0.048	26.00	193	3	pClneo 600	0.062	-9.00	157	3	control	0.203	-9.00
151	0	control	0.044	26.00	194	3	pClneo 600	0.162	-9.00	158	3	control	0.039	-9.00
152	0	control	0.047	21.00	195	3	pClneo 600	0.046	-9.00	160	3	control	0.048	-9.00
153	0	control	0.034	27.50	196	3	pClneo 600	0.036	-9.00	161	6	pClneo 100	0.263	42.50
154	0	control	0.074	26.00	197	3	pClneo 600	0.082	-9.00	162	6	pClneo 100	0.244	47.00
155	0	control	0.094	33.50	198	3	pClneo 600	0.092	-9.00	163	6	pClneo 100	0.383	50.00
156	0	control	0.051	29.50	199	3	pClneo 600	0.119	-9.00	164	6	pClneo 100	0.555	44.00
157	0	control	0.162	22.50	200	3	pClneo 600	0.174	-9.00	165	6	pClneo 100	0.290	48.00
158	0	control	0.043	25.00	201	3	pClneo 1200	0.073	-9.00	166	6	pClneo 100	0.139	45.50
160	0	control	0.047	30.00	202	3	pClneo 1200	0.103	-9.00	167	6	pClneo 100	0.127	51.50
161	3	pClneo 100	0.172	-9.00	203	3	pClneo 1200	0.148	-9.00	168	6	pClneo 100	0.102	38.00
162	3	pClneo 100	0.267	-9.00	204	3	pClneo 1200	0.095	-9.00	169	6	pClneo 100	0.155	45
163	3	pClneo 100	0.274	-9.00	205	3	pClneo 1200	0.129	-9.00	170	6	pClneo 100	0.108	34.00
164	3	pClneo 100	0.234	-9.00	206	3	pClneo 1200	0.162	-9.00	171	6	pClneo 100	0.211	44.00
165	3	pClneo 100	0.182	-9.00	207	3	pClneo 1200	0.109	-9.00	172	6	pClneo 100	0.118	33.50
166	3	pClneo 100	0.209	-9.00	208	3	pClneo 1200	0.104	-9.00	173	6	pClneo 100	0.164	47.50
167	3	pClneo 100	0.188	-9.00	209	3	pClneo 1200	0.187	-9.00	174	6	pClneo 100	0.421	43.00
168	3	pClneo 100	0.043	-9.00	210	3	pClneo 1200	0.173	-9.00	175	6	pClneo 100	0.087	47.00
169	3	pClneo 100	0.250	-9.00	211	3	pClneo 1200	0.376	-9.00	176	6	pClneo 100	0.051	37.50
170	3	pClneo 100	0.228	-9.00	212	3	pClneo 1200	0.178	-9.00	177	6	pClneo 100	0.117	38.50
171	3	pClneo 100	0.164	-9.00	213	3	pClneo 1200	0.171	-9.00	178	6	pClneo 100	0.106	44.50
172	3	pClneo 100	0.061	-9.00	214	3	pClneo 1200	0.112	-9.00	179	6	pClneo 100	0.116	42.00
173	3	pClneo 100	0.133	-9.00	215	3	pClneo 1200	0.031	-9.00	180	6	pClneo 100	0.169	41.50
174	3	pClneo 100	0.156	-9.00	216	3	pClneo 1200	0.059	-9.00	181	6	pClneo 600	0.102	38.50
175	3	pClneo 100	0.106	-9.00	217	3	pClneo 1200	0.054	-9.00	182	6	pClneo 600	0.063	48.00
176	3	pClneo 100	0.062	-9.00	218	3	pClneo 1200	0.136	-9.00	183	6	pClneo 600	0.278	44.50
177	3	pClneo 100	0.126	-9.00	219	3	pClneo 1200	0.089	-9.00	184	6	pClneo 600	0.136	49.50
178	3	pClneo 100	0.090	-9.00	220	3	pClneo 1200	0.093	-9.00	185	6	pClneo 600	0.111	35.00
179	3	pClneo 100	0.087	-9.00	141	3	control	0.138	-9.00	186	6	pClneo 600	-9.000	-9.00
180	3	pClneo 100	0.139	-9.00	142	3	control	0.070	-9.00	187	6	pClneo 600	0.182	42.00
181	3	pClneo 600	0.126	-9.00	143	3	control	0.136	-9.00	188	6	pClneo 600	0.037	39.00
182	3	pClneo 600	0.120	-9.00	144	3	control	0.228	-9.00	189	6	pClneo 600	0.072	46.50
183	3	pClneo 600	0.289	-9.00	146	3	control	0.066	-9.00	190	6	pClneo 600	0.099	42.50

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
191	6	pClneo 600	0.048	56.50	154	6	control	0.121	46.50	196	9	pClneo 600	-9.000	-9.00
192	6	pClneo 600	0.118	34.00	155	6	control	0.071	54.00	197	9	pClneo 600	-9.000	-9.00
193	6	pClneo 600	0.049	50.00	156	6	control	0.098	46.50	198	9	pClneo 600	0.131	38.50
194	6	pClneo 600	0.059	50.50	157	6	control	0.243	37.50	199	9	pClneo 600	0.135	43.50
195	6	pClneo 600	0.076	38.50	158	6	control	0.054	40.50	200	9	pClneo 600	0.189	47.00
196	6	pClneo 600	0.065	33.50	160	6	control	0.044	53.00	201	9	pClneo 1200	0.201	48.00
197	6	pClneo 600	0.081	38.50	161	9	pClneo 100	0.795	41.00	202	9	pClneo 1200	0.102	49.50
198	6	pClneo 600	0.114	41.50	162	9	pClneo 100	0.273	49.50	203	9	pClneo 1200	0.256	53.50
199	6	pClneo 600	-9.000	-9.00	163	9	pClneo 100	1.005	54.00	204	9	pClneo 1200	0.232	37.00
200	6	pClneo 600	0.093	40.50	164	9	pClneo 100	0.719	46.00	205	9	pClneo 1200	0.439	44.50
201	6	pClneo 1200	0.085	51.50	165	9	pClneo 100	0.433	48.00	206	9	pClneo 1200	0.260	43.50
202	6	pClneo 1200	0.045	49.00	166	9	pClneo 100	0.188	45.50	207	9	pClneo 1200	0.340	33.50
203	6	pClneo 1200	0.203	51.50	167	9	pClneo 100	0.112	52.00	208	9	pClneo 1200	0.253	49.50
204	6	pClneo 1200	0.155	36.50	168	9	pClneo 100	-9.000	-9.00	209	9	pClneo 1200	0.570	45.50
205	6	pClneo 1200	0.098	40.50	169	9	pClneo 100	0.245	44.00	210	9	pClneo 1200	0.235	50.50
206	6	pClneo 1200	0.073	46.00	170	9	pClneo 100	0.128	34.50	211	9	pClneo 1200	0.448	46.50
207	6	pClneo 1200	0.612	33.00	171	9	pClneo 100	0.225	47.00	212	9	pClneo 1200	-9.000	-9.00
208	6	pClneo 1200	0.391	50.00	172	9	pClneo 100	0.131	37.50	213	9	pClneo 1200	0.448	42.00
209	6	pClneo 1200	0.201	46.00	173	9	pClneo 100	0.306	43.00	214	9	pClneo 1200	0.237	46.00
210	6	pClneo 1200	0.104	51.00	174	9	pClneo 100	1.409	45.00	215	9	pClneo 1200	0.588	44.00
211	6	pClneo 1200	0.144	45.00	175	9	pClneo 100	0.125	48.00	216	9	pClneo 1200	0.657	41.50
212	6	pClneo 1200	0.542	47.00	176	9	pClneo 100	0.090	39.50	217	9	pClneo 1200	0.121	44.50
213	6	pClneo 1200	0.263	44.50	177	9	pClneo 100	0.200	37.50	218	9	pClneo 1200	0.311	50.50
214	6	pClneo 1200	0.089	47.00	178	9	pClneo 100	0.191	44.50	219	9	pClneo 1200	0.034	47.00
215	6	pClneo 1200	0.063	44.00	179	9	pClneo 100	0.265	47.00	220	9	pClneo 1200	1.153	45.50
216	6	pClneo 1200	0.065	44.50	180	9	pClneo 100	0.643	49.00	141	9	control	0.204	43.50
217	6	pClneo 1200	0.058	44.50	181	9	pClneo 600	0.063	41.50	142	9	control	0.128	46.00
218	6	pClneo 1200	0.058	52.50	182	9	pClneo 600	0.138	49.00	143	9	control	0.238	34.00
219	6	pClneo 1200	0.052	43.00	183	9	pClneo 600	0.691	43.50	144	9	control	0.293	40.00
220	6	pClneo 1200	0.380	49.00	184	9	pClneo 600	0.246	46.00	146	9	control	0.246	45.00
141	6	control	0.174	49.00	185	9	pClneo 600	0.164	34.00	147	9	control	0.069	47.50
142	6	control	0.068	47.00	186	9	pClneo 600	-9.000	-9.00	149	9	control	0.239	46.50
143	6	control	0.185	30.00	187	9	pClneo 600	0.180	44.00	150	9	control	0.099	42.00
144	6	control	0.215	46.50	188	9	pClneo 600	0.073	38.00	151	9	control	0.136	31.50
146	6	control	0.048	44.00	189	9	pClneo 600	0.318	48.00	152	9	control	0.122	50.50
147	6	control	-9.000	49.00	190	9	pClneo 600	0.202	44.00	153	9	control	0.182	54.00
149	6	control	0.201	46.00	191	9	pClneo 600	0.152	52.00	154	9	control	0.180	43.50
150	6	control	0.060	44.50	192	9	pClneo 600	0.538	33.50	155	9	control	0.304	53.50
151	6	control	0.032	34.50	193	9	pClneo 600	0.071	48.50	156	9	control	0.159	40.50
152	6	control	0.052	46.00	194	9	pClneo 600	0.111	49.00	157	9	control	0.565	40.00
153	6	control	0.040	52.50	195	9	pClneo 600	0.116	43.50	158	9	control	0.197	43.50

*Ostrich	Time	TRT	RESP	Weight
160	9	control	0.082	53.50

*Ostrich-number of ostrich; TRT- Treatment received; Time- Time in weeks; RESP- Titre response; Weight- weight of specific ostrich

ANALYSIS OF VARIANCE

VR1020 1:500, 2015

Variable: Response to DNA vaccine VR1020_*oppA* (1:500)

Source	df	SS	MS	F-value	Pr>F
Total	299	50.836			
FAC_A	3	2.269	0.756	6.96	0.0002
FAC_B	3	12.918	4.306	39.60	0.0000
FAC_A by FAC_B	9	4.770	0.530	4.88	0.0000
Residual	284	30.879	0.109		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 0.248	R-squared = 0.3926	C.V. = 133.1%

C.V = coefficient of variance

LSD for FAC_A = 0.1060	S.E.D = 0.0538	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.10873

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
2	0.35	156.6	1 VR1020 600
3	0.31	172.3	2 VR1020 1200
1	0.20	119.9	3 VR1020 100
4	0.13	71.8	4 Control

LSD for FAC_B = 0.1060	S.E.D = 0.0538	r = 75.0
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.10873

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	0.61	113.7	1 Week 9
6	0.14	66.7	2 Week 6
0	0.13	73.9	3 Week 3
3	0.11	75.7	4

LSD for FAC_A*FAC_B = 0.2120	S.E.D = 0.1077	r = 18.8
T(2-sided a=0.050, 284 df) = 1.9684		MSE = 0.10873

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.117	0.000	0.000	0.132	0.000	0.000	0.427
2	0.000	0.000	0.159	0.000	0.000	0.139	0.000	0.000	0.997
3	0.000	0.000	0.142	0.000	0.000	0.166	0.000	0.000	0.810
4	0.000	0.000	0.114	0.000	0.000	0.105	0.000	0.000	0.203

ANALYSIS OF VARIANCE

VR1020 1:500, 2015

Variable: Weight

Source	Df	SS	MS	F-value	Pr>F
Total	224	21669.723			
FAC_A	3	420.716	140.239	5.91	0.0007
FAC_B	2	16075.611	8037.806	338.90	0.0000
FAC_A by FAC_B	6	121.659	20.277	0.85	0.5290
Residual	213	5051.736	23.717		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares.

Grand mean = 38.814	R-squared = 0.7669	C.V. = 12.55%

C.V = coefficient of variance

LSD for FAC_A = 1.8101	S.E.D = 0.9203	r = 56.0
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
1	40.88	27.0	1 VR1020 100
4	39.13	24.9	2 Control
3	37.81	25.0	3 VR1020 1200
2	37.44	24.2	4 VR1020 600

LSD for FAC_B = 1.5676	S.E.D = 0.7953	r = 75.0
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
6	45.04	11.7	1 Week 6
9	44.52	13.3	2 Week 9
0	26.88	13.4	3

LSD for FAC_A*FAC_B = 3.1352	S.E.D = 1.5905	r = 18.8
T(2-sided a=0.050, 213 df) = 1.9712		MSE = 23.71707

LSD = Least significant difference; S.E.D = Standard Error of Difference; MSE = Mean Squared Error

Two-way table for FAC_A*FAC_B, n=19									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.000	0.000	0.000	47.875	0.000	0.000	47.237
2	0.000	0.000	0.000	0.000	0.000	42.556	0.000	0.000	43.583
3	0.000	0.000	0.000	0.000	0.000	44.625	0.000	0.000	42.850
4	0.000	0.000	0.000	0.000	0.000	45.118	0.000	0.000	44.412

Data used for statistical analysis of the VR1020_*oppA* vaccine results using a 1:500 serum dilution factor. The data is arranged in columns, each containing the ostrich number, treatment received (trt), time (in weeks), response obtained from ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
221	0	VR1020 100	0.064	31.50	223	0	VR1020 100	0.093	27.50	225	0	VR1020 100	0.074	33.00
222	0	VR1020 100	0.136	29.00	224	0	VR1020 100	0.123	25.00	226	0	VR1020 100	0.061	27.50

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
227	0	VR1020 100	0.156	22.00	270	0	VR1020 1200	0.043	23.50	236	3	VR1020 100	0.197	-9.00
228	0	VR1020 100	0.049	29.50	271	0	VR1020 1200	0.186	28.50	237	3	VR1020 100	0.131	-9.00
229	0	VR1020 100	0.061	18.50	272	0	VR1020 1200	0.067	26.00	238	3	VR1020 100	0.203	-9.00
230	0	VR1020 100	0.034	26.00	273	0	VR1020 1200	0.238	23.00	239	3	VR1020 100	0.121	-9.00
231	0	VR1020 100	0.038	24.00	274	0	VR1020 1200	0.562	26.50	240	3	VR1020 100	0.107	-9.00
232	0	VR1020 100	0.223	25.00	275	0	VR1020 1200	0.103	19.50	241	3	VR1020 600	0.124	-9.00
233	0	VR1020 100	0.185	25.00	277	0	VR1020 1200	0.069	32.00	240	3	VR1020 100	0.107	-9.00
234	0	VR1020 100	0.126	31.00	278	0	VR1020 1200	0.050	26.50	241	3	VR1020 600	0.124	-9.00
235	0	VR1020 100	0.177	29.00	279	0	VR1020 1200	0.123	23.50	242	3	VR1020 600	0.124	-9.00
236	0	VR1020 100	0.073	28.50	280	0	VR1020 1200	0.097	21.00	243	3	VR1020 600	0.073	-9.00
237	0	VR1020 100	0.268	28.00	141	0	control	0.135	29.50	244	3	VR1020 600	0.121	-9.00
238	0	VR1020 100	0.107	32.00	142	0	control	0.122	32.50	245	3	VR1020 600	0.134	-9.00
239	0	VR1020 100	0.237	28.50	143	0	control	0.161	24.50	246	3	VR1020 600	0.165	-9.00
240	0	VR1020 100	0.092	30.00	144	0	control	0.265	30.00	247	3	VR1020 600	0.591	-9.00
241	0	VR1020 600	0.115	27.50	146	0	control	0.042	31.00	248	3	VR1020 600	0.024	-9.00
242	0	VR1020 600	0.029	30.50	147	0	control	0.054	29.50	249	3	VR1020 600	0.142	-9.00
243	0	VR1020 600	0.052	24.00	149	0	control	0.141	29.50	250	3	VR1020 600	0.094	-9.00
244	0	VR1020 600	0.083	28.00	150	0	control	0.048	26.00	251	3	VR1020 600	0.159	-9.00
245	0	VR1020 600	0.068	32.00	151	0	control	0.044	26.00	252	3	VR1020 600	0.156	-9.00
246	0	VR1020 600	0.091	24.50	152	0	control	0.047	21.00	253	3	VR1020 600	0.093	-9.00
247	0	VR1020 600	0.208	24.50	153	0	control	0.034	27.50	254	3	VR1020 600	0.079	-9.00
248	0	VR1020 600	0.024	28.00	154	0	control	0.074	26.00	255	3	VR1020 600	0.417	-9.00
249	0	VR1020 600	0.134	24.00	155	0	control	0.094	33.50	256	3	VR1020 600	0.034	-9.00
250	0	VR1020 600	0.046	15.00	156	0	control	0.051	29.50	257	3	VR1020 600	0.045	-9.00
251	0	VR1020 600	0.152	28.00	157	0	control	0.162	22.50	258	3	VR1020 600	0.406	-9.00
252	0	VR1020 600	0.217	26.50	158	0	control	0.043	25.00	260	3	VR1020 600	0.065	-9.00
253	0	VR1020 600	0.198	32.00	160	0	control	0.083	30.00	261	3	VR1020 1200	0.355	-9.00
254	0	VR1020 600	0.061	26.00	221	3	VR1020 100	0.084	-9.00	262	3	VR1020 1200	0.169	-9.00
255	0	VR1020 600	0.347	24.00	222	3	VR1020 100	0.098	-9.00	263	3	VR1020 1200	0.070	-9.00
256	0	VR1020 600	0.055	25.50	223	3	VR1020 100	0.108	-9.00	264	3	VR1020 1200	0.061	-9.00
257	0	VR1020 600	0.055	24.50	224	3	VR1020 100	0.071	-9.00	265	3	VR1020 1200	0.181	-9.00
258	0	VR1020 600	0.174	25.50	225	3	VR1020 100	0.061	-9.00	266	3	VR1020 1200	0.069	-9.00
260	0	VR1020 600	0.046	27.50	226	3	VR1020 100	0.074	-9.00	267	3	VR1020 1200	0.165	-9.00
261	0	VR1020 1200	0.102	28.00	227	3	VR1020 100	0.153	-9.00	268	3	VR1020 1200	0.092	-9.00
262	0	VR1020 1200	0.214	33.00	228	3	VR1020 100	0.059	-9.00	269	3	VR1020 1200	0.131	-9.00
263	0	VR1020 1200	0.051	25.50	229	3	VR1020 100	0.079	-9.00	270	3	VR1020 1200	0.047	-9.00
264	0	VR1020 1200	0.066	24.50	230	3	VR1020 100	0.026	-9.00	271	3	VR1020 1200	0.271	-9.00
265	0	VR1020 1200	0.176	27.00	231	3	VR1020 100	0.058	-9.00	272	3	VR1020 1200	0.238	-9.00
266	0	VR1020 1200	0.042	29.50	232	3	VR1020 100	0.099	-9.00	273	3	VR1020 1200	0.150	-9.00
267	0	VR1020 1200	0.104	21.50	233	3	VR1020 100	0.181	-9.00	274	3	VR1020 1200	0.297	-9.00
268	0	VR1020 1200	0.105	27.50	234	3	VR1020 100	0.247	-9.00	275	3	VR1020 1200	0.114	-9.00
269	0	VR1020 1200	0.125	29.00	235	3	VR1020 100	0.179	-9.00	276	3	VR1020 1200	0.044	-9.00

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
277	3	VR1020 1200	0.112	-9.00	244	6	VR1020 600	0.155	45.00	149	6	control	0.201	46.00
278	3	VR1020 1200	0.051	-9.00	245	6	VR1020 600	0.109	45.50	150	6	control	0.060	44.50
279	3	VR1020 1200	0.109	-9.00	246	6	VR1020 600	0.086	35.50	151	6	control	0.032	34.50
280	3	VR1020 1200	0.095	-9.00	247	6	VR1020 600	0.210	41.50	152	6	control	0.052	46.00
141	3	control	0.138	-9.00	248	6	VR1020 600	0.091	47.00	153	6	control	0.040	52.50
142	3	control	0.070	-9.00	249	6	VR1020 600	0.179	40.00	154	6	control	0.121	46.50
143	3	control	0.136	-9.00	250	6	VR1020 600	-9.000	-9.00	155	6	control	0.071	54.00
144	3	control	0.228	-9.00	251	6	VR1020 600	0.134	40.50	156	6	control	0.098	46.50
146	3	control	0.066	-9.00	252	6	VR1020 600	0.222	50.00	157	6	control	0.243	37.50
147	3	control	0.097	-9.00	253	6	VR1020 600	0.188	41.00	158	6	control	0.054	40.50
149	3	control	0.092	-9.00	254	6	VR1020 600	0.079	38.50	160	6	control	0.044	53.00
150	3	control	0.076	-9.00	255	6	VR1020 600	0.231	41.50	221	9	VR1020 100	0.086	63.00
151	3	control	0.288	-9.00	256	6	VR1020 600	0.131	44.00	222	9	VR1020 100	0.252	48.00
152	3	control	0.158	-9.00	257	6	VR1020 600	0.050	41.00	223	9	VR1020 100	1.412	44.00
153	3	control	0.061	-9.00	258	6	VR1020 600	0.225	41.00	224	9	VR1020 100	0.286	36.50
154	3	control	0.089	-9.00	260	6	VR1020 600	0.060	45.50	225	9	VR1020 100	-9.000	-9.00
155	3	control	0.073	-9.00	261	6	VR1020 1200	0.099	44.50	226	9	VR1020 100	0.331	45.50
156	3	control	0.056	-9.00	262	6	VR1020 1200	0.236	51.00	227	9	VR1020 100	0.189	42.50
157	3	control	0.203	-9.00	263	6	VR1020 1200	0.074	42.50	228	9	VR1020 100	0.136	54.00
158	3	control	0.039	-9.00	264	6	VR1020 1200	0.057	48.00	229	9	VR1020 100	1.084	34.50
160	3	control	0.048	-9.00	265	6	VR1020 1200	0.165	44.00	230	9	VR1020 100	0.076	43.00
221	6	VR1020 100	0.088	54.50	266	6	VR1020 1200	0.316	50.00	231	9	VR1020 100	0.126	41.00
222	6	VR1020 100	0.047	48.00	267	6	VR1020 1200	0.113	42.00	232	9	VR1020 100	0.413	47.50
223	6	VR1020 100	0.179	44.00	268	6	VR1020 1200	0.077	43.00	233	9	VR1020 100	0.163	40.00
224	6	VR1020 100	0.120	43.50	269	6	VR1020 1200	0.113	45.00	234	9	VR1020 100	0.611	52.50
225	6	VR1020 100	0.094	55.50	270	6	VR1020 1200	0.089	43.50	235	9	VR1020 100	0.223	54.50
226	6	VR1020 100	0.096	47.00	271	6	VR1020 1200	0.202	49.00	236	9	VR1020 100	1.022	46.00
227	6	VR1020 100	0.091	41.00	272	6	VR1020 1200	0.522	46.00	237	9	VR1020 100	0.750	51.00
228	6	VR1020 100	0.065	53.50	273	6	VR1020 1200	0.134	41.50	238	9	VR1020 100	0.523	43.50
229	6	VR1020 100	0.049	35.00	274	6	VR1020 1200	0.253	49.50	239	9	VR1020 100	0.193	56.00
230	6	VR1020 100	0.018	45.00	275	6	VR1020 1200	0.070	38.50	240	9	VR1020 100	0.242	54.50
231	6	VR1020 100	0.084	44.50	276	6	VR1020 1200	0.027	38.00	241	9	VR1020 600	1.767	42.00
232	6	VR1020 100	0.113	44.50	277	6	VR1020 1200	0.198	54.50	242	9	VR1020 600	1.570	37.00
233	6	VR1020 100	0.102	45.50	278	6	VR1020 1200	0.101	43.50	243	9	VR1020 600	0.174	42.50
234	6	VR1020 100	0.252	53.00	279	6	VR1020 1200	0.151	42.00	244	9	VR1020 600	1.969	42.50
235	6	VR1020 100	0.223	49.00	280	6	VR1020 1200	0.312	36.50	245	9	VR1020 600	1.835	49.00
236	6	VR1020 100	0.157	51.50	141	6	control	0.174	49.00	246	9	VR1020 600	1.870	34.00
237	6	VR1020 100	0.157	53.50	142	6	control	0.068	47.00	247	9	VR1020 600	0.849	48.00
238	6	VR1020 100	0.455	47.00	143	6	control	0.185	30.00	248	9	VR1020 600	0.116	45.00
239	6	VR1020 100	0.171	52.00	144	6	control	0.215	46.50	249	9	VR1020 600	0.639	41.00
242	6	VR1020 600	0.104	44.50	146	6	control	0.048	44.00	250	9	VR1020 600	-9.000	-9.00
243	6	VR1020 600	0.154	40.00	147	6	control	-9.000	49.00	251	9	VR1020 600	0.190	37.50

*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight	*Ostrich	Time	TRT	RESP	Weight
252	9	VR1020 600	0.321	54.00	268	9	VR1020 1200	0.492	38.00	143	9	control	0.238	34.00
253	9	VR1020 600	2.590	47.00	269	9	VR1020 1200	0.170	46.50	144	9	control	0.293	40.00
254	9	VR1020 600	0.199	42.50	270	9	VR1020 1200	0.275	44.50	146	9	control	0.246	45.00
255	9	VR1020 600	0.548	44.00	271	9	VR1020 1200	0.724	44.50	147	9	control	0.069	47.50
256	9	VR1020 600	0.478	45.50	272	9	VR1020 1200	2.892	42.00	149	9	control	0.239	46.50
257	9	VR1020 600	0.093	46.50	273	9	VR1020 1200	0.518	43.00	150	9	control	0.099	42.00
258	9	VR1020 600	1.483	41.00	274	9	VR1020 1200	0.719	46.00	151	9	control	0.136	31.50
260	9	VR1020 600	1.243	45.50	275	9	VR1020 1200	0.265	38.50	152	9	control	0.122	50.50
261	9	VR1020 1200	0.173	42.50	276	9	VR1020 1200	0.179	37.50	153	9	control	0.182	54.00
262	9	VR1020 1200	0.966	55.50	277	9	VR1020 1200	0.273	45.00	154	9	control	0.180	43.50
263	9	VR1020 1200	0.228	44.00	278	9	VR1020 1200	0.149	39.00	155	9	control	0.304	53.50
264	9	VR1020 1200	0.457	43.50	279	9	VR1020 1200	1.298	34.50	156	9	control	0.159	40.50
265	9	VR1020 1200	0.559	43.50	280	9	VR1020 1200	2.832	39.00	157	9	control	0.565	40.00
266	9	VR1020 1200	2.631	45.00	141	9	control	0.204	43.50	158	9	control	0.197	43.50
267	9	VR1020 1200	0.402	45.00	142	9	control	0.128	46.00	160	9	control	0.082	53.50

*Ostrich-number of ostrich; TRT- Treatment received; Time- Time in weeks; RESP- Titre response; Weight- weight of specific ostrich.

7.2 Addendum B

Alignment of the *IgA*$_H$ gene to the partially sequenced *Struthio camelus australis* genome scaffold (NW_009271630.1). The part of the scaffold shown is from position 19081 to position 23141. The segments indicated in green match the genome scaffold whereas the segment indicated as yellow does not match the genome scaffold which might be as a result of an incomplete genome sequence. The *IgA*$_H$ is in the reverse compliment orientation in order to match the genome sequence which is expected as it was found that the IgA gene of duck, chicken and goose are in reverse orientation on the respective chromosomes.

```
Ostrich      GTAGACAAGACCCTTCCAGTAGACAAGACCCTTCCAGTAGACTAGCTCAG
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      TTGGCTCTTCCAGTAGGCTAGACCCTTCCAGTAGACAAGACCCTTCCAGT
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      AGACCAGCCCAGTTGGCTCTCCTAGTAGACTACACCCTTCCTGTAGTCAA
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      GACCCTTCCAGCAGCCTGGCCCAGTTGGCTCTCCCAGGAGGCTAGACCCT
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      TCCAGTAGTCAAGACCCTTCCAGCAGCCGAGACGCTTCCAGCAGCCTGGT
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      CCAGTTGGCTCTCCCAGTAGACTACATCCTTCCAGTAGACAAGACCCTTC
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      CAGCAGCCTGGTCCAGTTGGCTCTCCCAGGAGGCTAGACCCTTCCAGTAG
IgA_H        ~~~~~~~~~CAGTAGCAGGTGATGTCGGCGTCGGCCAGGACCACCGAGAC

Ostrich      GCTAGACCCTTCCAGTAGATGAGACCCTTCCAGCAGCCTGGCCCAGTTGG
IgA_H        GTTGACGGCGGTGGGTTTACCCGAAGCCTTGTCCAGGCTCTTGTGGACGA

Ostrich      CTCTCCCAGGAGGCTAGACCCTTCCAGTAGCCTAACTGGAACGGCCCACT
IgA_H        AGGTGATGGGGATGCCCTCGTGCCCCACCACGCAGGCGAAGGAGTCGCCC

Ostrich      TGACCCTCCCAGCAGGCTAGACTCTCCCAGTAGACTAACCCCTTCCCATG
IgA_H        CGCTGCCACTCGTCCAATGGGACGCTCAGCTTGCTGTAGACCGTGTAGCC

Ostrich      GCCTGGCCCAGTCGACTCTCCCAGTAGCCTAGGCTCTCCCAGCAGCCCTT
IgA_H        CCCGCCGTCCTCCTTGGGGCCGAAGACGGCGAAGCTGTCGGGGGGACACGG

Ostrich      CCCAGTTGACTCCCCGAGTCGATTCGACTCTTCCAGCCAACTGGCCTAGA
IgA_H        GGCGCTCTTGCTGCGTCCAGGTCACCAGGATGTCCTGAGGCCGGAAGCCC

Ostrich      CCTGCTCTTCCCTCGCTGCCGACCCCGCTGTGCCCCCGGGGTGGGGGGCT
IgA_H        GTGGCCAGGCAGGTCAAGGTGGCCATCTCCCCCCGGGCCAGCTCCTCCGC

Ostrich      CAGAGCGGGGCAGAGGAGAAGGGCAGGAAAGGGTGAGCGCCTACCTAGTT
IgA_H        TGGCGGGGCGAAGACGTAGACGGAGGGCGCCTGCACGGAGACGGCTAGTT

Ostrich      CCTTCTGTGCAGATTTGGTGACGGGCGAGGAGAGCTCGGAGCTGGCCACG
IgA_H        CCTTCTGTGCAGATTTGGTGACGGGCGAGGAGAGCTCGGAGCTGGCCACG

Ostrich      GTGCAGGTGAAGACCTCGCCGGAGTTCCACTCCTCGGCGCACACCTTCAG
IgA_H        GTGCAGGTGAAGACCTCGCCGGAGTTCCACTCCTCGGCGCACACCTTCAG

Ostrich      CACGCTGGTGACGCGGTAGAGGCCATCGCCTTGCTGCACCGGCTCCTCGG
IgA_H        CACGCTGGTGACGCGGTAGAGGCCATCGCCTTGCTGCACCGGCTCCTCGG

Ostrich      AGGTCACGTCCAGAGCGCTGCCCTTCTGCCGCTGCCAGGAGAACCTGACG
IgA_H        AGGTCACGTCCAGAGCGCTGCCCTTCTGCCGCTGCCAGGAGAACCTGACG

Ostrich      TCGGCGGGCGTCTTGACGTTGGCCACCACGCAGGTGATGCTGGCGTTCTG
IgA_H        TCGGCGGGCGTCTTGACGTTGGCCACCACGCAGGTGATGCTGGCGTTCTG

Ostrich      GCTGAGGTAGAGGTCCTCCAAGGAAGGCGGGAGGATGGAGACCACGGGCA
IgA_H        GCTGAGGTAGAGGTCCTCCAAGGAAGGCGGGAGGATGGAGACCACGGGCA

Ostrich      GGGCGATATCTGGGTGGAGGGAGCAGCGTTGAGCGGGGGCTTGGCTCCCC
IgA_H        GGGCGATAT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      GGGCCCCGGCGCGCCCCGAACGCCCTTCCGTTTTGCAGGCGCGGCAACAG
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      TTTTCCCCACTGGAGCGTCCACGGCGGCTAGATCGCAGGGCGTTAGTACC
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich      TACCCCAGCAGGCAGGAGCAAGATGCTTTTCAAGAAACTCTCTAGAGCAT
IgA_H        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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Ostrich     CGTTTTTGTTAAGTTGTTTTTTTCATCCGAGGATCGCTCAGTCCATCCAC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     TTCCTTAATATGCTCTTTCGGTCTTCTGTTTTGTCTCCGAAGCTTTTTTT
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     TTTTTTTTTTTTTTTAAACAAACAGTTACAACATGAGACAGTTTCTCAGC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     TTTAGTAAACAGGATTGTTCTGCTGACCTGGATTGAGTGTAGAGTCTGCC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     GTGCGTTCATGCAGCTTCTAATCCGCCCGTGCATCTGTCCATCCATCCAC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CCAGCCAGCCAGCCACCAACCCACCCAGTCACCAACCCAACCATCCATCC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     ACCAACCCATCCATCCATCCATCCATCCATCCACCAAACCATCCATCCAT
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CCATCTGTCCATCCGTCTGTCCATCCATCCATCCATCCATCCATCCATTC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     ACCAAACCATCCATCCATCCATCCATCCATCCATCCATCCATCCACCCAA
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CCATCCATCCATCCCTCCATCTGTCCATCCANNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     NNNNNNNNNNNNNNNNNNNNNTCCATCCATCCATCCACCAACCCATCCAT
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CCATCTGTCCGTCCATCCATCCGTCCATCCATCCATGCAGTCAATGCATG
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CTCCCACTCATCCATCCTTGCATCCATTCGTGACCGCTAGCAAACCACGA
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CCTCCCCGTTGCACCATGCCAGGGCCGCGTTCCCCACCTCGGACCCTCAC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CTGGGCAGGAGTTGCTGATGGTGCGCATGATCGGCTCCGTCAGCGACGCG
IgA_H       CTGGGCAGGAGTTGCTGATGGTGCGCATGATCGGCTCCGTCAGCGACGCG

Ostrich     TGGGACACTCGGCAAGCGAACTCGGAGCCCCGGTCCCAGCTTTGTCGGCT
IgA_H       TGGGACACTCGGCAAGCGAACTCGGAGCCCCGGTCCCAGCTTTGTCGGCT

Ostrich     GACGTTGACCTGACTCGACTGCACGGCCTGGTTGTTGGAGCACTTGTCGC
IgA_H       GACGTTGACCTGACTCGACTGCACGGCCTGGTTGTTGGAGCACTTGTCGC

Ostrich     AGCTGTAGTCCACCTGGGGCAGCGTCACCACCTCTCGGTTCTTCAGCCAT
IgA_H       AGCTGTAGTCCACCTGGGGCAGCGTCACCACCTCTCGGTTCTTCAGCCAT

Ostrich     TCCACTGTGGCCGGGCCGTCTGGGAGTCCCTCGATGAGGCAGAGCAGCAG
IgA_H       TCCACTGTGGCTGGGCCGTCTGGGAGTCCCTCGATGAGGCAGAGCAGCAG

Ostrich     GACCTCATCATTATTCTCTGAAAAGCTCAGGATTTTTACCTGTGGGTTGC
IgA_H       GACCTCATCATTATTCTCTGAAAAGCTCAGGATTTTTACCTGTGGGTTGC

Ostrich     TGCATAAGTCTGCAGAAAAAACAAGGAAATAACTGTAGTGATCAACCAAG
IgA_H       TGCATAAGT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     ACCTCGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAG
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     GAAATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CAGCACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CAGGAGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCGTGCAATGC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CGGGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAATGACCAAGAC
IgA_H       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich     CTCGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGA
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IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        AATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCA
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        GCACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCA
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        GGAGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        GGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAATGACCAAGACCT
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        CGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAA
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        TGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCAGC
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        ACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCAGG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        AGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCGGG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        AGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        TGCAATGCCGGGAGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAATG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        ACACCAAGACCTCGTGTTGCTGCATAAGTCTGCAGAAAAAACAAGGAAAT
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        ACCTCTAGGAAATGACCAAGACCTCGTGCAATGCCGGGAGCAAGGCCAGG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        AGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCGTGCATTGCCGGG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        AGCAAGGCCAGGAGCTACCCCAGCACCTCTAGGAAATGACCAAGACCTCG
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        TGCATTGCCGGGAGCAAGGCCAGGAGCAGCTCTAGCCCATTTAGGAGTCA
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        ACCAAGACGTCATGCAATGCTGGGAGCAACCCAGTTCCCCTTTGCCTTTC
IgA_H          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Ostrich        TCACCTCCGATGTTCTTGGAGATTTCCGTGCTTGTGGGGCTGTGGTTCAC
IgA_H          ~~~~CCCCGATGTTCTTGGAGATTTCCGTGCTTGTGGGGCTGTGGTTCAC

Ostrich        GTTGCACTGGATGGCGTTGCCCTGCAGGTTGCTGGTGGGCGTCGTGAGCT
IgA_H          GTTGCACTGGATGGCGTTGCCCTGCAGGTTGCTGGTGGGCGTCGTGAGCT

Ostrich        GGCTGCTGAGGCTGTACAGGCTGTTGCTGACCACCACGGCCGGGTAGGTC
IgA_H          GGCTGCTGAGGCTGTACAGGCTGTTGCTGACCACCACGGCCGGGTAGGTC

Ostrich        AAGACCTCGTAGGGCTCGCTGGAAACCCAGGTGACGGTGGCGGTCTCGGG
IgA_H          AAGACCTCGTAGGGCTCGCTGGAAACCCAGGTGACGGTGGCGGTCTCGGG

Ostrich        GAAGAAGTCGTTCACGAGGCAGGCCACGGTGACGTTTCCCTCGTCGCAGC
IgA_H          GAAGAAGTCGTTCACGAGGCAGGCCACGGTGACGTTTCCCTCGTCGCAGC

Ostrich        CGCAGGGCTTGAGCGCGTAGACGTCGGGCGCTTTGGCCGTGGCTGCAAGG
IgA_H          CGCAGGGCTTGAGCGCGTAGACGTCGGGCGCTTTGGCCGTGGC-------

Ostrich        AGAAGGGAGAGGGCTGCGAGGGCGCCGGGGCTCGGGGTCTGCCGAAGGCC
IgA_H          --------------------------------------------------

Ostrich        GCGAGCTTTCTGCAAGCTTTTGGGGCATCGCACGGATTGAAAACGAGCGT
IgA_H          --------------------------------------------------

Ostrich        TGCGGGCACAGCTCGAAGCCGAGCATCGCGAGCACGGCTGGAAGATGATA
IgA_H          --------------------------------------------------

Ostrich        ACCGTGTGCAT
IgA_H          -----------
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7.3 Addendum C

Alignment of the IgA heavy chain constant secretory gene (secretory) (Accession number: AFA41928.1) with the IgA heavy chain constant region transmembrane gene (transmembrane) (Accession number: AFA41929.1). The end parts of the two genes that do not align with each other represents the secretion-coding sequence and membrane coding sequence respectively. The secretory IgA_H consists of four domains where domain one consists of amino acid 2-103, domain two consists of amino acid 102-216, domain three consists of amino acid 204-302 and domain four of amino acid 308-422.

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                    10        20        30        40        50        60
                    |         |         |         |         |         |
Secretory           ATAKAPDVYALKPCGCDEGNVTVACLVNDFFPETATVTWVSSEPYEVLTYPAVVVSNSLY
Transmembrane       ............................................................

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Secretory           SLSSQLTTPTSNLQGNAIQCNVNHSPTSTEISKNIGDLCSNPQVKILSFSENNDEVLLLC
Transmembrane       ..........................A.................................

                    130       140       150       160       170       180
                    |         |         |         |         |         |
Secretory           LIEGLPDGPATVEWLKNREVVTLPQVDYSCDKCSNNQAVQSSQVNVSRQSWDRGSEFACR
Transmembrane       ............................................................

                    190       200       210       220       230       240
                    |         |         |         |         |         |
Secretory           VSHASLTEPIMRTISNSCPDIALPVVSILPPSLEDLYLSQNASITCVVANVKTPADVRFS
Transmembrane       ............................................................

                    250       260       270       280       290       300
                    |         |         |         |         |         |
Secretory           WQRQKGSALDVTSEEPVQQGDGLYRVTSVLKVCAEEWNSGEVFTCTVASSELSSPVTKSA
Transmembrane       ............................................................

                    310       320       330       340       350       360
                    |         |         |         |         |         |
Secretory           QKELAVSVQAPSVYVFAPPAEELARGEMATLTCLATGFRPQDILVTWTQQERPVSPDSFA
Transmembrane       ..........................................................P.....

                    370       380       390       400       410       420
                    |         |         |         |         |         |
Secretory           VFGPKEDGGGYTVYSKLSVPLDEWQRGDSFACVVGHEGIPITFVHKSLDKASGKPTAVNV
Transmembrane       ..........................................................DCVFV.LG

                    430       440       450       460
                    |         |         |         |
Secretory           SVVLADADITCYX
Transmembrane       EDAP.EGEEDVTNLWTAVTFVLLFLLSLCYSTGVTLFKVLX
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