Evaluation of DNA vaccines developed against *Mycoplasma struthionis* sp. nov. str. Ms01 in ostriches

by
Bertus de Wet

Thesis presented in fulfilment of the requirements for the degree of Master of Science (Biochemistry) in the Faculty of Natural Science at Stellenbosch University

Supervisor: Dr. A Botes
Co-supervisor: Prof. D. U. Bellstedt

March 2016

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Bertus de Wet

Date: March 2016

Abstract

The vast demand for ostrich meat has made South Africa the leader in not only the production of ostrich meat but also ostrich associated products such as feathers and leather. Ostrich specific mycoplasmas (referred to as Ms01, Ms02 and Ms03) cause respiratory tract infections with subsequent reduction in physical growth rate and therefore reduced production. To date no vaccines are available to combat these infections in ostriches. In this laboratory three DNA vaccines (pCI-neo, VR1012 and VR1020) have been developed with each containing the Ms01 *oppA* gene as antigen. The aim of this study was to evaluate these developed DNA vaccines in a mammalian cell culture based system as well as an ostrich vaccination trial.

COS-1 cells were transfected with the three developed DNA vaccines. Transcription of the *oppA* gene was proven for all the plasmids. Translation into the OppA protein was shown to be limited to the VR1020_*oppA* plasmid. The protein was visualised by SDS-PAGE and detected by western blot using chemiluminescence.

Two of the vaccines, VR1020_*oppA* and pCI-neo_*oppA*, were administered in three concentrations (100 µg/ml, 300 µg/ml and 600 µg/ml) to ostriches during a vaccination trial followed by a booster injection. The ability of the vaccines to elicit anti-OppA antibodies was measured using ELISA. The pCI-neo_*oppA* vaccine failed to induce an immune response against the antigen after both the first and booster vaccinations. The VR1020_*oppA* vaccine on the other hand was able to elicit an anti-OppA immune response.

Opsomming

Die groot aanvraag vir volstruisvleis het Suid-Afrika die leier in nie net die produksie van volstruisvleis nie, maar ook volstruis-geassosieerde produkte soos vere en leer gemaak. Volstruis-spesifieke mikoplasmas (verwys na as Ms01, Ms02 en Ms03) veroorsaak lugweginfeksies wat lei tot ‘n afname in fisiese groei tempo en gevolglike daling in produksie. Tot op hede is daar geen entstowwe beskikbaar om hierdie infeksies in volstruise te bekamp nie. In hierdie laboratorium is drie DNS-entstowwe (pCI-neo, VR1012 en VR1020) ontwikkel wat elk die Ms01 *oppA* geen bevat as antigeen. Die doel van hierdie studie was om hierdie DNS-entstowwe in 'n selkultuur-gebaseerde sisteem, sowel as 'n volstruis inentingproef te evalueer.

COS-1-selle was getransfekteer met die drie DNS-entstowwe. Transkripsie van die *oppA* geen is bewys vir al die plasmiede. Translasie na die OppA proteïen was getoon, maar was beperk tot die VR1020_*oppA* plasmied. Die protein was gevisualiseer deur middel van SDS-PAGE en opgespoor deur middel van western-klad met behulp van chemiluminessensie.

Twee van die entstowwe, VR1020_*oppA* en pCI-neo_*oppA*, was toegedien in drie konsentrasies (100 µg/ml, 300 µg/ml en 600 µg/ml) in volstruise tydens 'n inentingsproef, gevolg deur 'n skraagdosis. Die vermoë van die entstowwe om anti-OppA teenliggame te ontlok is gemeet deur middel van ELISA. Die pCI-neo_*oppA* entstof was nie daartoe in staat om na beide die eerste en skraag inentings 'n immuunrespons teen die antigeen te induseer nie. Die VR1020_*oppA* entstof daarenteen, was daartoe in staat om 'n anti-OppA immuunrespons te ontlok.

Acknowledgements

I would like to thank the following people/organizations for the technical, financial and emotional support. Without the following the thesis would not have been possible:

Dr. A. Botes, Thank you for the opportunity given to me to complete this degree. Thank you for all the patience, time and your continuous efforts to assist me. The time spend here was an absolute joy and I learnt so much from you.

Prof. Dirk U. Bellstedt, Thank you for all the advice, support and leadership throughout my degree.

Mrs Coral de Villiers I want to thank you for not only the technical guidance and the welfare of our laboratory but also for the encouragement, life lessons and help during field trials. It made the time within the Laboratory an absolute joy.

Mr W. Botes for the statistical analysis of the ELISA data.

I would like to thank everyone within the **"Struismoere"** Laboratories and in particular **Mariet Wium** and **Hestie Jonker** for not only the "Sit die ketel aan" moments, but the friendships, support and encouragements throughout the degree. It made the environment very pleasant and day to day activities a lot of fun.

National Research Foundation and **Ostrich chamber**, for the financial support throughout the degree.

I want to say a special thanks to my **Family** for the support, love, encouragements as well as example they gave me to complete this degree.

A big thank you to **Samantha Jane Aldum**, for the continuous support, love, care and encouragement. I appreciate everything you do, and look forward to the next book I'm writing that will be our life journey together.

The biggest thank you must go to the **Lord Jesus Christ**, my rock and my best friend for the energy, strength and joy given every day (Psalm 106:1).

Abbreviations

ABC	ATP-binding cassette
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-suphonic acid)
Ag	antigen
*amp*R	ampicillin resistance gene
APC	antigen presenting cell
ATP	adenosine triphosphate
bp	base pair(s)
kb	kilobase pairs
BSA	bovine serum albumin
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DAFF	department of agriculture, forestry & fisheries
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
ELISA	enzyme-linked immunosorbent assay
G+C	guanine and cytosine
GOI	gene of interest
GST	glutathione S-transferase
g	gram(s)

h	hours
Ig	immunoglobulin
IPTG	isopropyl β-D-1-thiogalactopyranoside
*kan*R	kanamycin resistance gene
kDa	kilo Dalton
LB	Luria-Bertani
M	molar
mM	milimolar
µg	microgram
mg	milligram
ml	milliliter
µl	microliter
MHC	major histo-compatability complex
min	minutes
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information
Opp	oligopeptide permease
ORF	open reading frame
ori	origin of replication
PAP	peroxidase anti-peroxidase
PBS	phosphate buffered saline

PBS-T	phosphate buffered saline tween
PCR	polymerase chain reaction
RE	restriction endonuclease
rRNA	ribosomal ribonucleic acid
rpm	refs per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
%T	acrylamide present
%C	amount of cross-linker
SV40	simian virus 40
TB	terrific broth
TEMED	tetramethylethylenediamine
Th	T helper cell
UV	ultraviolet
X-ray	X-radiation

1 Table of Contents

1 General introduction....1
South Africa's ostrich industry....1
2 Literature Review....4
2.1 Introduction....4
2.2 The immune system....4
2.2.1 Introduction....4
2.2.2 Innate Immune system....4
2.2.3 Adaptive immune system....5
2.3 Vaccines....7
2.3.1 Introduction....7
2.3.2 Live attenuated vaccines....8
2.3.3 Killed or inactivated microbes as vaccines....8
2.3.4 Subunit vaccines....8
2.3.5 DNA vaccines....9
2.4 The characteristics, metabolism and pathogenicity of mycoplasmas....18
2.4.1 Introduction....18
2.4.2 Origin and taxonomy....19
2.4.3 Cell size and morphology....19
2.4.4 Genome....20
2.4.5 Distribution....21
2.4.6 Ostrich-infecting mycoplasmas....22
2.5 Metabolism of mycoplasmas....24
2.5.1 Introduction....24
2.5.2 ABC - transporters....24
2.5.3 The Opp operon of mycoplasmas – structure and function of OppA....25
2.6 Mycoplasma pathogenicity....27

2.6.1 Adherence to the target tissue....28
2.6.2 Invasion of the host....29
2.6.3 Evasion of the host immune system....29
2.6.4 Damage to the host....30
2.7 Pathogenicity of the ostrich-infecting mycoplasmas....30
2.8 Control of mycoplasma infections....31
2.8.1 Control by means of prevention....31
2.8.2 Control by means of medication....32
2.8.3 Control by means of vaccination....33
2.8.4 Control of mycoplasma infections in ostriches....33
2.9 The progress of DNA vaccine development against ostrich-infecting mycoplasma Ms01....34
2.9.1 Vaccine vectors pCI-neo, VR1012 and VR1020....35
2.10 *In vitro* expression of DNA vaccines....36
2.10.1 Animal cell culture....36
2.10.2 Cell choice....37
2.10.3 Transfection....38
2.10.4 Plasmid expression vectors....38
2.10.5 Cell culture medium....39
2.10.6 Detection of expressed protein....39
2.11 ELISA....39
3 Expression, isolation and the purification of the OppA protein of *Mycoplasma struthionis* sp. nov. str. Ms01 and antibody production in rabbits....42
3.1 Introduction....42
3.2 Materials and methods....42
3.2.1 Expression of the Ms01 OppA-GST protein in *Escherichia coli* cells....42
3.2.2 Isolation of the expressed OppA-GST protein using a glutathione agarose column....44

3.2.3 SDS-PAGE and western blot analysis of isolated OppA-GST protein.....45
3.2.4 Antibody production against OppA in rabbits.....46
3.2.5 Determination of anti-OppA antibody levels in a rabbit serum.....47
3.3 Results.....48
3.3.1 Expression of the Ms01 OppA-GST protein in *E. coli* cells.....48
3.3.2 Isolation of the expressed OppA-GST protein using a glutathione agarose column and its subsequent SDS-PAGE and western blot analysis.....49
3.1.1 Development and validation of rabbit anti-OppA antibodies.....51
3.4 Discussion.....53
4 Evaluation of the expression of the *oppA* gene using a mammalian cell culture based system.....56
4.1 Introduction.....56
4.2 Materials and methods.....56
4.2.1 Preparation of three DNA vaccine plasmids for transfection.....56
4.2.2 Transfection of COS-1 cells.....59
4.2.3 Lysis of cells after transfection.....62
4.2.4 Analysis of gene transcription.....63
4.2.5 Analysis of gene translation.....64
4.3 Results.....66
4.3.1 Preparation of three DNA vaccine plasmids for transfection.....66
4.3.2 Transfection and lysis of COS-1 cells.....68
4.3.3 Analysis of gene transcription.....68
4.3.4 Analysis of protein translation.....69
4.4 Discussion.....73
5 Vaccination trial.....76
5.1 Introduction.....76
5.2 Materials and methods.....77
5.2.1 Preparation of DNA vaccines.....77

5.2.2 Trial animals 78
5.2.3 Vaccination trial 79
5.2.4 Ethical clearance and approval for vaccine trial 77
5.2.5 Analysis of trachea swabs by PCR 79
5.2.6 Weight measurements during trial 81
5.2.7 Biotinylation of rabbit anti-ostrich antibodies 81
5.2.8 Optimization of ELISA for analysis of anti-OppA immune response 82
5.2.9 ELISA analysis of vaccination trial 83
5.3 Results 84
5.3.1 Preparation of DNA vaccines 84
5.3.2 Analysis of trachea swabs by PCR 85
5.3.3 Ostrich weights during the trial 89
5.3.4 Optimization of ELISA for analysis of anti-OppA immune response 90
5.3.5 ELISA analysis of vaccination trial 91
5.4 Discussion 94
6 Concluding remarks and future perspectives 97
7 References 99
8 Addendum A 112

1 General introduction

South Africa's ostrich industry

South Africa is the world leader in ostrich production and with 75% of the global market share it provides the country's economy with an annual income of R1.2 billion. Domestication of ostriches started in 1860 in the Oudtshoorn district of the 'Klein Karoo' region of South Africa for feather production and later for leather. Today ostrich farms are found throughout South Africa, except in Kwazulu–Natal Province. The dry climate conditions, as well as the winter rainfall encountered in the Western Cape province, provide perfect breeding conditions for ostriches, making Oudtshoorn the capital of the ostrich industry in South Africa. More than 588 ostrich farms are registered in South Africa, the Western Cape has 453, 103 in the Eastern Cape and 33 farms in the rest of the country. Most importantly, the ostrich industry provides about 20 000 jobs (DAFF, 2012).

The ostrich industry produces three main products in the following ratios according to income: 45% ostrich meat, 45% leather and 10% feathers. Ostriches are slaughtered at 14 months of age as this is the time when the bird presents the best meat, feathers and leather (Balog Ai and Paz, 2007). It is said that one slaughter ostrich produces as much as 27 kg of meat, 4.2 m^2 of leather and 1 kg of feathers. Ostrich meat production only started to increase in the 1990's and is strongly dependant on the high quality of the nutrition given to the ostriches (Majewska *et al.*, 2009). The industry is a strongly export orientated industry. More than 8.3 million kg of ostrich meat, 90% of total production, is exported annually. The largest consumers of ostrich meat are European Union countries such as Belgium, Germany, the United Kingdom and the Netherlands, as the health benefits of the meat make it very popular. The rest is exported to the Far Eastern countries such as China (DAFF, 2012)

To ensure that the high market demand is met, farmers have been forced to increase production that has resulted in an increase in the number of ostriches per stocking area. It is this increase in the stocking density of ostriches that results in the spread of pathogen related infections. These pathogens include mycoplasmas which take their toll as the ostriches are introduced into feedlot systems where they live in close proximity to each other. Mycoplasma infections lead to disease states associated with upper respiratory tract infections (Botes *et al*., 2005). A consequence of these infections is a reduction in the physical growth rate of the ostriches. Besides the influence these mycoplasma

infections are having on the growth of ostriches, they can also make ostriches more susceptible to secondary infections which can place further constraints on optimal production and thereby the export of ostrich products. In order for South Africa to keep up as the world leader in ostrich production, it is important that such diseases be controlled.

Botes *et al.* (2005) identified three *Mycoplasma* species that specifically infect ostriches in South Africa and these were provisionally named Ms01, Ms02 and Ms03. Langer (2009) described the Ms01 and Ms03 mycoplasmas isolated from ostriches in Namibia and proposed the names *Mycoplasma struthionis* sp. nov. str. 237IAT and *Mycoplasma nasistruthionis* sp. nov. str. 2F1AT, respectively. These descriptions have not been formally published and are yet to be accepted. In order to distinguish the South African isolated strains from those described by Langer, the mycoplasmas will be referred to as *Mycoplasma struthionis* sp. nov. str. Ms01, *Mycoplasma* sp. Ms02 and *Mycoplasma nasistruthionis* sp. nov. str. Ms03 in this thesis.

To control mycoplasma infections it is important to separate sick from healthy ostriches and secondly, to treat existing infections with antibiotics. Antibiotics however are not always effective, as they do not eliminate mycoplasmas and some ostriches may appear healthy but act as carriers of the organism. Thus antibiotics have to be administered over a longer period of time, which is expensive and furthermore renders the meat unsuitable for export as it contains levels of antibiotics that are unexcitable. A different approach is thus needed to combat these infections in a cost effective manner. The era of molecular genetics has led to the development of DNA vaccines. The motivation to develop DNA vaccines against these ostrich-infecting mycoplasmas was that they do not require large-scale cultivation of organisms, are inexpensive, easy to manufacture and safe to use.

In 2009, Pretorius initiated the process of developing three DNA vaccines against Ms01. The *oppA* gene from the oligopeptide permease transport system was identified and cloned into the vaccine plasmids pCI-neo, VR1012 and VR1020. Attempts by Brandt (2012) and van Tonder (2013) to evaluate the ability of these vaccines to elicit an immune response were unsuccessful. Besides both trials being compromised by avian influenza outbreaks during the trial period, several other factors were given as possible reasons for the lack of an immune response. These included amongst others stress due to frequent handling during the course of a vaccination trial, a too low dose of vaccine and possibly insufficient expression of the *oppA* vaccine candidate gene.

In an attempt to overcome previously encountered problems, the goal of the current study was to re-evaluate the DNA vaccines developed against Ms01 by evaluating the *in vitro* expression of the DNA vaccine plasmids as well as to determine the ability of different vaccine doses to elicit an anti-OppA immune response in ostriches. To this end the following objectives were set:

- Objective 1: The expression and isolation of the Ms01 OppA protein for use in the production of rabbit anti-OppA antibodies and as coating antigen for ELISA analysis of immune responses.
- Objective 2: The production of anti-OppA antibodies in rabbits using the OppA protein adsorbed to the naked bacteria for use in western blot analysis.
- Objective 3: To determine *in vitro* transcription and translation of the Ms01 *oppA* gene in transfected COS-1 cells using reverse transcriptase polymerase chain reaction (RT-PCR) and SDS-PAGE followed by western blot analysis, respectively.
- Objective 4: To perform a vaccination trial in ostriches using the pCI-neo_*oppA* and VR1020_*oppA* DNA vaccines at three different doses, together with a boosting dose.
- Objective 5: To analyse the anti-OppA antibody responses using ELISA.

A detailed literature review on the role of the immune system in reaction towards an infectious organism is given in Chapter 2 as well as background on vaccines, mycoplasmas and specifically ostrich-infecting mycoplasmas and their control. The experimental techniques used during this study are also presented. The expression and isolation of the Ms01 OppA protein and its use in the production of rabbit anti-OppA antibodies are described in Chapter 3. Both of these are used in downstream analyses. The *in vitro* expression of the Ms01 *oppA* gene within a mammalian cell culture system is examined in Chapter 4. The vaccination trial in ostriches and the evaluation of anti-OppA antibody responses using an ELISA developed for this purpose are described in Chapter 5. Finally, a brief conclusion is provided in Chapter 6 together with future perspectives. An Addendum is given at the end of the thesis that contains the statistical analyses of the data accumulated in this thesis.

2 Literature Review

2.1 Introduction

The focus of this thesis is the control of mycoplasma infections by means of DNA vaccination. To this end, this literature review gives a brief background of the immune system and how it responds to infectious organisms. This is followed by an overview of the different vaccine types and how they can elicit immune responses. Specific attention was given to DNA vaccines as this was the vaccine of choice for use against ostrich-infecting mycoplasmas in this study. Furthermore, the characteristics, metabolism and pathogenicity of mycoplasmas are discussed. To evaluate the transcription and translation of DNA vaccines, an *in vivo* cell culture system was used, for which reason a brief introduction into mammalian cell culture is given. Background on the enzyme-linked immunosorbent assay (ELISA) technique for the evaluation of humoral responses elicited by vaccination is also provided.

2.2 The immune system

2.2.1 Introduction

The immune system can be divided into innate immunity that provides initial protection against an infectious microbe and adaptive immunity or specific acquired immunity in which a more specific and longer acting response is induced against infecting microbe. Adaptive immunity can in turn be divided into the humoral immunity and cell mediated immunity.

2.2.2 Innate Immune system

Innate immunity serves as the first line of defense that helps to fight off infectious microbes in the body. The innate immune system relies on pathogen-associated molecular patterns (PAMPs) in order to recognise microbes. These PAMPs are patterns that are only found on pathogens and not on any eukaryotic cells of the body and are thus identified as foreign. The innate immune system also recognises molecules that are released from damaged or necrotic cells termed damage-associated molecular pattern molecules (DAMPs). Innate immunity has certain receptors that recognise these PAMPs and DAMPs and are called pattern recognition receptors (PRR). The receptors are expressed on dendritic cells, phagocytes, epithelial and endothelial cells, lymphocytes and macrophages. The PRRs can be expressed intracellularly or extracellularly. Examples of these receptors include Toll-like receptors (TLRs) and NOD-like receptors

(NLRs). The TLRs are specific for different components of the microbe. For instance TLR-4 recognises bacterial lipopolysaccharides (LPS) and TLR-9 recognises unmethylated CpG oligonucleotides in microbial DNA. The NLRs are cytokine receptors for DAMPs and PAMPs in the cytoplasm. Examples are the NLRP-3 (NOD-like receptor family, pyrin domain containing 3) that sense microbial products that indicate cell damage and death, and NOD-2 that is specific for bacterial peptides that have entered the cytosol (Murphy *et al.*, 2008; Abbas *et al.*, 2012).

2.2.3 Adaptive immune system

A brief overview of the adaptive immune system is shown in Figure 2.1. The activation of the adaptive immune system starts when a pathogen enters the body and is ingested by dendritic cells that are located in the epithelia of the body. The pathogen is then digested of which protein fragments are displayed on the surface of these dendritic cells via the major histocompatibility complex (MHC). The dendritic cells are also known as antigen presenting cells (APC). The APC's can then either elicit a humoral immune response by the activation of the B-lymphocytes (B-cells) with subsequent antibody production, or it can lead to a cellular immune response by the activation of Cytotoxic T lymphocytes (CTL) and/or the T helper (Th) lymphocyte cells (Oshop *et al.*, 2002; Browning *et al.*, 2011). B-lymphocytes can also ingest antigens and present them on MHC II molecules. The ability to raise antibody, Th and/or CTL responses determines the efficacy of a vaccine (Robinson and Torres, 1997; Liu, 2011).

The Th cell receptors specifically recognise the antigens presented on the MHC II of the APC's and the co-receptors ($CD4^+$) specifically recognize the MHC II and are thereby activated. Th cell activation leads to cell proliferation followed by the release of certain cytokines and cell surface proteins that serve as an activation signal for the proliferation of B-cells and CTL. B-cells can also serve as APC that can then be activated by the Th cell. When B-cells are activated by Th cells, they start to differentiate into millions of either effector B-cells (plasma cells) or memory B-cells. The plasma cells produce antibodies that are secreted into the blood and can bind to the surface of the pathogen that allows recognition for destruction by macrophages and complement (Robinson and Torres, 1997). Memory B-cells are also produced and can survive for a long period of time (Klinman *et al.*, 1998; Abbas *et al.*, 2012; Njongmeta *et al.*, 2012).

All normal nucleated cells in the body can express MHC I proteins. When normal cells in the body are infected by an intracellular pathogen, it digests them and parts of the

pathogen are displayed on the cell surface via the MHC I. The cytokines released by the antigen activated Th cells and the fragments displayed on the surface of the MHC I activate the CTL (Gurunathan *et al.*, 2000). The activation of CTL leads to the proliferation and differentiation into millions of specific CTL. CTL receptors recognise the pathogenic fragments on the MHC I and the co-receptor $CD8^+$ recognises the MHC I of normal infected cells and bind to the cell. Thereafter the CTL releases certain chemicals that kill the infected cells subsequently killing the pathogen (Robinson and Torres, 1997; Gurunathan *et al.*, 2000; Murphy *et al.*, 2008; Abbas *et al.*, 2012).

The adaptive immune system is very specific and contains a highly diverse group of lymphocytes that can distinguish a specific antigen from millions of different antigens. The specificity of the adaptive immune system is obtained by recombination of the gene regions of the antibody and T-cell receptor genes. This means that lymphocytes consist of many different clones, where each clone expresses a receptor different from that expressed by other clones. Another important characteristic of the adaptive immune system is the fact that after the primary immune response, the immune system produces memory lymphocytes for future similar infections. This will ensure a rapid and longer secondary immune response that can eliminate the pathogen (Abbas and Lichtman, 2011).

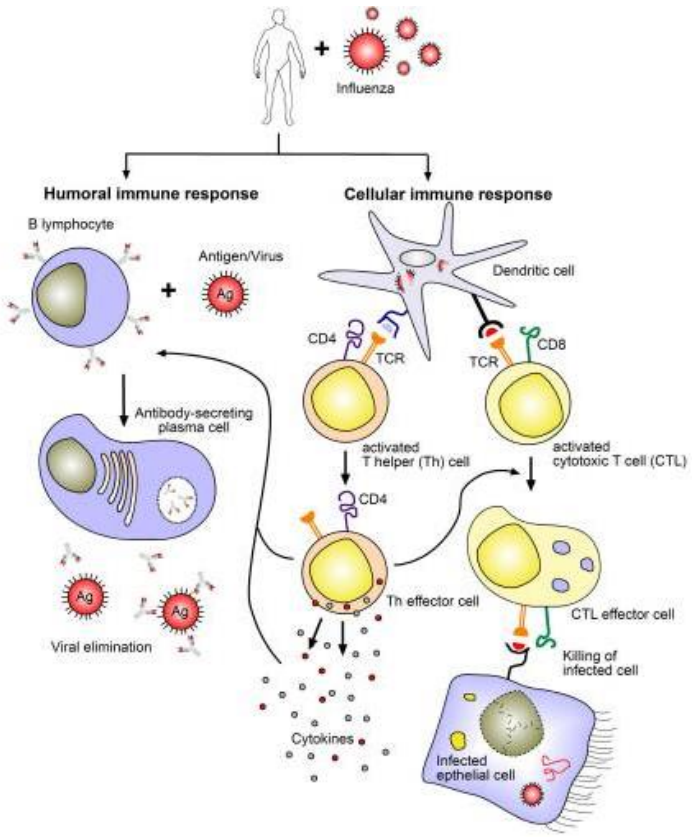

Figure 2.1 A brief overview of the main cells involved in a humoral and cellular immune system (obtained from https://betournay.wikispaces.com/The+Immune+System).

2.3 Vaccines

2.3.1 Introduction

The concept of vaccination was postulated by Edward Jenner in 1798 in his publication on how to prevent smallpox by means of inoculating with the pus from cowpox, which contained the *Vaccinia* virus. It was, however, only 100 years later in 1880 that the next vaccine was developed by Louis Pasteur, which was the killed attenuated *Pasteurella multocida* vaccine for use in poultry (as referenced by Makela, 2000; Plotkin, 2005). The development of vaccines has been fuelled not only by the need for prevention rather than treatment, but also by the technology itself and the continuous increase in knowledge of this field of study.

The immune system has specifically evolved to defend the body against onslaughts of microbes. The adaptive immune system needs to be taught to recognise the different pathogens before it can protect the host when attacking the pathogen. It is on this basis that a vaccine functions. The vaccine contains an antigen derived from the pathogen that will stimulate the production of specific antibodies, Th cells and/or cytotoxic T-cells that serve as the front line of defence against the pathogen. Vaccination thus gives a high

specificity to the induced immunity. Bacteria and viruses can circumvent this by antigenic variation, whereby even the slightest change in the target molecule can cause the immune system to not recognise it (Makela, 2000).

This can include the following types of vaccines: live attenuated vaccines, inactivated (killed) vaccines, purified subunit vaccines (proteins or glycoproteins of the pathogen) and DNA vaccines (Evensen and Leong, 2013).

2.3.2 Live attenuated vaccines

Attenuated vaccines are mutant forms of the microbe that are prepared in such a way that they do not have the ability to cause disease but are still immunogenic. The microbe is treated under conditions that differ in nutrition and surface to the host, making it unfavourable for its growth. Safety is regarded as a big disadvantage as the mutant can revert back to the disease causing state (Makela, 2000). An advantage of this type of vaccine is that it leads to both a cellular and humoral immune response, and also generally induces immunity with a prolonged memory immunity (Ferraro *et al.*, 2011).

2.3.3 Killed or inactivated microbes as vaccines

Killed or inactivated vaccines were developed for the first time in the 1890s where at the time it was the only means to prevent diseases. Killed vaccines work on the basis that the disease causing microbe is killed and used as an immunogenic vaccine target. The problems associated with these vaccine types are both their efficacy and safety. The efficacy of some of these vaccines (cholera and meningococcal vaccines) could never be proven (Makela, 2000). They also gave minimal protection to the host due to protein denaturation or disintegration of the microbe (Mora *et al.,* 2003). Another disadvantage is the fact that they can revert back to the activated microbe (Makela, 2000; Mora *et al.,* 2003).

2.3.4 Subunit vaccines

A difficulty that is associated with all of the abovementioned vaccines is the cultivation of the pathogen. There are many microbes that cannot be grown or are difficult to grow under *in vitro* conditions (Makela, 2000). These include some bacteria, most mycoplasmas and several viruses (Makela, 2000). A real breakthrough in the development of vaccines came with the introduction of genetic technology in which specific genes could be isolated and expressed as a recombinant product in an easily grown organism (Uzzau *et al.,* 2005).

Subunit vaccines were introduced in the 1920s and are a much more sophisticated product. These vaccines only use the immunogenic parts of the microbe as a vaccine target, rather than the whole microbe. This ensures that the reversion of the mutant microbe back to the disease causing state is no longer possible and therefore not a problem. The big advantage of this type of vaccines is their specificity. The immune response is directed towards the subunit itself, rather than the whole microbe. A problem that can be associated with these vaccines is that some pathogens undergo antigenic variation that changes the subunit to such an extent that it is not immunogenic any longer (Makela, 2000; Mora *et al.*, 2003; Uzzau *et al.*, 2005).

2.3.5 DNA vaccines

The concept of transfecting mammalian cells with naked DNA was first reported in 1960 and this formed the basis for DNA vaccines (Pardoll and Beckerleg, 1995). A DNA vaccine is a eukaryotic expression plasmid (circular ring of DNA) containing a specific gene of interest of the pathogen under the control of a eukaryotic promoter (Oshop *et al.,* 2002). When it is injected into a host, it can lead to the transcription as well as translation of the desired encoded protein and induce a specific immune response (Corr *et al.*, 1996; Robinson and Torres, 1997; Dufour, 2001; Oshop *et al.*, 2002).

DNA vaccines, also known as third generation vaccines, have attracted much attention since the 1990s when Wolff *et al.* (1990) inoculated the skin of mice with a plasmid DNA containing the *β–galactosidase* gene and observed that it induced an antibody response (Wolff *et al.*, 1990; Ferraro *et al.*, 2011). Wolff *et al.* indicated for the first time that a recombinant plasmid when it was injected *in vivo* had the ability to directly enter mammalian cells to express the specific protein in the cell (Mir and Kamili, 2012). The application of DNA vaccines has been well studied. Up to 2011 there have been four DNA vaccines licenced for veterinary use: the West Nile virus in horses (Davis *et al.*, 2001), the infectious haematopoietic necrosis virus in salmon (Alonso and Leong, 2013), melanoma in dogs (Bergman *et al.*, 2003) and DNA vaccines for sows (Khan *et al.*, 2010) that are aimed to increase litter survival (Liu, 2011). No further DNA vaccines have since been licenced. A variety of clinical trials are currently under way against a variety of different pathogens in humans as well as in animals (Dhama *et al.*, 2008).

2.3.5.1 **Construction of a DNA vaccine vector**

DNA vaccines elicit good levels of immune responses when plasmids are used that contain the following: a eukaryotic plasmid containing a desired target gene, an effective

viral/eukaryotic promoter and a polyadenylation signal sequence (poly-A) (Dhama *et al.*, 2008; Dufour, 2001; Evensen & Leong, 2013; Faurez *et al.*, 2010). The Cytomegalovirus (CMV) promoter is commonly used as a strong promoter to drive the *in vivo* transcription and translation of the target gene (Glenting & Wessels, 2005; Okuda *et al*., 2014). The plasmid also contains a bacterial origin of replication so that the plasmid can be multiplied within a bacterial mass production culture. It also contains an antibiotic resistance gene allowing for selection during the mass production of the plasmid in a bacterial culture (Dhama *et al.*, 2008). Although some antibiotic resistance genes have been shown to be beneficial to humans, kanamycin which is predominantly used in this thesis does not influence humans (Bengtsson-Palme and Larsson, 2015). Figure 2.2 illustrates the different components that make up a DNA vaccine.

Before a DNA vaccine can be developed, the genome of the pathogen has to be studied in order to identify an appropriate candidate gene. This candidate gene is then isolated and inserted into the plasmid in which the promoter and the poly–A signal will ensure stability as well as effective transcription and subsequent translation of the candidate gene (Gurunathan *et al.*, 2000; Dhama *et al.*, 2008).

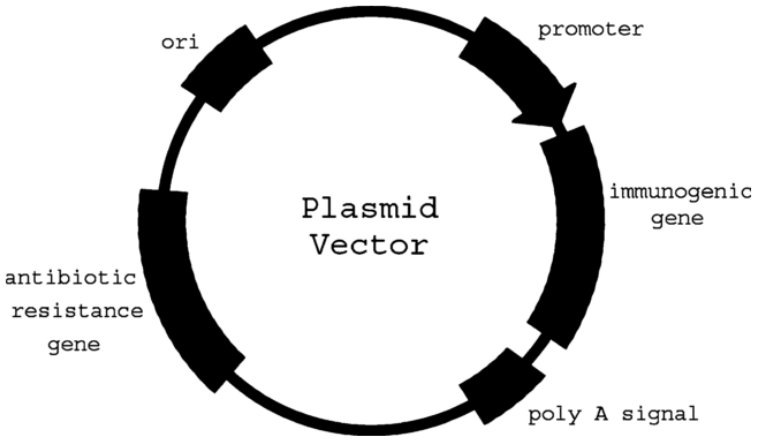

Figure 2.2 A diagram of a typical DNA vaccine plasmid containing a promoter, the immunogenic candidate gene, a poly-A signal, an antibiotic resistance gene and the ori of replication (Dhama *et al.*, 2008).

2.3.5.2 **Advantages of DNA vaccines**

Whole organism vaccine types rely on the development of immunity through the administering of live attenuated or killed inactivated pathogens. DNA vaccines on the other hand are much more specific due to the fact that the plasmid can be constructed to

function with encoded safety features and still have the specificity of subunit vaccines. DNA vaccines are non-live which means that one does not have to work with a virulent pathogen during preparation or application of the vaccine. They are also non-replicating as well as non-spreading which contributes to the fact that there is little risk in the vaccine gene reverting back to the disease causing state or causing secondary infections. Most importantly, DNA vaccines lead to both humoral and cellular immunity inducing all three arms of adaptive immunity namely antibodies, helper T cells (Th) and cytotoxic T lymphocytes (CTL) (Robinson and Torres, 1997; Gurunathan *et al.*, 2000; Ferraro *et al.*, 2011). DNA vaccines can be produced rapidly, a critical attribute for the production of DNA vaccines against emerging pandemic threats (Le *et al.*, 2000; Liu, 2011). DNA vaccines are also easy to produce and are regarded as stable at room temperature in contrast to the live vaccines that require a cold chain. The vaccine candidate gene can code for proteins that have been modified to eliminate deleterious regions, thereby improving the safety and immunogenicity of the proteins delivered (Le *et al.*, 2000; Oshop *et al.*, 2002; Liu, 2011)

2.3.5.3 **Potential safety concerns**

The initial concern associated with DNA vaccines was the potential for genomic integration, the development of anti-DNA immune responses, and activation of oncogenes or inactivation of tumour repressor genes (Dhama *et al.*, 2008; Ferraro *et al.*, 2011). There is a large volume of research that has shown that the probability of naturally occurring mutations to be about three orders higher than the probability of mutations that might result from plasmid insertion (Schalk *et al.,* 2006). This indicates that the integration of a DNA vaccine after injection is an extremely rare event and is not a safety concern (Gurunathan *et al.,* 2000; van Drunen Littel-van den Hurk *et al.*, 2001; Oshop *et al*., 2002; Zhang *et al.,* 2005; Loots *et al.,* 2006; Schalk *et al.,* 2006). The vaccines that are currently in preclinical trials do not show any form of integration into the DNA of the host (Kutzler and Weiner, 2008).

Although plasmid DNA has shown that it can induce the formation of anti-DNA antibodies, no evidence has been found that DNA vaccines induce systematic autoimmune disease in animals (van Drunen Littel-van den Hurk *et al.*, 2001; Schalk *et al.*, 2006). Other potential concerns regarding DNA vaccines may include the low levels of immunogenicity especially in larger animals and also tolerance against the DNA vaccine but this has not been observed to date (van Drunen Littel-van den Hurk *et al.*, 2001; Oshop *et al.*, 2002).

2.3.5.4 **Environmental effects**

Very little attention has been given to the risk of plasmid DNA release into the environment. This may be due to the fact that the risk is so small and does not pose any significant threat (Schalk *et al.*, 2006). The possible concerns that can be anticipated are the spread of the plasmid by shedding or the consumption of vaccinated food animals. If there is any shedding of plasmid DNA into the environment, the DNA will be degraded quickly and will cause no harm to any other animal or humans that come in contact with it (Blum *et al.*, 1997; de Vries *et al*., 2003). The fact that DNA vaccines are non-live, non-replicating and non-infectious also means that they hold little environmental risk.

2.3.5.5 **Consumption of plasmid DNA vaccinated food**

Ingesting meat from animals that have been injected by plasmid DNA has long been a big safety concern and a regulatory hurdle to overcome, but research is proving that it does not have any effect on the consumer (Loots *et al.*, 2006). The degradation rate of supercoiled plasmid DNA after injection was 86.8% after one day, indicating that most injected DNA was degraded (Zhang *et al.*, 2005). Donnelly *et al.* (2003) indicated that the dose of DNA needed to obtain an immune response in humans is about 1 – 5 mg, thus the amount of plasmid consumed from vaccinated animals is orders of magnitude less than the dose given to humans. Schalk *et al.* (2006) also indicated that the consumption of vaccinated animals poses no greater risk to the consumer than the consumption of natural DNA. Furthermore, cooking the meat before it is consumed would further lead to the degradation of plasmid DNA making it safe to consume. Two of the licenced DNA vaccines, one aimed at increasing the litter survival in sows, and other to treat salmon, are used in animals that are a food source and there have been no effects shown when consuming these meats (Giese, 2012).

2.3.5.6 **Different delivery systems of DNA vaccines**

DNA uptake by the vaccinated host's cells *in vivo* is inefficient due to the fact that these vaccine formulations lack the structures utilized by viruses to infect cells. The concentration of DNA vaccines have to be high enough that it protect the plasmid from enzyme degradation and also to increase the ability of the vaccine to transfect the cells of the host (Liu, 2011).

There are a variety of different methods used to get the vaccine antigen in contact with the immune system. These can include, amongst others injecting DNA vaccines directly

using a carrier system, and electroporation as well as a gene gun (Okuda *et al.,* 2014). Injecting DNA vaccines directly into the host, also termed “naked” DNA vaccines, is where the DNA is injected intramuscularly (i.m.) into the skeletal muscle which leads to both Th1 and antibody responses (Robinson and Torres, 1997; Dufour, 2001). Another means is intradermal (i.d.) injection into the extracellular spaces or into the cells using a hypodermic needle, which also lead to Th1 responses (Faurez *et al.*, 2010; Okuda *et al.*, 2014). The factors influencing the immune response after i.m. and i.d. injection may be affected by the needle type, muscle type, age of the animal and the speed of injection (Okuda *et al.*, 2014).

The DNA vaccines can also be delivered to the intestines of the host by transforming the DNA vaccine into bacterial vectors such as muted *Salmonella* (*Salmonella enterica serovar typhimurium SL3261*) and administrating them orally. The advantage gained by using bacterial carriers and administering them orally, is that this results in mucosal immunity (Liu, 2011).

Electroporation involves the process whereby pores are temporarily induced in cells by an electric field, permitting the DNA to traverse membranes. This method was shown to increase the immune response in mice (Lei *et al.*, 2011), rabbits (Medi *et al.,* 2005), monkeys (Luckay *et al.*, 2007), sheep (Scheerlinck *et al.*, 2004) and pigs (Sardesai *et al.*, 2012). An increase in the amount of antigen that is produced due to more efficient penetration of cells by the plasmid was also found (Liu, 2011). The disadvantages that come with using this technique are that it is expensive and the apparatus required may limit its use (Liu, 2011; Ferraro *et al.*, 2011).

The gene gun method utilises gold particles coated with the DNA and introducing them, using compressed helium as an accelerant, to shoot them directly into the cells of the host (Okuda *et al.,* 2014). When inside, the DNA dissociates from the beads and the gene it encodes is expressed. The advantage of this method is its effectiveness as it takes advantage of the molecular weight and the safety of gold. Using mice as model, naked needle injections require about 2-20 µg DNA per animal, whereas the gene gun only requires 1-3 µg DNA to evoke an effective immune response (Okuda *et al.,* 2014). The disadvantages of using the gene gun method is that it is expensive and difficult to develop (Lin *et al.*, 2000; Okuda *et al.,* 2014).

2.3.5.7 **Dose requirements of DNA vaccines**

Biodistribution studies showed that the number of plasmid DNA molecules surviving to transfect the target cells after i.m. injection was only a small fraction of the total DNA injected (Oshop *et al.*, 2002). The amount of antigen produced from this was in the picogram to nanogram range. Despite this, an efficient antibody response was still induced (Oshop *et al.*, 2002). It is believed that 90% of the DNA vaccine administered never reaches the cytoplasm and of the 10% that does reach it, less than 1% enters the nucleus in order to be expressed (Babiuk *et al.,* 2003).

The dose administered is dependent on the route of delivery, type and age of the animal. The dose typically administered to birds ranges from 0.5-1500 µg when injected intramuscularly (Kodihalli *et al.*, 1997; Oshop *et al.,* 2002). According to Dunham, (2002) the amount of DNA required for i.m. administration in mice is 10-100 µg, 100-300 µg in smaller animals and 500-2500 µg in larger animals. This is much higher when compared to the gene gun method where the concentration ranges from 0.1-1 µg of plasmid. Animals usually receive a booster vaccination after about 3 weeks (Dhama *et al.*, 2008). The dose administered to different animals is given in Table 2.1.

Table 2.1 DNA vaccine dose administered to different animals.

Animal	Dose (µg)	Reference	Boosting
Pigs	4000	(Gorres *et al.*, 2011)	3 weeks
Pigs	400	(Borrego *et al.*, 2011)	2 weeks
Monkey	1250	(Raviprakash *et al.*, 2006)	Every 4 weeks
Monkey	1500-5000	(Schadeck *et al.*, 2006)	(Every 4 weeks) x2
Cattle	1000	(Skinner *et al.*, 2003)	3 weeks
Cattle	150	(Capozzo *et al.*, 2011)	4 weeks
Turkey	200-750	(Chen *et al.*, 2013)	2 weeks
Turkey	100	(Vanrompay *et al.*, 1999)	3 weeks
Duck	200	(Yao *et al.*, 2010)	2 weeks
Duck	250-750	(Triyatni *et al.*, 1998)	(Every 3 weeks) x2
Mice	100	(Corr *et al.*, 1996)	(Every 2 weeks)
Mice	25	(Muthumani *et al.*, 2008)	(Every 2 weeks)
Chickens	100	(Fang Yan *et al.*, 2013)	2 weeks
Chickens	100	(Song *et al.*, 2010)	1 week
Chickens	200	(Gong *et al.*, 2013)	(Every 2 weeks) x3
Dogs	100-1500	(Bergman *et al.*, 2003)	(Every 2 weeks) x4
Dogs	100	(Osorio *et al.*, 1999)	7 weeks
Horse	200	(Fischer *et al.*, 2003)	4 weeks
Horse	4000	(Ledgerwood *et al.*, 2011)	(Every 4 weeks) x2
Cats	200	(Cupillard *et al.*, 2005)	-
Cats	100-300	(Osorio *et al.*, 1999)	7 weeks
Sheep	100	(Hiszczyńska-Sawicka *et al.*, 2011)	4 weeks
Sheep	100-500	(Kennedy *et al.*, 2006)	4 weeks

2.3.5.8 **The mechanism of action and immune responses elicited by DNA vaccines**

The exact mechanism by which DNA vaccines initiate an immune response is unknown, but there are several theories (Oshop *et al.*, 2002). The responses elicited resemble those of live attenuated vaccines, but without the need for a replicating pathogen (Ferraro *et al.*, 2011). The proposed mechanism of action of DNA vaccines is illustrated in Figure 2.3. When the DNA vaccine is delivered into the muscle, the plasmid uses the host's cellular machinery in order to reach the nucleus or to transfect local cells (Kutzler and Weiner, 2008). There are generally three obstacles to overcome before gene expression: firstly, to cross the plasma membrane; secondly, to cross the cytoplasm; and finally to cross the nuclear membrane. When the DNA vaccine has reached the nucleus, the immunogenic gene is transcribed into mRNA that is transported into the cytoplasm (Liu, 2011). There the mRNA is translated into its encoded protein, which is the required signal needed to activate the immune system for an adaptive immune response (Faurez *et al.*, 2010). Along with the somatic cells, APC such as the dendritic cells are found to be the cell types that are efficiently transfected by the plasmids leading to the production of the encoded antigen (Dunham, 2002; Faurez *et al.*, 2010; Liu, 2011).

Once the APC's are transfected the immune response can follow one of the following paths: it can either lead to a humoral immune response by the activation of the B-cells and subsequent antibody production; or it can lead to a cellular immune response by the activation of CTL as well as Th cells (Oshop *et al.*, 2002; Browning *et al.*, 2011). The ability to raise both antibody, Th and CTL responses is important for the efficacy of the vaccine and both also lead to memory against future infections as well (Robinson and Torres, 1997; Liu, 2011).

2.3.5.9 **Fate of the plasmid DNA after intramuscular injection**

The biodistribution and persistence of DNA plasmids is dependent on several factors such as route of injection, number of injections, amount given, age and type of animal (Schalk *et al.*, 2006; Loots *et al.*, 2006). In a mouse model 90% of intramuscular injected plasmid was found in the muscle of the mice 5 min after injection, and no more than 10% of injected plasmid was present in observed organs at any given time (Levy *et al.*, 1996). Zhang *et al.* (2005) indicated that the highest concentration of plasmid after injection was at the site of injection, but PCR analysis also revealed the presence of DNA in the thymus, spleen, lymph node, kidney, heart, liver and lungs. It has been reported that the concentration of DNA plasmid is at its maximum several minutes after administration and

decreases within hours to trace amounts (Schalk *et al.*, 2006). Faurez *et al*. (2010) indicated that more than 98% of the plasmid DNA is eliminated 9 hours after injection into mice. This was also indicated by Kim *et al.* (2003) where they found that 90 minutes after injection there was less than 1% plasmid left in mice.

The remaining plasmid molecules that do not enter the nucleus for expression are free and have to be eliminated from the body. Endonucleases found in the muscle mediate the degradation of up to 98% of DNA plasmid within 90 min of injection (Faurez *et al.*, 2010). The percentage of plasmid that actually reaches the blood in pigs was found to be below 10% (Faurez *et al.*, 2010). Zhang *et al*. (2005) indicated that the degradation of supercoiled plasmids in mice blood after intramuscular injection was 20.9% after 10 min, 34% after 1 h, 86.8% after 1 day and 97.8% after 1 week. The persistence of the plasmid is also dependant on the age of the animal, Loots *et al.* (2006) showed 4 – 6 week old mice to have a higher expression level when compared to 10 week old mice. On the other hand, it was found that the plasmid can persist for 54 days in sheep, 10 weeks in turkeys and for more than 2 years in mice (Faurez *et al.*, 2010).

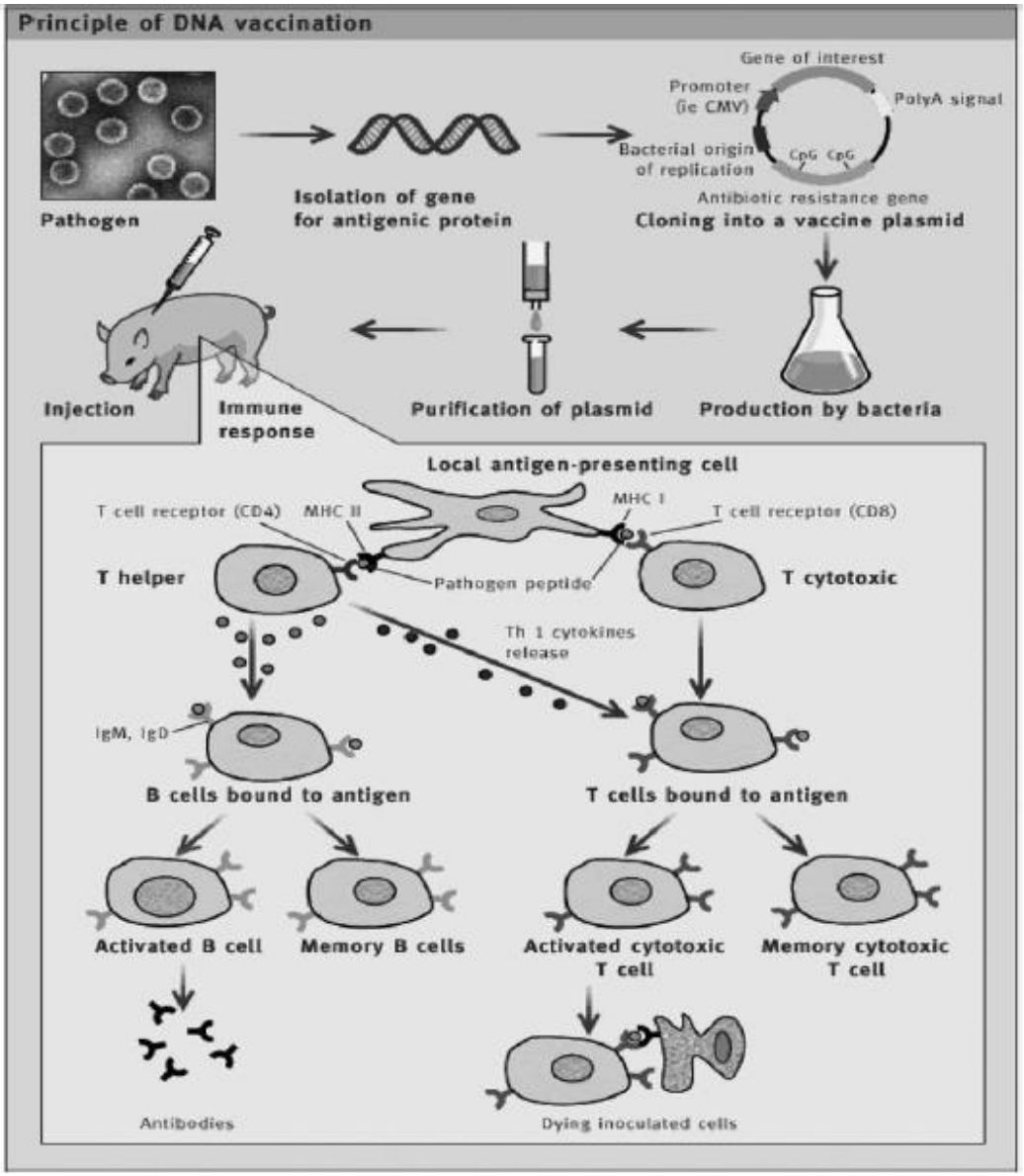

Figure 2.3 The proposed mechanism of action of a DNA vaccine. A candidate gene is identified from a pathogen, isolated and subsequently inserted into a eukaryotic DNA expression plasmid. This is then administered to the host and can lead to both a humoral immune response by the activation of the B-cells and subsequent antibody production or it can lead to a cellular immune response by the activation of T-helper and cytotoxic T-lymphocyte cells (CTL). Taken from Dufour, 2001).

2.4 The characteristics, metabolism and pathogenicity of mycoplasmas

2.4.1 Introduction

The term mycoplasmas was first used in 1889 by Albert Bernhard Frank in his description of the structures in the root nodules of legumes due to their morphological similarities to fungi (*mykes*-fungus; *plasma*-formed) (Razin, 2010). It was then used in 1898 by Nowak to describe an organism causing bovine pleuropneumonia, which was later classified as

Mycoplasma mycoides (Adamu *et al.*, 2013). However the first mycoplasma was cultivated *in vitro* by E. Dujardin-Beaumetz in 1900 (Razin, 2010; Taylor-Robinson and Jensen, 2011). The first isolation of mycoplasmas only came in 1981 when Tully *et al.* successfully isolated a mycoplasma from two men with non-gonococcal urethritis which were analysed and termed *M. genitalium.*

The on-going research on mycoplasmas is bringing us much closer to the goal of defining the minimal machinery of a self-replicating cell (Razin *et al.,* 1998; Manhart *et al*., 2011). The fact that they lack a cell wall makes them ideal for studies on membrane structure and function. Unique characteristics that identify mycoplasmas are their small genome, low guanine + cytosine (G+C) content, cholesterol requirement for growth, optimum growth at 37°C as well as being very host specific (Kleven, 2008).

2.4.2 Origin and taxonomy

Mycoplasmas are prokaryotic organisms that are classified as part of the family Mycoplasmataceae within the order Mycoplasmatales (Razin *et al.*, 1998). The genus *Mycoplasma* is a member of the Class *Mollicutes* of which the name is derived from the latin ‘mollis’ meaning soft and ‘cutis’ meaning skin (Razin *et al.*, 1998). Mycoplasmas are taxonomically distinguished from their closest relatives by their minute size and the lack of a conventional bacterial cell wall. They are therefore known as “naked bacteria” as they are only encapsulated by a single plasma membrane (Razin *et al*., 1998; Rottem, 2003; Kleven, 2008; Taylor-Robinson & Jensen, 2011). It is only since the introduction of 16S rRNA gene sequence data for phylogenetic inference, that mycoplasmas can today be defined as descendents of Gram-positive bacteria (Rottem, 2003; Razin & Hayflick, 2010; Thompson *et al*., 2011). It was suggested that ancestral mycoplasmas arose from the genus *Streptococcus* about 600 million years ago (Razin, 2010; Taylor-Robinson and Jensen, 2011). Today more than 200 species of the genus *Mycoplasm*a are recognised that are distributed over several taxonomic groups in the class *Mollicutes* and more are still being added (Bradbury, 2005; Le Roux & Hoosen, 2010; Razin & Hayflick, 2010; Adamu *et al*., 2013). Due to the specificity of the mycoplasmas they usually get their name from either their host, the site of infection or alternatively, symptoms associated with infections.

2.4.3 Cell size and morphology

Mycoplasmas are the smallest self-replicating organisms known with sizes as small as 0.2 – 0.7 µm (Le Roux and Hoosen, 2010). *M. genitalium* is the smallest with a size of

0.6 – 0.7 µm in length and 0.3 – 0.4 µm wide. They were initially thought to be viruses due to the fact that they could pass through filters meant to trap bacteria, but were later accepted as bacteria when viruses were better defined in the 1930's (Freundt, 1979; Le Roux and Hoosen, 2010; Taylor-Robinson and Jensen, 2011; Thompson *et al.*, 2011).

The absence of a cell wall is what gives *Mycoplasma* species unique properties such as formation of the peculiar fried-egg shaped colonies, sensitivity to osmotic shock as well as resistance to antibiotics that are targeting the cell wall for destruction (Morozumi *et al.*, 2010; Razin & Hayflick, 2010; Taylor-Robinson & Jensen, 2011). The absence of a cell-wall allows for close proximity between the adherent mycoplasma cell and the cytoplasmic membrane of the host.

It is proposed that a mycoplasma has a simple cellular structure comprising of a nucleoid, ribosomes and a double stranded circular DNA molecule in a cytoplasm surrounded by a plasma membrane that is about 10 nm thick. The cytoplasmic membrane consists of lipids, cholesterol and a large number of proteins. The membrane and membrane associated proteins play an important role in the importation of nutrients that are used for the growth and function of the cell (Le Roux and Hoosen, 2010). The membrane associated lipoproteins that are found include the cytoadhesins that play a role in the motility of the mycoplasma as well as its virulence (Adamu *et al.*, 2013).

Mycoplasmas can have many different shapes with the most dominant shape being a sphere due to the fact that it is only surrounded by a cell membrane. Others include helical filaments, pear-shaped or flask-shaped cells with a terminal tip structure and filaments of varying length (Razin *et al.*, 2010). The fact that these different cell shapes can be maintained without a cell wall indicates the presence of a cytoskeleton. Detergent treatment of a variety of mycoplasmas reveals the presence of filamentous threads and rods. The cytoskeleton is also important in cell division (Razin *et al.,* 2010).

2.4.4 Genome

Research done on mycoplasmas in the 1960s and 1970s regarding cell structure, genome size and metabolic pathways has led us to recognize that mycoplasmas are the smallest and simplest self-replicating organisms found today (Razin *et al.,* 2010). The size of the mycoplasma genome can range from 0.58 – 2.20 Mb, compared to *Escherichia coli* with a size of 4.64 Mb (Rottem, 2003). *M. genitalium* was the organism with the smallest known genome size of 580 kb until *M. parvum,* a non-pathogenic mycoplasma was found in pigs. It was identified by Nascimento *et al.* in 2013 and has a genome size

of 0.56 Mb. Due to their small genome, mycoplasmas were one of the first bacteria of which the genome was fully sequenced (Manhart *et al.*, 2011). The genome sizes may vary not only within the genus, but also among strains of the same species. This may be due to the frequent occurrence of repetitive elements, consisting of segments of protein genes that differ in size and number.

A small genome size gives mycoplasmas limited metabolic options for replication and survival that makes them dependent on their host for their required nutrients (Glass *et al.,* 2006). Despite this small genome size, there are studies that suggest that the genome still carries double the number of genes that are required for minimal cellular function (Glass *et al.,* 2006). The essential genes are described as the genes that are indispensable for the survival of an organism and are therefore the basis of life for a particular organism. Glass *et al.* (2006) identified 382 genes of the *M. genitalium* genome to be essential. These essential genes include coding regions for DNA replication, transcription, translation, DNA repair, cellular transport and energy metabolism (Le Roux and Hoosen, 2010).

The genomes of mycoplasmas are also characterised by their low G+C content, possessing considerably less guanine and cytosine bases than adenine (A) and thymine (T). The G+C content of mycoplasma ranges between 24% and 33% (Razin *et al.*, 1998; Le Roux & Hoosen, 2010). Another unique characteristic of mycoplasmas is their use of the UGA codon that codes for tryptophan instead of a stop codon as in the universal genetic code (Taylor-Robinson and Jensen, 2011).

2.4.5 Distribution

Mycoplasmas in nature are widespread parasites living on reptiles, fish, mammals and humans and more hosts are being discovered regularly (Razin *et al.* 1998; Thompson *et al.* 2011). Mycoplasmas enter a specific host where they can multiply and survive for long periods of time (Rottem, 2003). The mucus surfaces of the urogenital and respiratory tract, the eyes, alimentary canal, joints and mammary glands are the primary environments for human and animal mycoplasmas. Christensen *et al.* (1994) found that mycoplasmas can survive on absorbent surfaces (wood, cotton) rather than smooth surfaces. The highest survival was on feathers followed by cotton, hair and feed.

Mycoplasmas are said to be host specific and usually exhibit a strict tissue or host specificity, which is reflected in their exact nutritional requirements as well as parasitic mode of life (Kleven, 1998). However, several mycoplasmas are found in different hosts

or tissues to hosts where they are known for to be. One example is of *M. pneumonia* which is known to cause respiratory disease in humans, but was also found to infect and cause similar symptoms in chimpanzees, albeit that chimpanzees are the closest relatives of humans (Razin *et al.*, 1998). The specificity of mycoplasmas towards their host makes them very difficult to cultivate in a laboratory where their usual host supply of specific nutrients needs to be imitated.

2.4.6 Ostrich-infecting mycoplasmas

In 2005, Botes *et al.* identified three unique *Mycoplasma* species infecting ostriches in the main ostrich producing areas in South Africa and these were provisionally named Ms01, Ms02 and Ms03. In 2009, Langer described two mycoplasmas which were isolated from ostriches in Namibia. The 16S rRNA sequences of these isolates matched those of Ms01 and Ms03 and the names *Mycoplasma struthionis* sp. nov. and *Mycoplasma nasistruthionis* sp. nov. respectively, were subsequently proposed for them.

Langer (2009) determined that *M. struthionis* sp. nov. isolated from lungs and *M. nasistruthionis* sp. nov. isolated from the nostrils of ostriches appear to have typical fried egg-shape colonies that are non-helical and non-motile. They were both determined to have a requirement for serum and cholesterol for growth and were also filterable through a membrane with a pore size of 0.22 µm (Langer, 2009).

From a phylogeny derived from 16S rRNA sequences (Figure 2.4) it appears that the three ostrich-infecting species fall into two different clades, but all three form part of the hominis group (Botes *et al.*, 2005). In this phylogeny, Ms02 and Ms03 are more closely related to each other than to Ms01. The genomes were sequenced using 454 pyrosequencing technology and a draft genome was obtained (estimated to have 99.7% accuracy). A summary of the genome information of the three ostrich specific mycoplasmas is given in Table 2.2.

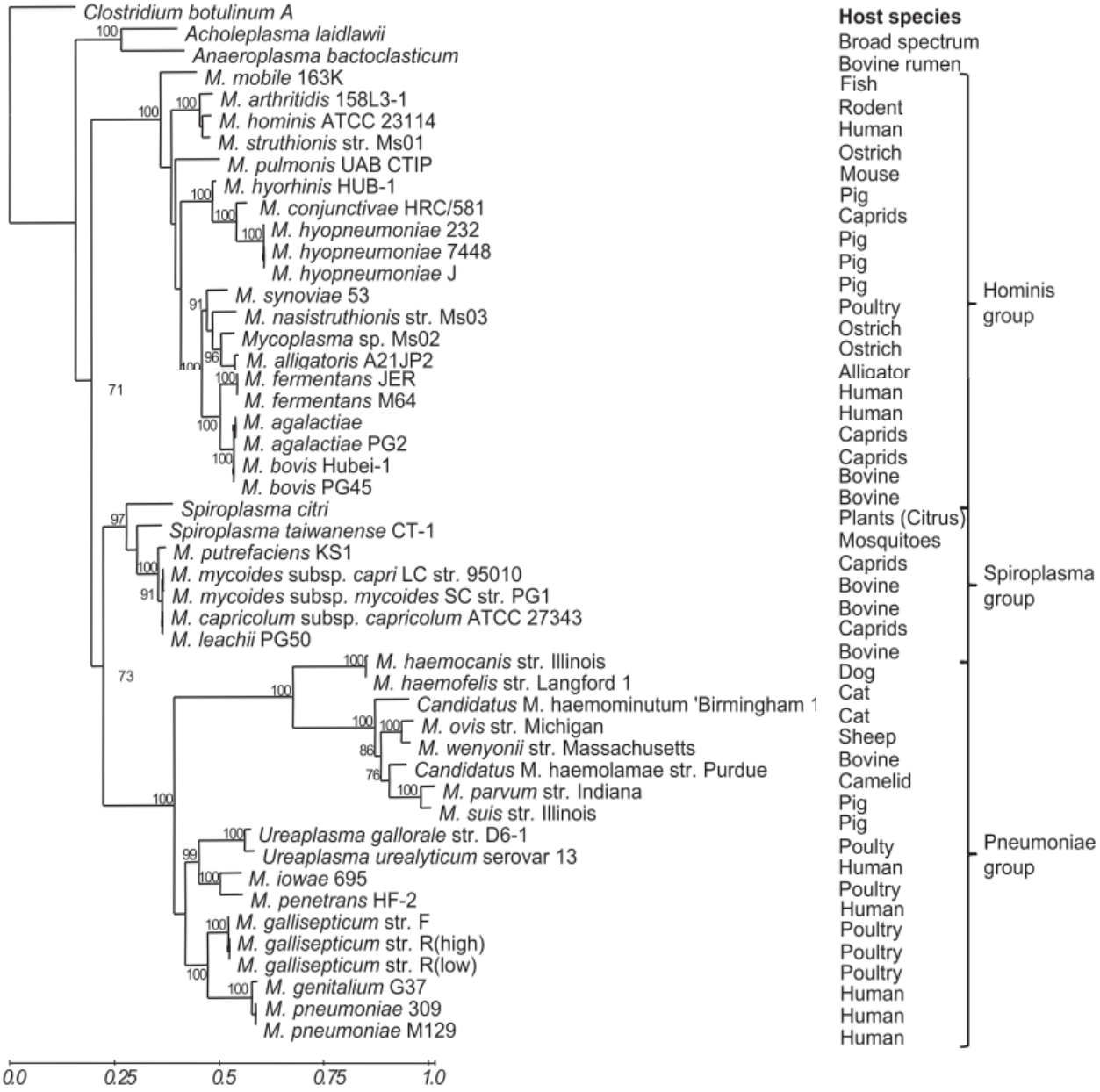

Figure 2.4 The phylogeny of different *Mycoplasma* species based on 16S rRNA sequences. The three ostrich-specific *mycoplasma* species fall within the hominis group (adapted from Wium *et al.*, 2015).

Table 2.2 The genomic information of the three ostrich-infecting *Mycoplasma* species

***Mycoplasma* species**	**Approximate Genome sizes (kbp)**	**G+C content**	**Authors**
Ms01	700	27.0%	(Pretorius, 2009)
Ms02	900	32.0%	(Steenmans, 2010)
Ms03	900	28.7%	(Wium *et al.*, 2015)

2.5 Metabolism of mycoplasmas

2.5.1 Introduction

Mycoplasmas have adopted a parasitic lifestyle within eukaryotic hosts from which they obtain their nutrient requirements (Hames *et al.,* 2009). This has led to a degenerative genome evolution and resulted in a severe genome reduction with a loss of certain essential pathways for the production of macromolecules. The absence of essential pathways make them dependent on the host for the supply of cholesterol, amino acids, fatty acids, purines and pyrimidines (Henrich *et al.,* 1999). Pathways specific to the pathogen and absent in the host are classified as unique pathways, and the essential proteins involved in these pathways can be considered as potential vaccine targets. Butt *et al*. (2012) identified 67 essential proteins in *M. genitalium*, of which six were mapped to unique pathways and 61 to common pathways.

Mycoplasmas lack a complete tricarboxylic acid cycle as well as quinones and cytochromes and therefore do not use oxidative phosphorylation as an adenosine triphosphate (ATP)-generating mechanism. For this reason, the energy yielding pathways of mycoplasmas produce low ATP yields (Razin *et al.*, 1998). Based on their carbohydrate metabolism they are divided into fermentative and non-fermentative organisms. Fermentative mycoplasmas gain energy by means of the pyruvate dehydrogenase pathway and they produce acids from carbohydrates. Non-fermentative mycoplasmas gain energy by the arginine di-hydrolyse pathway (Razin *et al.*, 1998). It has been reported that *M. struthionis* sp. nov. uses arginine from its host as energy source, whereas *M. nasistruthionis* sp. nov. uses glucose as its energy source (Langer, 2009).

Mycoplasmas still contain a large number of genes that code for proteins that transport essential nutrients from the host into the organism. Three different types of transport systems were found in *M. genitalium* namely the ATP-Binding cassette (ABC-transporters), the phosphoenolpyrovate-dependent sugar phosphotransferase (PTS) transport system and facilitated diffusion by transmembrane proteins (Razin *et al.*, 1998). The genes that encode for many of the lipoproteins found in the membrane appear to be in operons encoding for ABC-transporters (Browning *et al.,* 2011; Adamu *et al.,* 2013).

2.5.2 ABC-transporters

The ABC-transporters represent one of the largest superfamilies of active membrane transport proteins. They are found in all species, from the smallest microbe to man and

are of special significance in mycoplasmas (Henrich *et al.*, 1999; Higgins, 2001; Linton, 2007). However, there are fundamental differences between prokaryotic and eukaryotic ABC-transporters and therefore major sequence differences in the genes that encode them. The presence of ABC-transporters in *Mollicutes* was first indicated by Dudler *et al.* (1998) when he was studying *M. hyorhinus.* He found a deduced amino acid sequence to have significant homology with ABC-transporter proteins. Nicolás *et al.* (2007) found that ATP-dependent transporters represent more than 50% of all the membrane transport systems and ABC-proteins represent up to 91.7% of all the ATP-dependent proteins found in mycoplasmas. The basic unit of an ABC-transporter consists of five core domains: two hydrophobic transmembrane domains (TMDs) facilitating the translocation of molecules, two highly conserved hydrophilic ATP-binding domains, which bind and hydrolyse ATP thus supplying energy for the translocation of molecules, and a peptide binding domain that is associated with surface-anchored lipoproteins (Higgins, 2001; Nicolás *et al.*, 2007; Linton, 2007; Locher, 2009). The exact mechanism of this action remains unclear and various mechanistic models have been proposed.

The ABC-transport system can be classified into two main classes: carbohydrate transporters and the di- and oligopeptide transporters (Gao *et al.,* 2012). It was first discovered to be involved in the import of nutrients but is now known to import and export a variety of large and small substrates such as sugars, peptides, toxins, inorganic ions, complex polysaccharides, proteins and metabolic waste products (Razin *et al*., 1998; Higgins, 2001). ABC-transporters are very specific towards their substrates and there is an ABC-transporter for essentially every type of molecule that crosses the membrane. Although most have high substrate specificity, there are some that are multi-specific, such as the oligopeptide transporter which can handle all di- and tripeptides (Higgins, 2001). There is an increased interest in the ABC-transporters of mycoplasmas due to their potential as targets for the development of vaccines (Nicolás *et al.*, 2007).

2.5.3 The *opp* operon of mycoplasmas – structure and function of OppA

The oligopeptide permease (Opp) ABC-transporters are known for transporting oligopeptides not only for cell nutrition but also for signalling processes (Hopfe and Henrich, 2004; Nicolás *et al.*, 2007). Figure 2.5 shows an illustration of the Opp transporter. The transport system is a multicomponent system which comprises of two homologous pore-forming TMDs (OppB and OppC), two homologous cytoplasmic ATP-binding domains (OppD and OppF) that function in binding and hydrolysing ATP and an

extracellular substrate binding domain (OppA). Henrich *et al.* (1999) found that OppB and OppC carry six transmembrane-spanning segments and a hydrophilic motif in their C-terminals. Interestingly, OppF appears larger in size in mycoplasmas compared in other bacterial species. The cytoplasmic domains are considered to be the motors to transport substrates across the membrane for which purpose ATP is utilised (Hopfe and Henrich, 2004; Hopfe and Henrich, 2008). The genes that encode for OppA, B, C, D and F domains are often found within a single polycistronic operon which is under the control of a single promoter (Henrich *et al*., 1999; Nepomuceno *et al.*, 2007; Hopfe & Henrich, 2008).

OppA is considered to be the surface-exposing domain and plays a role in substrate binding but also, in the case of *M. hominis*, cytoadherence (Henrich *et al.,* 1999). The OppA proteins are unique within prokaryotes, differing in their amino acid sequence (Gao *et al.*, 2012). Hopfe *et al.* (2004) stated that the OppA of *M. hominis* acts as the main ATP hydrolase on the surface of the cell since it contains an ATP-binding P-loop in the C-terminal region of OppA. A lysine residue within the P-loop structure was found to be involved in ATP hydrolysis as well as the binding of nucleotides. A tryptic digestion pattern reveals that the *M. hominis* OppA has a high affinity for ATP and ADP and less affinity for GTP and CTP, and only has a high ATPase activity and very low GTPase (8%) and CTPase (6%) activity (Henrich *et al.*, 1999; Hopfe and Henrich, 2008). The *opp* operons have been identified within the respective genomes of ostrich infecting mycoplasmas: Ms01 contains only one *opp* operon and Ms02 and Ms03 each contains two (Wium, 2015).

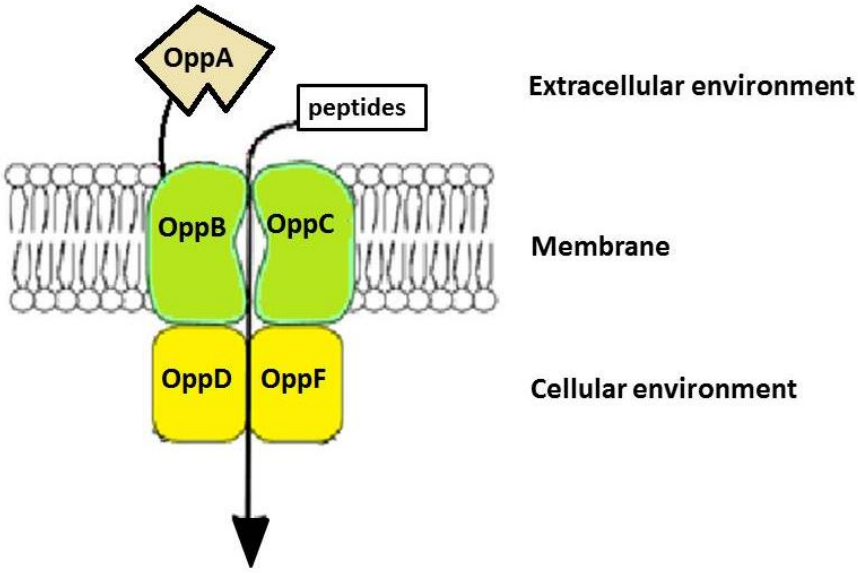

Figure 2.5 Schematic illustration of the different protein domains that make up the oligopeptide permease transport (Opp) system (http://www.uark.edu/campus-resources/mivey/ressum.html).

2.6 Mycoplasma pathogenicity

A variety of *Mycoplasma* species have been shown to be pathogenic to humans, animals, plants, and insects and particularly intracellular pathogens in animals such as poultry and swine (Thompson *et al.*, 2011). Interactions between the mycoplasma and its host, as well as the host and its environment, can lead to disease causing states within the host. Environmental factors that can influence the immunity of the host and therefore its susceptibility to respiratory infections caused by mycoplasmas include temperature, ventilation, humidity, atmospheric ammonia as well as dust (Kleven, 1998).

Mycoplasmas have over the years developed numerous ways of interacting and attaching themselves to the host in order to breach the host's defences to gain its nutrition. For this to occur mycoplasmas first have to enter the host (Rottem, 2003; Bradbury, 2005; Browning *et al.*, 2011). Mycoplasmas are said to enter the host mainly through inhalation, but can spread from one generation to the next through the eggs. Cross-infection from the colonised air sac to the adjacent ovaries of the female poultry animals can infect the developing embryo, which leads to egg transmission. In *M. meleagridis* and *M. iowae,* which infects turkey, they can also spread through the venereal route (Bradbury, 2005).

After the mycoplasma has entered the host it has to identify its target tissue. Mycoplasmas attach predominantly to the epithelial surfaces of the respiratory or urogenital tract. After attaching itself, the pathogen must multiply and acquire nutrients from the host whilst evading the host's immune systems. With the limited number of

genes in mycoplasmas as well as the limited survival time outside the host, this seems a daunting task (Rottem, 2003; Bradbury, 2005).

Mycoplasmas possess a unique way of movement which has been termed 'gliding motility'. It is this gliding motility of the mycoplasma that enables the organism to reach its target tissue as well as allowing the pathogen to breach the host's defences and immune systems (Taylor-Robinson and Jensen, 2011). These defences include the mucin layer in the respiratory tract and ciliary activity. *In vitro* studies have also shown that the gliding ability of the mycoplasma is influenced by the age of the culture as well as the incubation medium and temperature thereof (Le Roux and Hoosen, 2010).

Entering different non-phagocytic cells of the host can be advantageous to the pathogen, as it can escape many of the immune response mechanisms developed by the host against extracellular pathogens (Bradbury, 2005). The exact mechanism whereby mycoplasmas enter the cell is still unclear, some internalise by using a unique tip like structure, others internalise without them (Razin *et al.*, 1998).

2.6.1 Adherence to the target tissue

After the pathogen has reached the target tissue, the next step in successfully infecting the host is achieved by adhering to the tissue of the host. This step is considered to be the first in pathogenesis and a prerequisite for colonisation and infection (Rottem, 2003; Hopfe and Henrich, 2008; Le Roux and Hoosen, 2010). The adherence of mycoplasmas has been well documented. Due to the absence of cell wall associated structures that are involved in adhesion, this process is mediated through the cell membrane bound components termed the adhesins (Le Roux and Hoosen, 2010).

Some *Mycoplasma* species have developed unique features called tip structures to adhere to the target tissue (Browning *et al.*, 2011). In *M. genitalium* the expression and translation of both the MgPa and P32 proteins, which make up the terminal tip structure, are required to successfully adhere to the host (Rottem, 2003; Taylor-Robinson and Jensen, 2011). Some mycoplasmas have a flask-like shape that is formed by specialised terminal-tip structures which help them to not only adhere to the host but also functions as the leading end in *M. pneumoniae* gliding-type motility (Rottem, 2003; Bradbury, 2005). The cytoskeleton is of significant importance in that it functions in localising adhesins, gliding motility as well as to adjust the shape of the mycoplasma cell (Razin *et al.*, 1998). There are also unique adhesin-like proteins that have been developed by species that enable them to bind to the host. These proteins are not adhesins, but are

required by the organism for cytoadherence (Rottem, 2003; Taylor-Robinson and Jensen, 2011).

Mycoplasmas can attach to a wide range of tissues, for example *M. genitalium* that has shown to attach to the following cell types: erythrocytes, fallopian tube cells, respiratory cells and spermatozoa (Le Roux and Hoosen, 2010). Specific enzymes have also been found to play a role in attachment of the organism. Alvarez *et al.* (2003) found that the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plays a role in the attachment of *M. genitalium* in human vaginal and cervical mucin diseases. Here the GAPDH acts as a ligand to the mucin and fibronectin receptors (Rottem, 2003; Le Roux & Hoosen, 2010).

2.6.2 Invasion of the host

After respiratory specific mycoplasmas successfully adhere to the epithelial tissue of the respiratory tract, they have to invade the respiratory system of the host. They do this with the assistance of other respiratory proteins. After adhesion, these mycoplasmas can penetrate the epithelial cells of the host by causing the membrane of the target cell to invaginate into the target cell similar to the clathrin-coated pit mechanism of endocytosis. Once the organism reaches the inside of the cell, it appears to reside in membrane-bound vacuoles close to the target cell nucleus. This process can take up to 30 minutes in *M. genitalium* (Le Roux and Hoosen, 2010). The basis of mycoplasmal invasion is the ability to bind fibronectin or sulphated polysaccharides, forming a molecular bridge between the mycoplasma and host cell surface proteins (Rottem, 2003; Taylor-Robinson and Jensen, 2011). Several invasive bacteria have shown that when they come into contact with the host cell surface they trigger cytoskeletal rearrangements which facilitate bacterial internalisation. The lack of a mycoplasma cell wall allows for direct contact between the mycoplasma membrane and the cytoplasmic membrane of the host, which can lead to invasion of the host cell (Rottem, 2003).

2.6.3 Evasion of the host immune system

The pathogenesis of mycoplasmas is dependent on close contact between the organism and the host, the ability to evade the host immune system, adapt to the environment and change phenotypically (Le Roux and Hoosen, 2010). The ability of mycoplasmas to sustain and persevere in the presence of the host's immune system can be achieved in a few unique ways. Some mycoplasmas enter the host to protect themselves from the innate immune system (Rottem, 2003). It is thought that the mycoplasma can undergo

molecular mimicry and antigenic variation. The *Mollicutes* do this by generating intragenomic variation in nucleotide sequences or DNA arrangement at selected chromosomal loci of membrane proteins expressed on the cell surface. They can do this without compromising the function of the specific protein (Le Roux and Hoosen, 2010). There are unique multigene families encoding for proteins that are involved in generating antigenic variation, phase variation as well as antigenic switching, allowing for immune invasion during an infection. Examples of such proteins showing antigenic variation include the P140 and P110 cell surface protein and the PvpA surface antigen. Alternatively the organism can also mimic the host cell antigens as a survival mechanism (Bradbury, 2005; Le Roux and Hoosen, 2010).

2.6.4 Damage to the host

When the mycoplasma has successfully attached itself to the host and therefore infected it, the mycoplasmas can cause injury and cytopathic effects (Rottem, 2003). This can involve the innate and acquired immune responses. Most of the *Mycoplasma* species attach themselves to the epithelial surfaces of the host which is then the main area of damage to the host. As mycoplasmas are dependent on the host for their nutrition, they compete for biosynthetic precursors that can disrupt the host cell integrity and alter host cell function (Rottem, 2003). Nonfermenting mycoplasmas utilise the arginine di-hydrolyse pathway for their source of ATP, which depletes the host of arginine thereby affecting protein synthesis and host growth (Rottem, 2003). In mycoplasmas that use glycerol as their energy source, the metabolism of glycerol can lead to host damage through hydrogen peroxide production by glycerol-3-phosphate oxidase (Hames *et al.*, 2009).

The attachment of these mycoplasmas to the host surface also affects various host cell processes such as membrane receptors, transport mechanisms and K^+ channels. During the fusion process some toxic material and mycoplasma components such as various superoxide radicals, ammonia, hydrolytic enzymes and nucleases of the adhering mycoplasma cause the membrane to become vulnerable and therefore affect the normal function of the cell (Rottem, 2003; Bradbury, 2005)

2.7 Pathogenicity of the ostrich-infecting mycoplasmas

In ostriches, mycoplasmas lead to disease states associated with respiratory tract infections causing inflammation of the trachea, nostrils and air sacs (Botes *et al.,* 2005). Mycoplasma infections in ostriches also have indirect consequences such as a reduction

in physical growth rate, downgrading of carcasses, increased veterinary costs and also increasing their susceptibility to secondary infections of pathogens such as *E. coli* and other Gram negative bacteria (Botes *et al.*, 2005). These secondary infections can lead to an increased mortality rate. The clinical symptoms associated with these infections in ostriches include nasal exudates, swollen sinuses, foamy eyes and shaking of their heads. Factors such as the weather, stress and an underdeveloped biosecurity can enhance the occurrence of these infections. Infections are especially high during the months of the year with extreme temperature fluctuations and during the cold and windy winters (Botes *et al.*, 2005).

2.8 Control of mycoplasma infections

The financial impact poultry mycoplasmas have on poultry production has motivated the development of methods to control these infections. The control of mycoplasma infections, specifically in poultry, can be divided into three categories: control by prevention, medication and vaccination.

2.8.1 Control by means of prevention

An important consideration in prevention is the creation of an effective biosecurity system whereby animals infected with mycoplasmas are separated from uninfected animals. The vast market demand for different poultry species has forced farmers to increase their flocks through increased farming densities (Dr. Adriaan Olivier, research veterinarian, Klein Karoo international, personal communication). The problem thus arises that these animals live in such close proximity to each other that it can increases the chance of mycoplasma spread. It is thus of importance to have a consistent monitoring system for early detection of mycoplasma infections. It is preferred to rather monitor fewer samples more frequently, than to measure more samples less frequently (Glatz *et al.*, 2005). Studies were performed on the survival of mycoplasmas on different materials, and in 1994 Christensen *et al.* found that mycoplasmas can survive for two days on rubber and cotton, indicating the importance of using disinfectant foot baths as well as changing of overalls when moving between different flocks. It was also found that the mycoplasmas could survive longer on the unwashed hair of workers.

Mycoplasmas can spread horizontally, meaning mainly through the air from one animal to another and can quickly infect the entire flock if the infected animals are not removed. After these infected animals are separated from the flock it is important to house the remaining uninfected animals in a different cage. Some *Mycoplasma* species can survive

in the environment for longer periods that can lead to an indirect infection. These mycoplasmas can also form biofilms in drinking water, increasing their environmental survival (Glatz *et al.*, 2005). It is therefore important to clean the cage where these animals were housed before reintroducing any new animals (Glatz *et al.*, 2005).

Mycoplasmas can also be transmitted vertically, meaning from one generation to the next through the eggs (Kleven, 2008). A process called 'egg dipping' is where the eggs get dipped or can be injected with antibiotics in an attempt to destroy mycoplasmas. This is an essential step in preventing the spread of the disease to subsequent generations. In some cases if the genetic lines or grandparent flocks are infected, the entire flock will have to be eliminated. Although it can be a costly process, it is still more favourable than to treat the animals and have the risk that the mycoplasma infections will reoccur (Kleven, 2008).

2.8.2 Control by means of medication

Another manner of treating infected animals is by means of medication. The use of antibiotics started in the 1950's to assist with the increasing demand for food (Allen *et al.*, 2013). The fact that mycoplasmas lack a cell wall makes them resistant to any β-lactamic antibiotics that target the cell wall, such as penicillin (inhibits Gram-positive and negative bacteria), cephalosporins and thallium acetate (Razin *et al.*, 1998; Morozumi *et al.*, 2010). Antibiotics are mainly used to treat eggs to minimise egg transmission of mycoplasmas (Kleven, 2008). They can, however, also be administered to the birds. Some antibiotics such as enrofloxaxin or tilmicosin are highly effective, but are not approved for use in infected breeding stock.

Antibiotics such as Oxytetracycline, Doxycycline, Chlortetracycline, Tylosin and Lincomycin have been used with success in treating ostriches with mycoplasma infections, but at a cost. Once the bird is infected, secondary infections often worsen its condition and increase the combinations of medication needed. The different antibiotics also have limited periods in which they can be administered. Using antibiotics can potentially reduce the clinical signs of mycoplasmas as well as spreading to neighbouring flocks, but often do not completely eliminate infections. This should therefore not be considered as a long term solution to control mycoplasma infections. In addition to this, the continuous usage of antibiotics can result in antibiotic resistance of the *Mycoplasma* species (Kleven, 2008).

2.8.3 Control by means of vaccination

The best way to control *Mycoplasma* infections in animals is by preventing infections through immunisation (Makela, 2000). To date there have been various attempts to generate vaccines against mycoplasmas especially in chickens. Commercially available live attenuated vaccines against *Mycoplasma gallisepticum* (MG) include the ts-11 (ts11MG), 6/85 attenuated vaccines and F-strain (FMG) that are for use in chickens (Whithear, 1996; Nicholas *et al.*, 2009; Jacob *et al.*, 2014). A commercially available live vaccine against *Mycoplasma synoviae* (MS) includes the strain MS-H (Bayatzadeh *et al.*, 2014). The inactivated vaccines (Bacterins) used today include the MG-Bacterin (MG-Bac) and MS-bacterin (MS-Bac) that consist of a concentrated suspension of the MG and MS that are emulsified in an oil adjuvant (Whithear, 1996). Various other adjuvants can also be used such as aluminium hydroxide, liposomes and iota carrageenan. A Bacterin for MG has been licensed in the United States (Nicholas *et al.*, 2009). Subunit vaccines are less frequently used than live and attenuated vaccines. There are some subunit vaccine developed using the MGC1 and MGS2 proteins and were evaluated against MG in chickens (Moura *et al.*, 2012).

2.8.4 Control of mycoplasma infections in ostriches

Controlling mycoplasmas infections in ostriches has proven to be expensive. Monitoring these infections within a commercial ostrich farm starts with a biosecurity system whereby monitoring and early identification of the pathogen are of utmost importance. The biosecurity system further includes: decreasing any stress causing factors, to clean their enclosures and water troughs regularly, and to have an area that provides shelter for the birds against wind and rain. It is also important to monitor the weight of each bird regularly. When the symptoms are visible, sick birds have to be separated from the rest of the flock. This generally also determines the type and period of treatment given (Botes *et al.*, 2005; Dr Adriaan Olivier, personal communication).

The use of antibiotics further does not eliminate infections, but only contains them (Dr. Adriaan Olivier, research veterinarian, Klein Karoo international, personal communication). Thus animals that do not exhibit symptoms of infections are found but they act as carriers of mycoplasma thereby exacerbating the problem.

The financial and environmental implications of antibiotic use have necessitated an investigation into alternative methods of controlling mycoplasma infections in ostriches by means of vaccination.

Live attenuated and inactivated whole organism mycoplasma vaccines (MS-Bac and MG-Bac) for use in chickens were found to be ineffective in treating mycoplasmosis in ostriches (Pretorius, 2009). The Bacterin vaccines that are suspended in oil emulsion further produces granulomas in ostriches. To date there is no vaccine registered for use against mycoplasma infections in ostriches. The production of traditional live or inactived whole organism vaccines is problematic given the difficulty and expense of cultivating the ostrich-infecting mycoplasmas in large quantities (Johan Gouws, Laboratory manager, Onderstepoort Veterinary Institute, personal communication). These problems can, however, potentially be overcome by the development of suitable DNA vaccines.

2.9 The progress of DNA vaccine development against ostrich-infecting mycoplasma Ms01

The availability of genome information of an organism allows the identification of potential antigen genes. The genome of *M. struthionis* sp. nov. str. Ms01 was previously partially sequenced (Pretorius, 2009), and the *oppA* gene identified as an antigen gene. The *oppA* gene codes for the substrate binding domain (OppA) of the oligopeptide permease transport system, as previously described. The OppA protein serves as a good potential antigen as it is predicted to be highly antigenic and is also a surface protein making it easier for the immune system to recognize it. Binding of antibodies to the OppA protein could inhibit the function of the OppA and therefore the flow of oligopeptides into the organism. This could in turn lead to cell death. In *M. hominis* the OppA protein also functions as the cytoadherence domain of the organism. If this is also the case for the Ms01 OppA protein, antibodies could prevent cytoadherence and in turn infections. Thus it can be concluded that the Ms01 OppA protein has an excellent potential to serve as a vaccine candidate gene.

The TGA codon encodes for tryptophan in mycoplasmas, whilst TGA is a termination codon in all other organisms (Yamao *et al.*, 1985). This can therefore lead to early termination of the gene containing these TGA codons in *E. coli* as well as in the vaccinated animal leading to an ineffective vaccine. These TGA codons therefore need to be modified to TGG codons by means of site directed mutagenesis (SDM) in order for the correct translation as a tryptophan residue within mycoplasma (Simionatto *et al.*, 2009). The *oppA* gene of Ms01 was previously cloned by Pretorius (2009). Ten TGA codons were identified by sequencing and these were modified to TGG codons by SDM (Pretorius, 2009).

2.9.1 Vaccine vectors pCI-neo, VR1012 and VR1020

Three DNA vaccines each containing the *oppA* gene of Ms01 were previously prepared by Pretorius (2009). The DNA vaccine vectors used were pCI-neo, VR1012 and VR1020 (Figure 2.6). The pCI-neo (Promega) expression vector contains a cytomegalovirus (CMV) immediate-early enhancer/promoter region promoting constitutive expression of the antigen gene within mammalian cells. The antigen gene was inserted into the multiple cloning site (MCS) downstream of the enhancer/promoter. It also contains a T7 and T3 RNA polymerase promoter, f1 ori of replication, SV40 polyadenylation signal, neomycin phosphotransferase gene and an ampicillin resistance gene. The SV40 late polyadenylation signal helps to increase the steady state of RNA. The neomycin phosphotransferase gene is a selectable marker for mammalian cells and is under the regulation of the SV40 enhancer and early promoter region (pCI-neo manual, Promega).

VR1012 and VR1020 (Vical Incorporated) both contain a polyadenylation sequence downstream of the MCS, a prokaryotic origin of replication and a kanamycin resistance gene. Both also express genes using a strong eukaryotic CMV promoter (Hartikka *et al*., 1996; Smooker *et al.,* 1999). The VR1020 plasmid has a tissue plasminogen activator (tPa) signal sequence upstream of its cloning site. This assists with the secretion of newly expressed proteins (Lobo *et al.*, 1999; Luke *et al*., 1997).

The ability of these DNA vaccines to elicit an anti-OppA immune response has previously been tested in vaccination trials, but due to unforeseen avian influenza infections, possible inefficient dose and handling of ostriches, results were limited (Brandt, 2012; van Tonder, 2013).

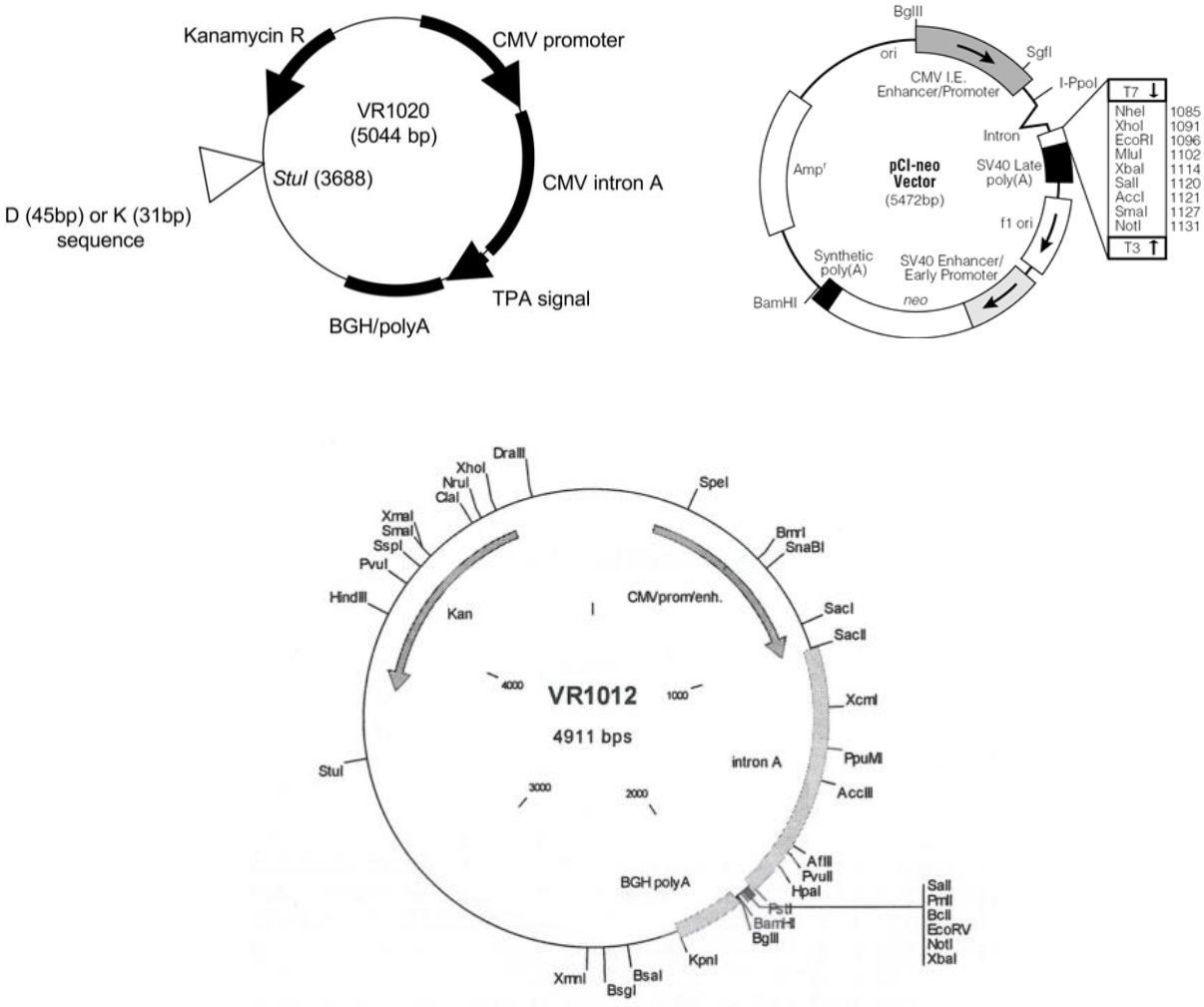

Figure 2.6 The vector maps of the three DNA vaccines pCI-neo, VR1012 and VR1020 used in the development of DNA vaccines.

2.10 *In vitro* expression of DNA vaccines

2.10.1 Animal cell culture

The term cell culture is the removal of prokaryotic and eukaryotic cells from *in vivo* plants or animals and subsequently growing them in a favourable artificial environment *in vitro*. The process further involves these single cells to act as independent units. These cells are either removed from tissues directly or they can be derived from an already established cell line or cell strain (Butler, 2005; Gibco & Thermo Fisher Scientific Handbook, 2015).

The first evidence of eukaryotic cell growth was when Wilhelm Roux maintained embryonic chick cells in saline solution (Butler, 2005). The beginning of cell cultivation *in vitro* began in 1907 when Ross Harrison, who many believe to be the father of cell culture, was first to indicate the maintenance and growth of frog nerve cells in a hanging drop for

up to 30 days (Harrison, 1912). A big drawback until 1955 was the stringent sterility controls needed until Earle and Eagle developed a chemically defined medium namely Eagle's minimum essential medium (EMEM) that could replace the biological fluids used (Butler, 2004). The first therapeutic protein from recombinant mammalian cells to be obtained for market approval was the human tissue plasminogen activator in 1966 (Kretzmer, 2002).

Today animal cell culture is an essential laboratory technique to study biochemical and physiological processes and up to 70% of all pharmaceutical recombinant proteins are produced in mammalian cells. One of the advantages of mammalian cell culture is the consistency and reproducibility of results obtained due to the fact that one can control the conditions of growth. In addition to this the culture normally contains one type of cell that may be genetically identical. Animal cells, however, are more vulnerable than bacterial cells and therefore require extreme sterile conditions as well as specific nutrients (Wurm, 2004; Butler, 2005).

2.10.2 Cell choice

For successful transfection, the choice of cell line is of significant importance to obtain efficient expression and should be highly susceptible to transfection. There are generally two types of cells available namely continuous cell lines and primary or finite cell lines. Normal cells can only divide a limited number of times before losing their ability to proliferate, known as senescence. A primary cell line refers to cells that only have a limited senescence. Some cell lines can become immortal through the process of transformation. Continuous cell lines are primary cell lines that undergo transformation where after they have the ability to divide indefinitely (Gibco & Thermo Fisher Scientific Handbook, 2015).

Among the most widely used cell lines for small scale expression are the African green monkey kidney CV-1 and COS cell lines (Aruffo, 1998). The COS-1 and COS-7 cell lines developed by Yakov Gluzman in 1981 were derived from the African green monkey CV-1 cell line that was integrated into the chromosomal DNA of COS cells. Before integration, the CV-1 cell line was transformed with an ultraviolet-irradiated, replication-defective SV40 virus (Strayer, 1996; Aruffo, 1998). Plasmids containing the SV40 ori and that express the SV40 large T antigen (TAg) can replicate to a high copy number in these cell lines (Pham *et al*., 2006). For large scale expression and industrial usage the Chinese

hamster ovary (CHO) and human embryonic kidney 293 (HEK 293) cells are commonly used (Pham *et al*., 2006; Baldi *et al.,* 2007).

In this thesis the expression of the OppA protein of Ms01 by different vaccine vectors was evaluated in a COS-1 cell line. In mycoplasma the OppA is a membrane bound protein. COS-1 cells were used since they are generally easy to transfect and have been used in various studies expressing membrane bound proteins (Zhou *et al.*, 1994; Harding *et al.*, 1999; Rios-Esteves & Resh, 2013).

2.10.3 Transfection

Cell transfection is the process in which plasmid DNA is introduced into a growing cell culture, leading to the expression of the plasmid DNA by the cells. This is usually done using a specific transfection reagent (Wurm, 2004; Pham *et al.*, 2006). The main aspects that influence transfection are the cell line, expression vector, DNA plasmid quality, transfection agent and medium (Pham *et al.*, 2006).

The expression of proteins usually follows a well-established scheme (Wurm, 2004). The gene of interest is inserted into a plasmid vector where after it can be multiplied within and DNA isolated from *E. coli* cells. A specific eukaryotic cell-line is cultured in media until it is growing at its optimal reproducibility. The plasmid is thereafter used to transfect the cell line together with a transfection agent where after the cells are grown kept in a controlled environment. After transfection the cells are lysed and the expressed proteins are detected in the cell lysate (Wurm, 2004; Matasci *et al.,* 2008).

2.10.4 Plasmid expression vectors

The choice of plasmid plays an important role in the production of recombinant proteins. A strong promoter/enhancer is the biggest influence driving the transcription of the gene (Matasci *et al.*, 2008). The CMV promoter is considered very powerful and highly active in most of the mammalian cell lines (Aruffo, 1998; Pham *et al.*, 2006). The presence of introns between the promoter and the coding sequence can also improve the expression by increasing the mRNA stability and export from the nucleus.

The quality of the plasmid DNA can also influence the transfection efficiency and therefore the expression of the inserted gene. Before transfecting it is important to verify whether the plasmid DNA is supercoiled as this conformation gives the highest transfection efficiency and therefore should produces the highest level of expression.

Some of the plasmid DNA will be converted into linear DNA inside the nucleus by the active endo- and exonucleases (Wurm, 2004).

2.10.5 Cell culture medium

The choice of medium can influence the efficiency of the transfection. The main function of the medium is to create the optimum artificial environment that will replicate the physicochemical environment for the cells so that they can grow and divide. The medium supplies the cells with the essential nutrients such as amino acids, vitamins, minerals, growth factors and hormones (Gibco & Thermo Fisher scientific Handbook, 2015). It also regulates the pH, temperature and osmotic pressure for optimum growth. Either serum free or medium containing serum can be used. Bovine serum is mostly used for the propagation of mammalian cells (Wurm, 2004; Matasci *et al.*, 2008;).

For optimal and rapid cell growth it is important to subcultivate (or passage) the cells whenever the cell confluency reaches 90%. Subcultivation involves transferring the cells to a new vessel with fresh growth medium. This will ensure that there is sufficient room for continued growth and also to remove the cell waste material (Gibco & Thermo Fisher scientific Handbook, 2015). The COS-1 cell line grows rapidly, therefore it requires a passage every 3-4 days (Aruffo, 1998).

2.10.6 Detection of expressed protein

Western blotting involve the use of highly sensitivity-enhanced chemiluminescence substrates, a technique developed by Mattson & Bellehumeur in 1996. These chemiluminecent techniques can detect protein levels in the femtogram range with high signal to noise ratio, making them more sensitive than the common colorimetric detection methods (Alegria-Schaffer *et al*, 2009). Chemiluminescence involves a chemical reaction whereby energy is released in the form of light. For example, when luminol is oxidised in the presence of a horseradish peroxidase (HRP) conjugated secondary antibody and peroxide (Alegria-Schaffer *et al.*, 2009).

2.11 ELISA

The enzyme-linked immunosorbent assay (ELISA) was first described independently by Engvall and Perlmann in 1971 who first termed immunoassays employing labelled enzymes, ELISA (Engvall and Perlmann, 1971; Clark *et al.*, 1986). Today there are various types of ELISA's available, which include amongst others: direct, indirect and sandwich ELISA's. For the purpose of this study, the focus will be on the indirect ELISA

method, which provides quantitative results and has a high sensitivity and specificity for high molecular weight proteins (Voller *et al.*, 1978). Figure 2.7 illustrates the indirect ELISA assay.

An indirect ELISA works on the basis of a microtitre plate being coated with an antigen by passive adsorption and covalent linkage (Snyder *et al.*, 2014). A microtitre plate is used as it contains 96-wells and therefore can accommodate 96 samples in a single analysis. There are two types of Nunc 96-well microtitre plates that are generally used for ELISA the Maxisorp and the Medisorp. Both plates are polystyrene surface treated, with Maxisorp having a high affinity for polar groups and Medisorp binding molecules of an intermediate hydrophobic/hydrophilic nature. The addition of a positive control at relevant antigen concentration levels is also recommended by the European Medicines Agency.

After the antigen binding step, the wells are saturated with a blocking agent, which will bind to all the areas that are not bound by the antigen. This is to prevent any non-specific binding of the secondary antibodies. This is followed by washing away the entire unbound blocking agent, a step that is essential in minimizing background. The serum (primary antibody) being tested during the study is thereafter incubated with the adsorbed antigen. This will allow the antibodies, in the serum, to attach to the antigen. After an appropriate incubation time the unbound serum components are washed away. This is followed by the secondary antibody that will specifically detect the primary antibody. Different detection methods are available, and one such method is the biotin/avidin method. Biotin is conjugated to the secondary antibody by a hydroxysuccinimide ester. The secondary antibody conjugated to biotin is left to incubate with the antigen-primary antibody complex. This will result in the secondary antibody attaching to the primary antibody. Washing again will remove any unbound antibody. With the biotin/avidin detection method, avidin in now added. Biotin has a high affinity for avidin which is a tetrameric protein that contains four binding sites. This results in more than one biotin molecule being bound to the avidin thereby increasing the signal. Streptavidin, that is isolated from *Streptomyces*, has the same affinity for biotin as avidin and is preferred as it is not glycosylated, and thereby less non-specific binding occurs (Chaiet and Wolf, 1964; Berman and Basch, 1980; Nerurkar *et al.*, 1984). The avidin can be conjugated to horseradish peroxidase (HRP), which will result in the subsequently added substrate being converted to a coloured product. This colour change can be measured using a

spectrophotometer and is an indication of the amount of conjugate bound, which is proportional to the primary antibody level in the serum sample (Voller *et al*., 1978; Snyder *et al.,* 2014).

There are a wide variety of substrates that can be used for ELISA that include amongst others: tetramethylbenzidine (TMB) and *p*-Nitrophenyl Phosphate (pNPP), both yielding yellow products that absorbs light at 405 nm (Caponi and Migliorini, 1999). In the case of HRP, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is an excellent substrate that gives a green product that absorbs light at 405 nm (Hay and Westwood, 2003).

For the determination of antibody responses elicited in a host, ELISA is an excellent assay due to its specificity, sensitivity, rapidity and cost effectiveness (Alegria-Schaffer *et al.*, 2009).

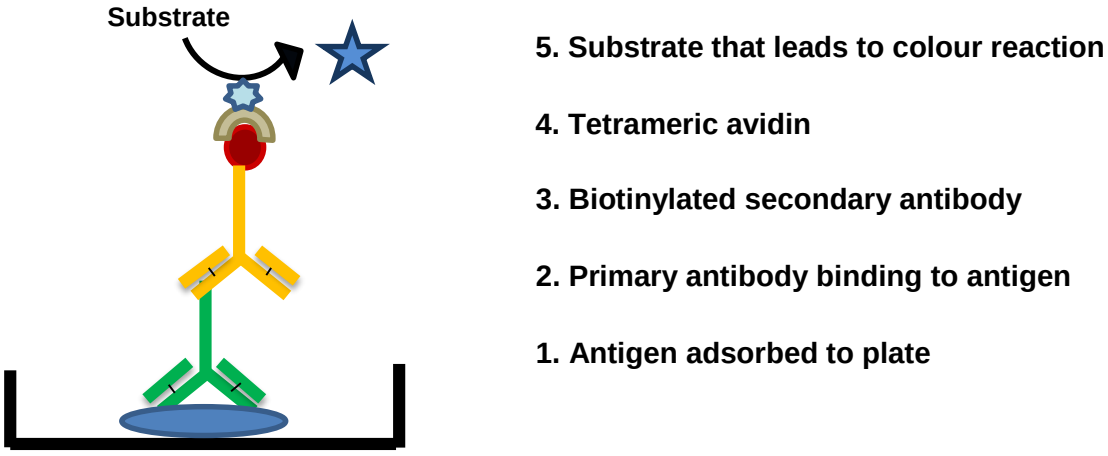

Figure 2.7 A schematic illustration of an indirect ELISA assay. The antigen is firstly incubated on the plate to bind to the plate by passive adsorption. Secondly the primary antibody binds specifically to the antigen. This is followed by a secondary biotinylated antibody that attach to the primary antibody. The addition of the substrate would lead to a colour reaction that is emitted.

3 Expression, isolation and the purification of the OppA protein of *Mycoplasma struthionis* sp. nov. str. Ms01 and antibody production in rabbits.

3.1 Introduction

The primary goal of this study was to re-evaluate the ability of previously developed DNA vaccines to elicit an immune response in ostriches. However, the *in vitro* expression of the *oppA* gene by these DNA vaccines was firstly determined using a cell culture system (Chapter 4). Assessing the *in vitro* expression of the *oppA* gene required a western blot analysis for which primary anti-OppA antibodies were needed. Furthermore, the ability of the DNA vaccines to elicit an immune response (Chapter 5) was assessed using an enzyme-linked immunosorbent assay (ELISA) whereby the humoral immune response of vaccinated ostriches was determined. For this ELISA, purified Ms01 OppA protein was required for coating purposes. Both the recombinant expression of OppA in *E. coli* using a pGEX plasmid containing the *oppA* gene, and the production of antibodies in rabbits against this recombinant OppA is described in this chapter.

3.2 Materials and methods

3.2.1 Expression of the Ms01 OppA-GST protein in *E. coli* cells

The o*ppA* gene was previously cloned into the prokaryotic pGEX-4T-1 (GE Healthcare, United Kingdom) expression vector and transformed into *E. coli* BL21(DE3)pLysS cells (Promega, USA), after which they were stored as freezer cultures at -80°C (van Tonder, 2012). To ensure high levels of protein expression, freezer stocks were not stored for longer than 6 months. For *E. coli* BL21(DE3)pLysS cultivation, Luria-Bertani/ampicillin (LB/amp) Petri dishes were prepared by adding 3.15 g bacteriological agar to 250 ml LB-medium (2.5 g Bacto-Tryptone, 1.25 g Yeast extract, and 1.25 g NaCl). After autoclaving, the mixture was allowed to cool before adding 100 µg/ml ampicillin. One hundred microliters of BL21(DE3)pLysS cells containing the pGEX_*oppA* were plated onto the LB/amp agar and incubated overnight (16-18 hours) at 37°C. BL21(DE3)pLysS cells containing the pGEX-4T-1 plasmid without the *oppA* gene were used as controls.

Colony PCR was used to verify the presence of the plasmid in the cells. The specific primer pairs are listed in Table 3.1. They were used to amplify the multiple cloning site (MCS) of the plasmid. All PCR reactions were carried out in 10 µl volumes containing 1 µl 10x Reaction Buffer, 0.4 µl dNTP (5 mM), 0.6 µl $MgCl_2$ (25 mM), 0.5 µl Pgex_F forward

primer (20 pmol/µl), 0.5 µl Pgex_R reverse primer (20 pmol/µl) (all primers/ sequences were obtained from the vector map and synthesised by Integrated DNA Technologies(IDT), United states) and 0.1 µl Supertherm Taq (JMR holdings, United States) (5 U/µl). As positive control 0.5 µl diluted (1/500) pGEX containing the Ms03 *oppA* gene was used. The thermal cycler was programmed to perform 25 cycles of each 94°C (30 sec), 55°C (30 sec), 72°C (30 sec) followed by an additional 7 min at 72°C. PCR products were separated on a 1% agarose gel. A 0.1 µl/ml Gelred™ Nucleic Acid Gel Stain (Biotium, USA) was used for UV visualisation of PCR products.

For protein expression a 14 ml Falcon® tube containing 5 ml Terrific broth-medium (Bacto-tryptone 12 g/l, Bacto-yeast extract 24 g/l, 2 M glycerol 4 ml/l), supplemented with glucose (20% m/v), ampicillin (100 µg/ml), chloramphenicol (34 mg/ml) and 100 ml/l phosphate buffer (1 M, pH 7.8) was thereafter inoculated with two positive colonies identified by colony PCR to contain the pGEX_*oppA* plasmid. Two colonies without the pGEX_*oppA* plasmid were used as controls, containing the same medium in a separate Falcon® tube. Both where incubated overnight at 37°C on an orbital shaker (150 rpm).

Expression cultures were made with 100 ml and 10 ml supplemented TB-medium for the pGEX_*oppA* plasmid and the pGEX without the *oppA* gene in Erlenmeyer flasks, respectively. The 100 ml flask was inoculated with 2 ml of the overnight culture and the 10 ml with 200 µl overnight culture. Both were incubated at 37°C on a shaker (200 rpm) until an OD_{600} value of 0.6 was reached. Expression of the plasmid was subsequently induced by adding 400 µl and 40 µl of 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) to the 100 ml and 10 ml volumes, respectively and left to incubate for 8 h. Samples were taken after induction at 2, 4, and 6 hours. The cells were harvested by centrifugation for 10 min at 10 000 x g (Beckman Coulter, Avanti® J-E). The bacterial pellet was resuspended in TEN 50 buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 0.1% Triton X-100, 0.2 M Dithiothreitol (DTT) and 10% (v/v) glycerol).

Table 3.1 pGEX specific primer pairs used for the amplification of the cloned insert and the respective band sizes produced from pGEX plasmids with and without the *oppA* insert.

Primer name	**Sequence**	**Product size (bp)**	
Pgex_F	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	With *oppA*	2979
Pgex_R	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	Without *oppA*	150

The resuspended pellet containing the harvested cells was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to identify whether the OppA-GST protein was expressed. Thirty microliters of resuspended sample was mixed with 30 µl of reducing treatment buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, pH 6.8) and 12 µl bromophenol blue (0.1% (w/v) in 100 mM NaOH). The sample mixture was boiled for 2 min and kept on ice until it was loaded on the gel. As molecular marker a PageRuler™ Unstained Protein Ladder (Thermo Scientific) was loaded containing 14 proteins spanning 10 to 200 kDa in size.

The SDS-PAGE apparatus was set up and filled with electrode buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3) before 20 µl of sample mixture was loaded. The expression products were separated on an acrylamide gel consisting of a 4.5% stacking gel containing 4.25 ml stacking buffer (125 mM Tris-HCl (pH 8.8), SDS (0.1% w/v), 0.75 ml monomer (30% T, 2.7% C), 15 µl TEMED (N,N,N‟N‟-tetramethylene diamine) and 30 µl ammonium persulphate (20% w/v)) followed by an 8% resolving gel, containing 8.8 ml resolving buffer (375 mM Tris-HCl (pH 8.8), SDS (0.1% w/v), 3.2 ml monomer (30% T, 2.7% C), 30 µl TEMED and 36 µl of ammonium persulphate (20% w/v)). The gel was electrophoresed for 2 h 30 min at a constant current of 25 mA. Afterwards the stacking gel was removed from the resolving gel and the latter incubated in staining solution (0.125% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 1 h at 37°C while shaking. Subsequent to that the gel was incubated in destain 1 (50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 1 h at 37°C while shaking before it was incubated overnight at room temperature in destain 2 (5% (v/v) methanol and 7% (v/v) glacial acetic acid).

The theoretical size of the OppA protein was predicted using Compute pI/Mw (Expasy).

3.2.2 Isolation of the expressed OppA-GST protein using a glutathione agarose column

The OppA-GST protein was isolated using a glutathione agarose column (Sigma-Aldrich, United States). The expressed OppA protein contains a GST-tag which binds with a high affinity to the glutathione resin, and can then be eluted from the column with reduced glutathione. The glutathione agarose lyophilized powder was allowed to swell in Milli-Q® water at 200 ml/g for 30 min at room temperature and left overnight at 4°C for complete swelling to occur. The column (10.5 mm in height and 2.5 mm in width) was then packed

with a bed volume of 10 ml and subsequently washed with 10 volumes of phosphate buffered saline (PBS) equilibrium buffer (10 mM phosphate, 150 mM NaCl, pH 7.4) to remove the lactose present in the lyophilized product and left overnight at 4°C.

The OppA protein expressed from the pGEX_*oppA* construct was resuspended in TEN 50 buffer and prepared for isolation by three cycles of freeze thawing (10 min at 37°C followed by 10 min at -80°C). Thereafter the sample was sonicated for 2 sec and kept on ice for 20 sec (repeated 5 times). The sample was then centrifuged at 10 000 x g for 10 min at 4°C (Labnet, PrismR). Four milliliters of clear supernatant were loaded onto the column at 4°C, and allowed to flow through the resin by gravity. It was passed through the column five times and thereafter incubated for 1 h on the column. The column was then washed firstly with 5 volumes of PBS-T (PBS (pH 7.2) and 1% Triton X-100) and followed by 5 volumes of PBS (pH 7.2). The OppA protein that was bound to the glutathione via its GST-tag was eluted with 3 volumes of elution buffer (10 mM reduced glutathione and 50 mM Tris-HCl, pH 9.5) and collected in 1 ml fractions. A total of 20 samples were collected. The resin was then rinsed with 5 volumes of cleaning buffer 1 (0.1 M boric acid, 0.5 M NaCl) followed by 5 volumes of cleaning buffer 2 (0.1 M sodium acetate, 0.5 M NaCl) before it was stored in a storage buffer (2 M NaCl, 0.2% Thiomersal) at 4°C.

A Bradford assay was used to determine the protein concentration of the eluted fractions. A standard curve of increasing protein concentrations 0, 0.25, 0.50, 0.75, 1, 1.25 and 1.5 mg/ml was prepared using bovine serum albumin (BSA) protein and the elution buffer. Five microliters of each isolated sample as well as the standards were pipetted into a 96-well microtitre plate (Greiner Bio-One, Germany) in triplicate. Two hundred and fifty micro litters of Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) phosphoric acid) was added to each well and incubated for 2 min before the absorbance was measured at 620 nm.

3.2.3 SDS-PAGE and western blot analysis of isolated OppA-GST protein

The purity of the isolated OppA protein was tested by 10% SDS-PAGE where after its identity was confirmed using western blotting. The gel assembly and electrophoresis conditions for the SDS-PAGE were the same as described in section 3.2.1. After electrophoresis the gel was transferred to a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, Sigma-Aldrich, United States) using a wet transfer system. The gel and membrane was packed for electrophoresis in the following order: perspex support

plate, sponge, 3 mm chromatography paper (Whatman®, England), SDS-PAGE gel, membrane, 3 mm chromatography paper, sponge and perspex support plate. The western blot apparatus was then set up and filled with electrode buffer (0.05 M Tris-base, 0.2 M glycine, 20% MeOH) and electrophoresis was performed at for 18 h at constant 120 mA. The membrane was then blocked with casein buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.5% casein, 0.02% (w/v) Thiomersal, pH 7.2) for 1 h at room temperature before it was washed with PBS containing 0.1% v/v Tween® 20 (PBS-Tween) 3 times for 5 min respectively at room temperature. A goat anti-GST antibody (GE Healthcare) was used as the primary antibody and diluted 1:10 000 in casein-Tween (casein buffer containing 0.1% (v/v) Tween® 20) in a volume that was sufficient to cover the membrane. The membrane was incubated for 1 h at 37°C while shaking at 6 rpm. The membrane was again washed with PBS-Tween and secondary antibody added. Horse-radish peroxidase (HRP) conjugated rabbit anti-goat antibody (Sigma-Aldrich, United States) was diluted in casein-Tween buffer (1:5000) and incubated for 1 h at 37°C while shaking at 6 rpm. The membrane was then washed three times with PBS-Tween. The substrate used was 0.05% (w/v) 4-chloro-1-naphtol, 16% methanol (cold), 1x PBS (cold) and 0.025% (v/v) H_2O_2 (cold). It was added to the membrane and left to develop while shaking. The development was stopped after 30 min by washing with Milli-Q® water.

3.2.4 Antibody production against OppA in rabbits

The isolated OppA-GST protein was adsorbed to acid treated, naked bacteria that were used as an immune carrier (Bellstedt *et al.*, 1987). To this end 2 mg naked bacteria was suspended in 0.5 ml Milli-Q® water in a 2.0 ml Eppendorf tube before the solution was homogenised using a pestle. The bacterial suspension was transferred to a 20 ml glass vial before the Eppendorf tube was washed with additional 0.5 ml Milli-Q® water that was also transferred to the vial. Thereafter 330 µl of 0.2 mg/ml expressed OppA-GST protein was added to the vial. Subsequently the mixture was dried overnight on a Savant™ SpeedVac™ concentrator (AEC Amersham, RSA). The OppA-GST protein that was now adsorbed to the naked bacteria was resuspended in 2.5 ml PBS before immunisation. All immunizations were done at the Animal Facility, Stellenbosch University, which also includes the ethics. A rabbit was injected with 500 µl of suspension on day 0, 4, 7, 14, 18, 21, 28, 32 and 35. Blood samples (2 ml) were collected before the immunisation started and again on day 14 and 28. On day 42 a final volume of 24 ml was collected. All blood samples were kept at 37°C for 30-45 min after collection and thereafter at 4°C overnight to improve clotting. The blood samples were then centrifuged for 20 min at 3000

x g (Hettich Universal, D-7200 Tuttlingen) and the serum transferred to a clean Eppendorf tube for storage at -20°C.

3.2.5 Determination of anti-OppA antibody levels in rabbit serum

To determine whether anti-OppA antibodies were successfully produced in the rabbit an antigen specific ELISA was developed. To this end two Maxisorp (Nunc, Denmark) 96-well microtitre plates were prepared by coating wells in columns 1-12 of plate one and 1-11 of plate two with the purified OppA-GST protein that was diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6). Concentrations of 1, 2 and 5 µg/100 µl/well were used. Column 12 of plate two was left uncoated to test the specificity of the ELISA and only received carbonate buffer (100 µl/well). The plates were then incubated overnight at 4°C.

On the following day the protein solution was decanted and the plates tapped dry on a paper towel. All of the wells of the 96-well plates were then blocked with 200 µl/well casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.02% Thiomersal and 0.5% casein, pH 7.6) and incubated at 37°C for 1 h. The excess casein buffer was subsequently removed and the plates were tapped firmly on a paper towel before all the wells was washed 3 times with PBS-Tween. The rabbit serum from day 0 (negative control), 28 and 42 was used as primary antibody. One hundred microliters of casein buffer containing 0.1% Tween (casein-Tween buffer) was added to column 1 and 3-11 of plate one and 1-11 of plate two. Thereafter 200 µl of the serum appropriately diluted 1:20 in casein-Tween buffer, was added to column 2 of plate one. A dilution series was then made by taking 100 µl of column 2 (plate one) and adding it to column 3, after mixing 100 µl was transferred to column 4. This was continued up until column 12 of plate one and the last 100 µl volume was transferred to column 2 of plate two. The dilution was continued up to column 11 of plate two and the final 100 µl was discarded. Column 12 of plate two received 100 µl of 1:20 diluted serum.

The plates were incubated at 37°C for 1 h and then washed 3 times with PBS-Tween before the secondary antibody (goat anti-rabbit IgG, Sigma-Aldrich), which was diluted 1:500 in casein-Tween, was added. Columns 1-12 of both plates received 100 µl of diluted antibody after which the plates were incubated for 1 h at 37°C. Plates were again washed 3 times with PBS-Tween. The tertiary antibody, a anti-goat HRP-conjugated antibody was subsequently diluted 1:1000 in casein-Tween, before 100 µl was added to each well. The plates were incubated for 1 h at 37°C and again washed 3 times with PBS-Tween. Substrate solution (0.006 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

(ABTS), 12 ml citrate buffer (100 mM citrate acid monohydrate and 0.1 M trisodium citrate dehydrate, pH 5) and 6 µl hydrogen peroxide (H_2O_2) was subsequently added (100 µl/well). The plates were thereafter left to incubate at 37°C for 30 min before the absorbance was measured at 405 nm on a Labysystem multiscan MS microtitre plate reader.

The anti-OppA antibodies were further analysed using western blotting to determine the ability of these antibodies to detect the OppA protein of Ms01 and the OppA-GST fusion protein. The OppA protein of Ms03 was used as control. The western blot was done similarly as in section 3.2.3 with the following differences. The rabbit anti-OppA-GST was diluted 1:2500, the Goat anti-rabbit secondary antibody (Sigma-Aldrich, United States) was diluted 1:1250 and the tertiary antibody, a anti-rabbit Peroxidase anti-peroxidase (Sigma-Aldrich, United States), was diluted 1:10000. All dilutions were done in casein buffer.

3.3 Results

3.3.1 Expression of the Ms01 OppA-GST protein in *E. coli* cells

Freezer cultures containing the BL21(DE3)pLysS cells with the pGEX_*oppA* plasmid were successfully grown on the LB/amp plates and yielded colonies containing pGEX_*oppA* as identified by colony PCR. Positive and negative colonies were identified as can be seen on the agarose gel electrophoresis image in Figure 3.1. A positive colony PCR produced a band size at about 3000 bp which corresponds to the 2979 bp size of the *oppA* gene. The control colony PCR produced a band size of 150 bp representing the multiple cloning site of pGEX. Two of the positive colonies identified were further used for protein expression.

SDS-PAGE analysis (Figure 3.2) of the samples of the expressed OppA-GST fusion protein produced a band at the appropriate size of 126 kDa (lane 3, 5 and 7) after induction of protein expression at 2, 4 and 6 hours respectively. This indicated that the OppA protein was being expressed since the same band was not visible at the corresponding position in the samples containing the plasmid without the *oppA* gene (lane 4 and 6). The size of the OppA protein was predicted with Compute pI/Mw (Expasy), to be 100 kDa. The OppA protein (100 kDa) is expressed as a fusion protein (126 kDa) with GST (26 kDa).

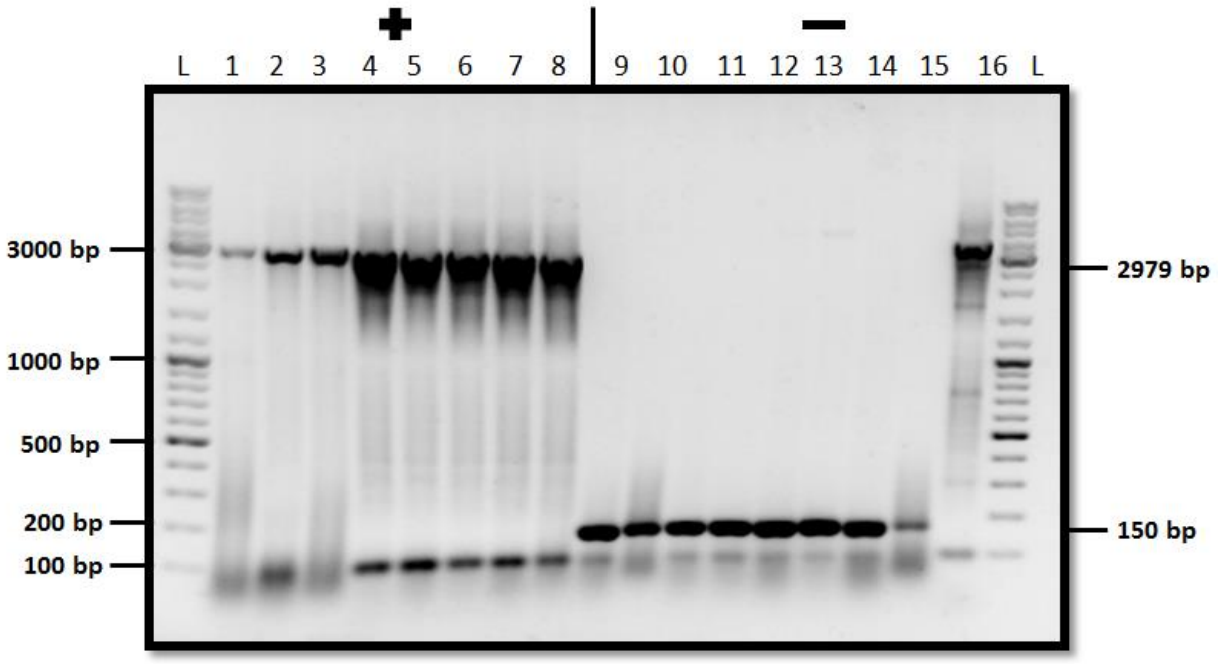

Figure 3.1 An image of a 1% agarose gel depicting the results of a colony PCR using colonies of *E. coli* BL21(DE3)pLysS cells transformed with the pGEX plasmid with and without the Ms01 *oppA* gene. Lane 1-8 shows 8 positive colonies identified that contain the *oppA* insert. Lane 9-15 on the right is 8 negative colonies identified that do not display the *oppA* insert. Lane 16 is a positive control, Ms03-*oppA* in pGEX. Lane L on the left and right contains a GeneRuler™ (Fermentas) ladder containing size standards of 100-10 000 bp. Gelred™ Nucleic Acid Gel Stain 0.1 µl/ml, was used for staining.

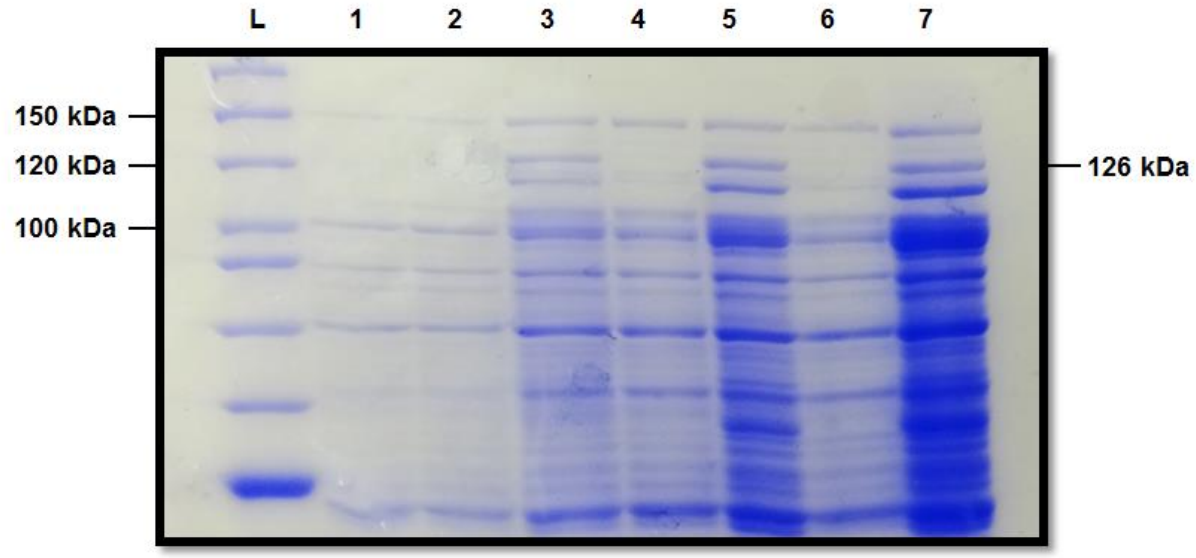

Figure 3.2 An image of a 10% SDS-PAGE gel showing expression of the OppA-GST protein 0, 2, 4 and 6 h after inducing with IPTG. Lane L: PageRuler™ Unstained Protein Ladder (Thermo Scientific). Lane 1: pGEX_*oppA* at 0 h expression. Lane 2: pGEX at 0 h expression. Lane 3: pGEX_*oppA* at 2 h expression. Lane 4: pGEX at 2 h expression. Lane 5: pGEX_*oppA* at 4 h expression. Lane 6: pGEX at 4 h expression. Lane 7: pGEX_*oppA* at 6 h expression.

3.3.2 Isolation of the expressed OppA-GST protein using a glutathione agarose column and the subsequent SDS-PAGE and western blot analysis

The solution containing the expressed Ms01 OppA protein fused to GST was subsequently harvested and loaded on a glutathione agarose column and eluted by the introduction of reduced glutathione. Figure 3.3 shows the elution profile of the 20 fractions collected as analysed using the Bradford assay. From the profile it can be seen that the protein starts to elute from sample 5 and that sample 7, 8 and 9 contained the highest protein concentration; determined as 0.455 mg/ml, 0.436 mg/ml and 0.342 mg/ml respectively. The majority of the bound protein was eluted by the time sample 12 was collected. An SDS-PAGE analysis of elution samples 7, 8, 9 and 10, showed protein bands with sizes of about 126 kDa, corresponding to the size of the OppA protein of 100 kDa attached to the GST of 26 kDa (Figure 3.4). The same band size was present in the sample before chromatography but not after addition to the column, indicating that the OppA-GST fusion protein successfully attached to the glutathione via the GST.

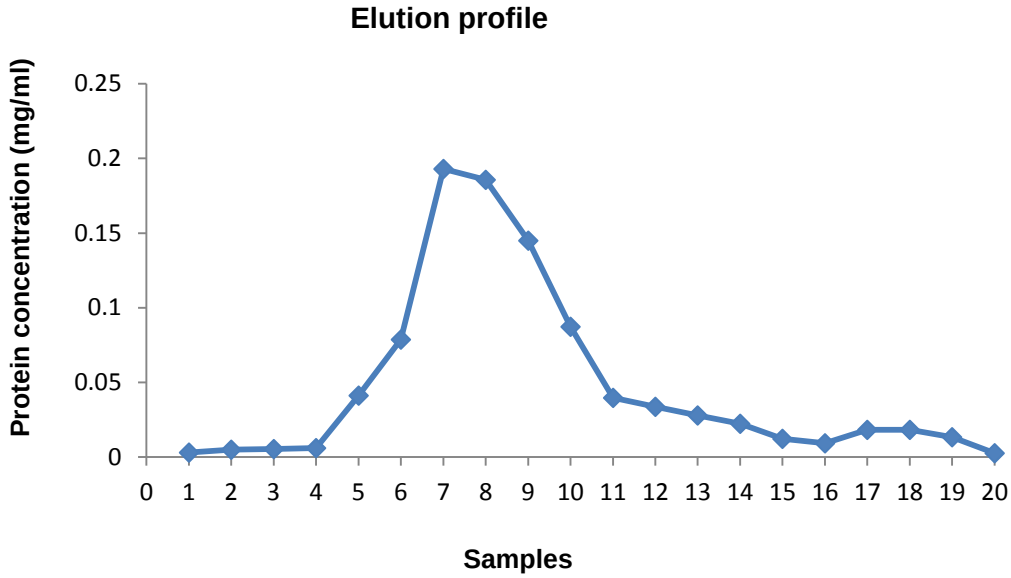

Figure 3.3 Graph illustrating the protein concentrations (mg/ml) as determined by the Bradford assay of the fractions that eluted from the glutathione agarose column onto which the expressed protein solution was loaded. The elution profile indicates that sample 7, 8 and 9 contains the highest amount of purified protein.

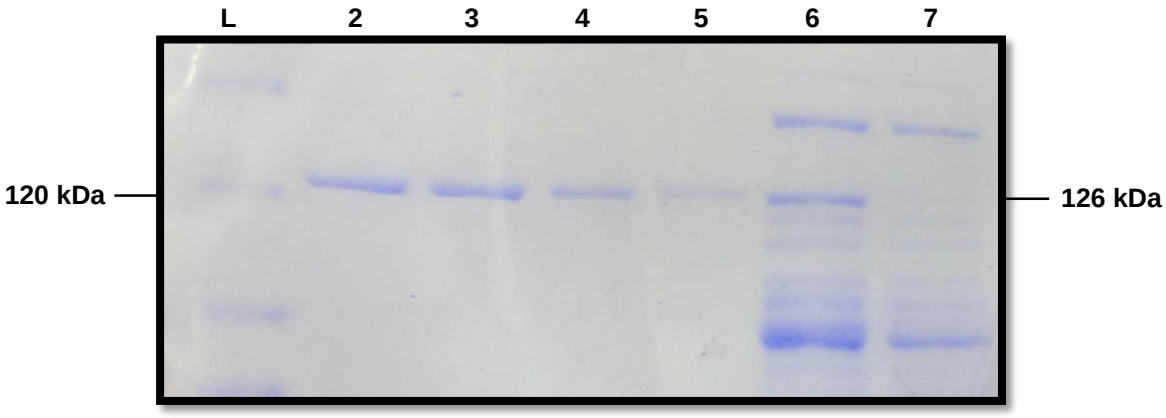

Figure 3.4 An image of an 8% SDS-PAGE gel of OppA-GST protein fractions eluted from the glutathione agarose column. Samples revealed a band at the appropriate size of 126 kDa. Lane L: PageRuler™ Unstained Protein Ladder (Thermo Scientific). Lanes 2-5 are the eluted samples 7, 8, 9, and 10 respectively. Lane 6 was a supernatant sample before it was loaded onto the column and lane 7 a sample taken from the flow through after it was loaded onto the column.

Four elution samples (7, 8, 9 and 10) were subsequently analysed with western blot (Figure 3.5) using an anti-GST antibody and a band at 126 kDa could be seen in all four elution samples (lane 2-5) as well as before addition to the column (lane 6) but not after addition (lane 7). The band size corresponds to the size of the OppA-GST protein.

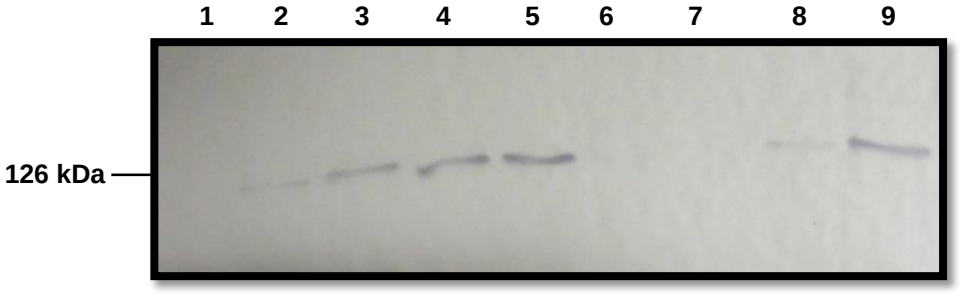

Figure 3.5 Image of a western blot analysis of four OppA-GST samples eluted from a glutathione agarose column. The samples were separated on an 8% SDS-PAGE gel and transferred to a 0.45 µm nitrocellulose membrane. Lane 1: Full range rainbow marker (Amersham), not visible on the figure due to inadequate transfer. Lane 2-5: eluted samples 7-10, respectively. Lane 6: after wash buffer 1. Lane 7: after wash buffer 2. Lane 8: before wash. Lane 9: before binding to the column.

3.1.1 Development and validation of rabbit anti-OppA antibodies

The OppA protein was adsorbed to the naked bacteria and used to immunise rabbits over a period of 6 weeks. Blood samples collected during this period were analysed by ELISA

to confirm the presence of anti-OppA antibodies (Figure 3.6). Column one which did not receive any serum was used as a blank and was colourless. Rabbit serum from day 0 resulted in no colour reaction, which indicated the absence of any OppA antibodies. Using a cut off titre value of 0.1 a colour reaction could be observed for day 28 and 42 serum up to a dilution of 1/327680 on plate two, indicating the presence of rabbit anti-OppA antibodies.

The ELISA results were used to generate a graph (Figure 3.7) as an illustration of the immune response generated by the rabbit in response to vaccination with the OppA-GST protein adsorbed to the naked bacteria. From the graph there is a consistent increase in titre values up to day 28 after which no increase in titre values was observed up to day 42.

The rabbit anti-OppA-GST antibodies were able to detect the Ms01 expressed OppA-GST protein with western blot analysis (Figure 3.8), but not the GST protein on its own nor the OppA-GST protein of Ms03. This indicates the specificity of the antibody towards the OppA of Ms01.

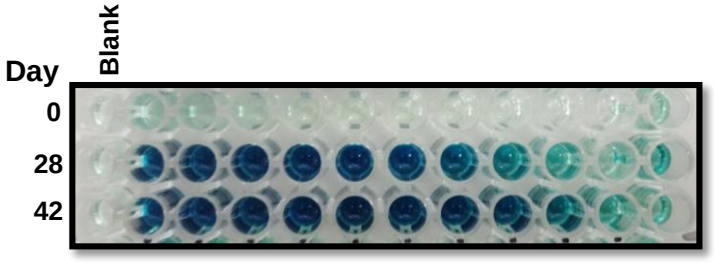

Figure 3.6 A 96 well Maxisorp plate showing ELISA results of rabbit serum immunised with the OppA-GST protein adsorbed to naked bacteria. Serum collected at days 0, 28 and 42 was compared using three concentrations 1 µg/100µl, 5 µg/100µl and 10 µg/100µl purified OppA-GST protein. This was followed by a serial dilution from column 3 up to column 12. Only results for the wells coated with 5 µg/100µl OppA-GST protein are shown.

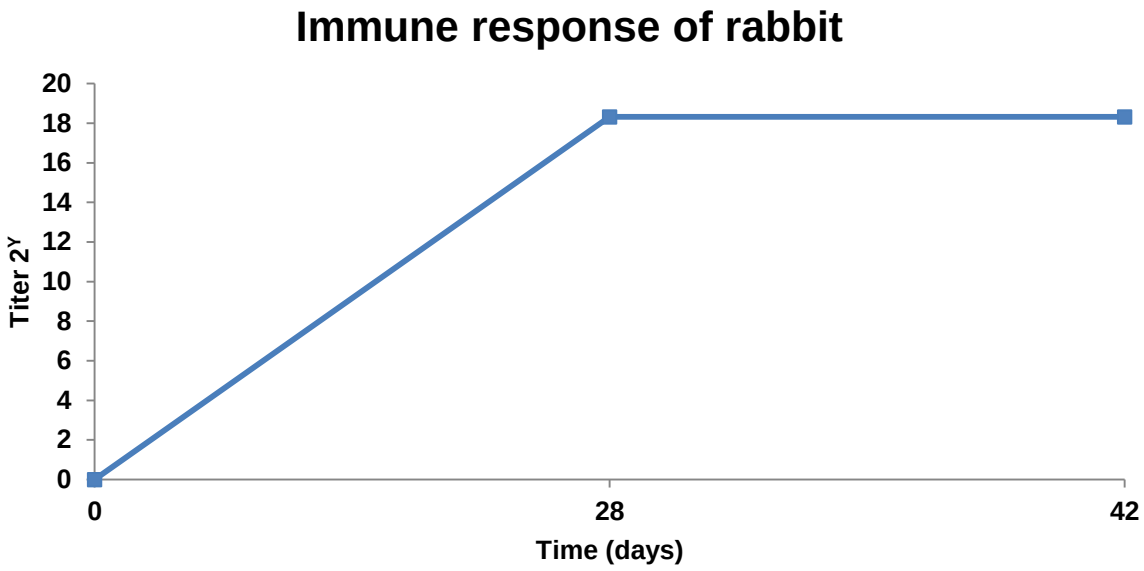

Figure 3.7 A graph that illustrates the rabbit anti-OppA-GST antibody response against the purified OppA-GST protein. Serum collected during days 0, 28 and 42 was compared at a protein concentration of 5 µg/100 µl. The graph illustrates day 0, 28 and 42 when an absorbance (OD_{405}) of 0.1 was reached.

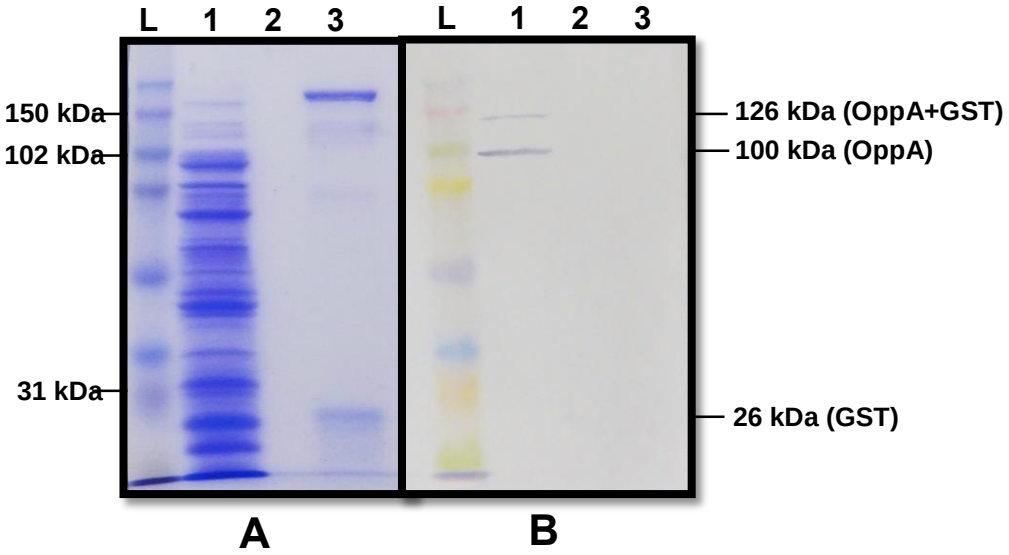

Figure 3.8 The corresponding SDS-PAGE (A) and western blot (B) analysis of the respective Ms01 and Ms03 OppA proteins. Lane 1: Full range rainbow marker (Amersham). Lane 2: Ms01 expressed OppA. Lane 2: open. Lane 3: isolated Ms03 expressed OppA.

3.4 Discussion

Several studies have used the pGEX expression system for successful expression of mycoplasma genes (Okamba *et al.*, 2007; Rosati *et al.*, 2000; Shil *et al.,* 2011). The pGEX system uses a *tac* promoter which allows inducible, high-level expression and synthesis

of proteins that are soluble, correctly folded and covalently bound to a GST moiety on their amino terminus (*GST Gene Fusion System Handbook*, 2002). The presence of a *lacIq* gene in the plasmid prevents expression of the fusion protein until the introduction of IPTG. This results in an increase of expressed fusion protein within the bacterial cytoplasm(*GST Gene Fusion System Handbook*, 2002). By employing the pGEX expression system in this study, successful expression of the OppA protein from Ms01 could be achieved as an OppA-GST fusion protein. A 126 kDa protein product was visible after SDS-PAGE that corresponds to the theoretical size calculated for the OppA-GST fusion protein.

The GST tag allows for the purification of the fusion protein by affinity chromatography, resulting in a GST fusion protein that contains preserved antigenicity as well as function (*GST Gene Fusion System Handbook*, 2002; Harper and Speicher, 2011). Using anti-GST antibodies, western blot analysis of the purified OppA-GST fusion protein confirmed the presence of the GST moiety as part of the 126 kDa protein and thereby confirming successful purification. The GST moiety was found to separate from the recombinant protein after storage at 4°C for about 3 weeks. This could be shown on an SDS-PAGE where the 126 kDa band will became less intense and the 100 and 26 kDa bands increased in intensity which corresponds to the sizes of the OppA and GST proteins respectively. The separation of the GST moiety can be explained by the natural protease enzymes found within the *E. coli* cells. The polylinker between GST and the recombinant protein is said to contain a specific protease cleavage site (Harper and Speicher, 2011). It might also be due to natural degradation of the linker between the protein and GST. Intact fusion protein was, however, always used for downstream applications since GST is a homodimeric protein that ensures solubility and the correct folding of the protein as well as avoiding denaturation of the protein (Harper and Speicher, 2011). The addition of a protease inhibitor could help prevent the GST from separating.

Successful production of rabbit anti-OppA-GST antibodies was achieved using the OppA-GST protein adsorbed to naked bacteria. The GST moiety was not removed from the fusion protein before immunisation of rabbits, since the GST protein is much smaller than the Ms01 OppA and is not likely to result in antibody production against the GST protein. This was confirmed by western blot analysis where the rabbit anti-OppA-GST antibodies were able to detect the OppA-GST and OppA protein but not the GST on its own in expressed samples that were not isolated using affinity chromatography. This indicates that the developed antibodies can be used in a western blot system to detect OppA

production. The ELISA assay further indicated the specificity of the rabbit anti-OppA antibodies as the antibody did not bind to any of the expressed lysate proteins in the absence of the Ms01 OppA protein.

Successful expression and purification of the OppA protein was achieved and this may therefore be used for ELISA analysis of anti-OppA antibody responses in DNA-vaccine trials in ostriches (Chapter 5). The anti-OppA-GST antibodies produced in rabbits was next used in western blot analysis to confirm the *in vitro* expression of OppA protein by the DNA vaccine vectors in a mammalian cell culture based study (Chapter 4).

4 Evaluation of the expression of the *oppA* gene using a mammalian cell culture based system.

4.1 Introduction

The ability of any DNA vaccine to elicit an immune response is evaluated through vaccination trials during which the vaccine is administered to live animals and the resulting immune response determined. The lack of an immune response can on the one hand be due to the inability of the vaccine to trigger the immune system. On the other hand, it may be due to the lack of expression of the antigen gene. The possible lack of expression of the *oppA* gene by the DNA vaccine vectors was given as a reason for the lack of success of previous Ms01 vaccination trials (van Tonder, 2013).

The ability of the DNA vaccine plasmid to express the vaccine antigen can be evaluated before an expensive DNA vaccination trial is undertaken by means of mammalian cell culture. The process of recombinant protein expression in the vaccinated animal can be mimicked by transfecting a mammalian cell with the DNA vaccine. During this process, the transfected cell culture may express the gene of interest over a period of time. This would confirm that the DNA vaccine is able to express the antigen gene and translate it to a protein.

In order to evaluate the *in vitro* expression of the previously developed DNA vaccines against Ms01 (Pretorius, 2009), the three DNA vaccine plasmids were prepared in large amounts and isolated for transfection. As a supercoiled configuration is preferred for transfection, the configuration of the isolated plasmids was evaluated using restriction enzyme digestion. The isolated plasmids were thereafter used for transfection of a COS-1 cell culture in 6 and 12 well plates. In order to determine if transcription resulted from the transfected plasmids, RNA was isolated and analysed using the reverse transcription polymerase chain reaction (RT-PCR). After cell lysis of the transfected cells using two different lysis buffers, the translation of the transcribed product was analysed using SDS-PAGE and western blot using chemiluminescence for sensitive detection.

4.2 Materials and methods

4.2.1 Preparation of three DNA vaccine plasmids for transfection

Frozen stocks of three DNA vaccine plasmids pCI-neo, VR1012 and VR1020 containing the *oppA* gene of Ms01 were previously prepared by van Tonder (2012). The plasmids were, however, transformed into *Salmonella enterica* serovar Typhimurium SL3261 cells.

In order to isolate the plasmids using a Nucleobond® Xtra Midi kit (Macherey-Nagel, Germany), the plasmids had to be transformed into *Escherichia coli* cells. One liter of Tryptic soy broth (TSB) (Merck, USA) was prepared by adding 38 g TSB medium to 1000 ml of Milli-Q® water and the solution autoclaved. In a 14 ml Falcon® tube, 5 µl ampicillin (100 µg/ml, Sigma-Aldrich, USA) was added to 5 ml TSB and inoculated with 4 µl of the *S. enterica* serovar Typhimurium SL3261 cells containing the pCI-neo_*oppA*. Twenty microliters of kanamycin (100 µg/ml, Biochemica, Fluka) was added to 5 ml TSB-medium in a 14 ml Falcon® tube for VR1012_*oppA* and VR1020_*oppA,* respectively. Thereafter these tubes were incubated for 16-18 h at 37°C while shaking at 200 rpm. Each of the plasmids were isolated from the *S. enterica* serovar Typhimurium SL3261 cells using an Invisorb® spin Plasmid mini two kit (Invitek, Germany) by following the manufacture's protocol. Briefly, the cells were first separated from the medium by centrifugation and the pellet resuspended, cells lysed and lysis buffer neutralized using the supplied Solution A, B and C respectively. Thereafter the cell suspension was loaded onto a column and the column cleaned using various washing buffers supplied as part of the kit. The plasmids were finally eluted from the column in 60 µl Milli-Q® water and their concentration determined as 25 ng/µl for pCI-neo_*oppA*, 51.2 ng/µl for VR1012_*oppA* and 55.1 ng/µl for VR1020_*oppA* using a Nanodrop® ND 1000 spectrophotometer (Novell®, USA).

Transformation into *E. coli* cells was achieved by adding 2 µl of each plasmid, pCI-neo_*oppA*, VR1012_*oppA* or VR1020_*oppA,* to 50 µl BL21 (DE3)pLysS *E. coli* competent cells in a 14 ml Falcon® tube and leaving the tubes to incubate on ice for 20 min. Thereafter the cells were treated with a heat shock for 45 sec at 42°C in a water bath and immediately returned to ice for 2 min. Nine hundred and fifty microliters of LB medium (10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 5 g/l NaCl) medium was subsequently added to the cells and left to incubate for 90 min at 37°C with shaking at 150 rpm.

LB Petri dishes were subsequently prepared by adding 15 g/l agar to LB-medium. The solution was autoclaved and allowed to cool before the addition of either ampicillin (100 µg/ml) for pCI-neo_*oppA* or kanamycin (100 µg/ml) for VR1012_*oppA* and VR1020_*oppA,* respectively. Fifty microliters of the LB-medium containing the *E. coli* cells with plasmid was plated out onto the LB-agar and incubated for 18 h at 37°C.

Ten colonies for each plasmid culture were chosen for further analysis by colony PCR. Primer pairs used during the PCR reaction was synthesised by IDT and is shown in Table

4.1. All primer pairs consisted of one plasmid specific primer and one primer specific for the *oppA* gene. All PCR reactions as well as the thermal cycler programming were done as described in section 3.2.1. All the PCR products were electrophoresed on a 1% (w/v) agarose gel. The staining of the gel was done by adding 0.1 µl/ml Gelred™ Nucleic Acid Gel Stain to the gel before casting for visualisation of the PCR products under a UV-light.

To ensure that the plasmid to be used for transfection was supercoiled, each plasmid was digested with specific restriction enzymes of which the restriction sites are situated in the multiple cloning site. The pCI-neo_*oppA* plasmid was digested with *Acc1* (Promega, USA) and VR1020_*oppA* with *BamH1* fast-digest (Fermentas, USA). VR1012_*oppA* was digested with *Sal1* and *Not1* fast-digest enzymes (Fermentas, USA). This was the two restriction enzymes that are situated at the opposite ends of the *oppA* gene. For the Promega enzyme (*Acc1*) a master mix of 16.3 µl Milli-Q® water, 2 µl 10x buffer, 0.2 µl BSA (10 mg/ml), 1 µl pCI-neo_*oppA* plasmid (1 µg/µl) and 0.5 µl of restriction enzyme was prepared and incubated for 2 h at 37°C, followed by a deactivation step of 5 min at 80°C. For the Fermentas enzymes (*BamH1, Not1 and Sal1*) 14 µl Milli-Q® water, 2 µl 10x fast-digest buffer and 1 µl of each restriction enzyme was mixed with 2 µl of the VR1012_*oppA* and VR1020_*oppA* plasmids (1 µg/µl) and left to incubate for 5 min at 37°C followed by an enzyme deactivation step of 5 min at 80°C. All the enzyme digested products were electrophoresed on a 1% (w/v) agarose gel. The staining of the gel was done as above.

Overnight cultures of positive colonies were prepared by inoculating a 14 ml Falcon® tube containing 5 ml LB-medium and 1 µl/ml ampicillin (100 µg/ml) for pCI-neo_*oppA* or 4 µl/ml kanamycin (100 µg/ml) for VR1012_*oppA* and VR1020_*oppA*, with the positive colony and incubating it for 16 h at 37°C on an orbital shaker (150 rpm). Thereafter a starter culture was prepared for pCI-neo_*oppA* in a 50 ml Erlenmeyer flask containing 10 ml LB medium and 10 µl of ampicillin (100 µg/ml) with 1 ml pCI-neo_*oppA* overnight culture. For VR1012_*oppA* and VR1020_*oppA* the starter culture contained 40 µl of kanamycin (100 µg/ml) together with the 10 ml LB medium and 1 ml of the respective plasmids overnight culture. These cultures were left to incubate at 37°C while shaking at 200 rpm until an OD_{600} of 0.6 was reached after 8 h. The respective starter culture mixtures were next added to a 1 L Erlenmeyer flask containing 200 ml LB-medium and 1 µl/ml ampicillin (pCI-neo_*oppA*) or 4 µl/ml kanamycin (VR1012_*oppA* and VR1020_*oppA*) and left to incubate for 16 h at 37°C while shaking at 200 rpm.

Plasmids were isolated with the Nucleobond® Xtra Midi kit, Macherey-Nagel following the manufacturers protocol. Plasmids were finally eluted in 800 µl Milli-Q® water and stored at 4°C until use. Frozen stocks of all three plasmids were also prepared for future plasmid isolations by adding 500 µl of overnight culture to 500 µl of an 80% glycerol solution in a 1.5 ml Eppendorf tube which was then stored at -80°C.

Table 4.1 The primer pairs used for colony PCR to confirm successful transformations.

Plasmid	Primer name	Sequence (5'-3')	Product size (bp)
pCI-neo	T3(EW)_F	AAGGCTAGAGTACTTAATACGA	722
	P1R	CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG	
VR1012	VR1012_F	CGCGCCACCAGACATAATAG	912
	P1R	CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG	
VR1020	VR1020_F	CGTCGACAGAGCTGAGATCCTACAG	955
	P1R	CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG	

4.2.2 Transfection of COS-1 cells

4.2.2.1 Preparation of cell culture medium

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by adding DMEM powder (Sigma-Aldrich), 700 ml sterilized Milli-Q® water, 0.1 g sodium pyruvate (0.1 g/l) (Sigma Aldrich) and 3.7 g sodium bicarbonate (3.7 g/l) (Sigma Aldrich) to a 1 liter Schott Bottle. The pH was adjusted to 7.2, volume adjusted to 1 L. Thereafter the medium was filter sterilized by passing the medium through a glass fibre pre-filter (Merck) followed by a second 0.22 µm filter (Millipore) before it was passed through a Sterivex bell filter (Millipore) using a Millipore easy-load® Masterflex® L/S® pump (Millipore). A sample of the medium was kept at 37°C overnight to test for any contamination of the medium. Subsequent to that the medium was supplemented with 10% heat inactivated fetal calf serum (PAA the Cell Culture Company) as well as 1% Penicillin-Streptomycin (100 µg/ml) (Sigma-Aldrich) and kept at 4°C for no more than one month.

4.2.2.2 **Maintenance of COS-1 cells for transfection**

African green monkey kidney cells (COS-1 cells), were purchased from ATCC and obtained from a stock cultivated at the Department of Biochemistry, University of Stellenbosch. In a laminar flow cabinet, 4 ml supplemented DMEM medium (preheated, 20 min, 37°C) was added to a 1 ml COS-1 (3 passages) frozen stock in a 50 ml Falcon® tube and centrifuged for 5 min at 3500 x g using an Eppendorf 5702 centrifuge. The supernatant was removed and the pellet resuspended in 5 ml supplemented DMEM medium. Thereafter the DMEM-cell mixture was added to a T75 cell culture flask (Nest Biotech, China) and incubated at 37°C (5.0% CO_2) and 90 % humidity, until the cells reached a confluency of 90%. The cells were transfected after three passages. A passage firstly involved removing the medium from the growing culture in the T75 flask. Thereafter 2 ml 0.05%/0.02% Trypsin/EDTA (Biochrom, UK) was added to the cell culture flask and left to incubate at 37°C (5.0% CO_2) for 3 min to loosen the cells from the base of the flask. Next 5 ml supplemented DMEM medium was added to the trypsin/cell solution to neutralize the trypsin, before it was transferred to a 50 ml Falcon® tube and centrifuged for 5 min at 3500 x g. The supernatant was removed and the pellet resuspended in 5 ml supplemented DMEM medium after which 1 ml was added to 19 ml supplemented DMEM medium in a new T75 cell culture flask. This was left to incubate at 37°C (5.0% CO_2) and 90 % humidity, until a confluency of 90% had been reached after which the process was repeated.

4.2.2.3 **Transfection conditions**

When 90% confluency was reached after the third passage, the medium was removed and the COS-1 cells detached from the T75 flask by adding 2 ml 0.05%/0.02% Trypsin/EDTA in PBS (Biochrom, UK) and incubated for 3 min at 37°C (5.0% CO_2). Thereafter 10 ml supplemented DMEM medium was added to the COS-1 cells to neutralise the trypsin and centrifuged for 5 min at 3500 x g. The pellet was subsequently resuspended in 10 ml supplemented DMEM.

COS-1 cells were counted and cell viability calculated using a Countess® automated cell counter (Life Technologies, USA). For this purpose a 10 µl resuspended cell sample was mixed with 10 µl, 0.4% Trypan Blue solution (Life Technologies, USA) and pipetted onto a Countess® cell counting chamber slide (Life Technologies, USA). The slide was then inserted into the cell counter to count the number of cells in the sample, as well as to

determine the viability. The COS-1 cells were seeded into 12 well cell culture plates at $1X10^5$ cells per well and incubated for 24 h at 37°C (5.0% CO_2) in 90 % humidity.

For transfection the X-tremeGENE HP DNA (Roche, USA) transfection reagent was used. Three different plasmid (pCI-neo_*oppA*) concentrations of 0.005 µg/µl, 0.01 µg/µl and 0.02 µg/µl were tested for transfecting COS-1 cells at three different transfection reagent to DNA ratios of 1:1, 3:1 and 4:1. A un transfected COS-1 cell sample, which did not receive any plasmid, was also included at each time point. For transfection, the plasmid was diluted to the appropriate concentration using unsupplemented DMEM (final volume of 100 µl). The appropriate amount of transfection agent was added to the solution and mixed by swirling. The plasmid-DMEM mixture was left to incubate for 15 min. Thereafter 100 µl of the mixture was added dropwise to each well and left to incubate at 37°C (5.0% CO_2) for 24 h, 48 h and 72 h.

The transfection was repeated and transfections with VR1012_*oppA* and VR1020_*oppA* using 0.01 µg/µl plasmid at a transfection reagent to DNA ratio of 3:1 were prepared. Controls in which pCI-neo, VR1012 and VR1020 without the *oppA* insert and an untransfected sample, were included.

4.2.2.4 **Transfection using 6 well culture plates**

In order to increase the total amount of expressed protein in the COS-1 cells the transfection was repeated using 6 well cell culture plates. Cell culture plates were prepared for three time periods: 24, 48 and 72 hours. COS-1 cells were again prepared as in section 4.2.2.2 and after three passages, and at a confluency of 90%, a final volume of 40 ml was prepared with $2X10^5$ cells in supplemented DMEM. Thereafter 2 ml of the cell suspension was added to each well of a 6 well cell culture plate (Greiner, Bio-One) and incubated for 24 h at 37°C (5.0% CO_2) before it was transfected. The transfections were performed in a similar way as in section 4.2.2.3 and included pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* (all at 0.01 µg/µl) and a un-transfected control at a transfection reagent to DNA ratio of 3:1. A total volume of 200 µl DNA/transfection reagent was added dropwise to each well and left to incubate at 37°C (5.0% CO_2) for the time periods specified above.

4.2.3 Lysis of cells after transfection

4.2.3.1 Reducing treatment buffer

After each of the 24, 48 and 72 h time periods in the 6 and 12-well plates, the adherent cells were lysed. This was achieved by adding 100 µl reducing treatment buffer (RTB: 0.125 M Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8) in each well of a 12-well plate and mixed with the cells using the pipette tip. The lysate was transferred into a 1.5 ml Eppendorf tube and were either boiled and analysed immediately using SDS-PAGE or stored at -20°C for analysis at a later stage. For 6-well plates the procedure was repeated as outlined above but 200 µl RTB was used.

4.2.3.2 RIPA buffer and protease inhibitor

Radioimmunoprecipitation assay (RIPA) buffer (Sigma, USA) was used as alternative to RTB due to concerns that the OppA protein was not being extracted from the insoluble cell lysis material by means of RTB. One protease inhibitor tablet (Complete ULTRA Tablets, Mini, Easypack, Roche) was added per 10 ml RIPA buffer to protect proteins against degradation. The medium was firstly removed from the wells and the attached cells were washed with 800 µl sterilised PBS buffer (pH 7.2) as the medium can influence the RIPA buffer. The PBS was removed and 125 µl of the RIPA buffer, containing the protease inhibitor, was added to each well of a 12-well plate. The buffer was left to incubate for 5 min on ice before the plates were swirled to loosen the cells. The cell mixture of each well was then transferred into a 1.5 ml Eppendorf tube and were either boiled and analysed immediately using SDS-PAGE or stored at -20°C for analysis at a later stage. For a 6-well plate the procedure was repeated as outlined above but 250 µl RIPA buffer was used.

4.2.3.3 Procedure for increasing the concentration of expressed protein in transfected cell cultures

In an attempt to increase the overall protein concentration of expressed OppA protein the cell contents of different wells within a cell culture plate were combined. This was done only for transfections using the VR1020 plasmid. Only 6-well plates were used for this purpose and the harvested cells from 3 wells were combined resulting in a volume of 750 µl. This was repeated using both RTB and RIPA buffer for cell lysis on the same plate. This combined volume was thereafter concentrated to a final volume of 250 µl using a Savant™ SpeedVac™ concentrator. This was done for transfected and untransfected samples. Protease inhibitor was again added to the RIPA buffer to limit protein

degradation during storage. In an attempt to further increase the concentration of the expressed OppA protein, transfected COS-1 cells were incubated for longer incubation periods up to 96 h. Expression levels of OppA were analysed using SDS-PAGE as described in section 4.2.5.1. Analysis was done both directly after lysis and after storage at -20°C.

4.2.4 Analysis of gene transcription

4.2.4.1 RNA isolation with the Nucleospin RNA midi kit

Reverse transcriptase-PCR (RT-PCR) was used to assess *oppA* gene transcription in cell culture lysates. As this involved a different cell harvesting method, transfection was repeated as described for the 12-well plates using the different plasmids. COS-1 cells were maintained as in section 4.2.2.2, and transfected as described in section 4.2.2.3. The medium was removed and the harvesting of the cells for RT-PCR was done by firstly washing the cells with PBS buffer before 0.3% trypsin/EDTA, diluted in PBS, was added to each well and incubated in a CO_2 (5.0%) oven at 37°C for 3 min to loosen the cells. Afterwards supplemented DMEM medium was added to each well, mixed and pipetted into a 1,5 ml Eppendorf tube. RNA was isolated using a RNA-Isolation Nucleospin RNA midi kit (Macherey-Nagel) according to the manufacturer's protocol. The RNA was finally eluted in 60 µl RNase-free water.

4.2.4.2 RNA isolation with Trizol®

An alternative to the RNA-Isolation Nucleospin RNA midi kit was also used to isolate RNA from the cultured cells. After the appropriate incubation period, the medium was removed before the cells were washed with 100 µl of sterilised PBS buffer (pH 7.2) to remove the excess medium. The PBS was subsequently removed and 400 µl Trizol® (Sigma, USA) added to each well of the 12-well plate and 800 µl to each well of a 6-well plate. The cells were lysed by pipetting up and down and subsequently pipetted into Eppendorf tubes. The Trizol-cell solution was left to incubate at room temperature for 5 min before 80 µl chloroform was added to each sample collected from a 12 well plate and 160 µl to each sample collected from a 6 well plate and mixed by inverting the tube. The mixture was subsequently centrifuged at 14 000 x g using a Labnet Prism R centrifuge for 15 min at 4°C. The clear aqueous phase was thereafter transferred to a clean Eppendorf tube before equal volumes of isopropanol were added to the Eppendorf tubes and mixed by inverting the tube. The solution was again centrifuged at 14 000 x g for 10 min at 4°C. The supernatant was removed whereafter the remaining pellet was washed with 500 µl

of 75% EtOH containing Diethylpyrocarbonate (DEPC) treated water. The solution was mixed using a vortex mixer for 1 min before it was again centrifuged at 8000 x g for 5 min at 4°C. The pellet was subsequently air dried for 10 min. The pellet containing the RNA was dissolved in 15 µl of DEPC-treated water and stored at -20°C until analysed.

4.2.4.3 **Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

The specific primer pairs listed in Table 4.2 were used to amplify a 541 bp gene-product of the *oppA* gene by RT-PCR in order to assess *oppA* gene expression. All PCR reactions were carried out in 25 µl volumes containing 15.12 µl Milli-Q® water, 2.5 µl 10x Buffer, 1.25 µl Dithiothreitol (DTT) (0.1 M), 1.0 µl dNTP (5 mM), 1.5 µl $MgCl_2$ (25 mM), 0.625 µl P2F forward primer (20 pmol/µl), 0.625 µl P3R reverse primer (20 pmol/µl), 0.25 µl Supertherm Taq (5 U/µl), 0.125 µl Superscript (200 U/µl, Invitrogen, USA) and 2 µl RNA sample. The thermal cycler was programmed as follows: 48°C (30 min) 35 cycles of each 94°C (30 sec), 55°C (30 sec), 72°C (30 sec) and an additional 7 min at 72°C. The PCR results were verified on a 1% agarose gel containing 0.1 µl/ml Gelred™ Nucleic Acid Gel Stain for UV visualisation.

Table 4.2 Primer pairs used for the amplification of a specific part of the *oppA* gene by means of RT-PCR.

Primer name	Base-pair sequence (5' - 3')	Product size (bp)
SDM- P2F	CTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTG	541
SDM-P3R	CTATTTGCTGTTACACCATACCAGTAAACACCTAATTTAGC	

4.2.5 Analysis of gene translation

4.2.5.1 **SDS-PAGE**

The expression of OppA protein in transfected cell cultures was analysed by SDS-PAGE. The gel was cast as described in section 3.2.1 and an 8% resolving gel was used to separate the proteins. The positive control used was the previously expressed and purified OppA protein (Chapter 3). Thirty microliters of the control was mixed with 12 µl of 0.1% (v/v) bromophenol blue and 30 µl RTB before it was boiled for 10 min in water over a Bunsen burner. For samples collected from cell culture plates using RTB, 0.1% (v/v) bromophenol blue was added to 30 µl of the sample followed by boiling for 10 min.

For samples collected from cell culture plates using RIPA buffer, 0.1% (v/v) bromophenol blue was added to 30 µl of the RIPA buffer-sample and 30 µl RTB followed by boiling for 10 min. Each sample mixture (30 µl) was loaded onto the 8% SDS-PAGE gel. Coomassie staining of the gel was performed as described in section 3.2.1.

4.2.5.2 **Western blot analysis**

Western blot using colorimetric detection

The proteins on the SDS-PAGE gel were transferred to a 0.45 µm nitrocellulose membrane and western blotting analysis performed as described in section 3.2.3. Briefly, the membrane was blocked using casein buffer. Next, rabbit anti-OppA serum (as prepared in Chapter 3, diluted 1:2 000 in casein-Tween buffer) was added to the membrane and incubated for 90 min at 37°C on a shaker (6 rpm). The membrane was washed twice for 10 min at 37°C with 0.1% PBS-Tween followed by the addition of the goat anti-rabbit (Sigma-Aldrich, USA) antibody (diluted 1:2500 in casein-Tween). The membrane was again incubated for 90 min at 37°C followed by a PBS-Tween wash. Rabbit peroxidase anti-peroxidase (PAP, Sigma-Aldrich, USA), diluted 1:5 000 in casein-Tween was then added and the membrane incubated for 1:30 h at 37°C on a shaker (6 rpm) followed by a PBS-Tween wash. Substrate (50% 4-chloro-1-naphtol, 16% MeOH 30 ml PBS and 9 µl H_2O_2) was finally added to the membrane which was allowed to incubate until a band could be detected.

Western blot using chemiluminescence detection

A chemiluminescence detection system was used as an alternative to detect expressed *oppA*. Chemiluminescence as detection method was chosen over the colorimetric method as it is much more sensitive. The oxidation of luminol by peroxide results in creation of an excited state product called 3-aminophthalate. This product decays to a lower energy state by releasing photons of light to which an X-ray film can be exposed (Odyssey imaging system, 2011). The different cell samples were firstly separated on an 8% SDS-PAGE gel before they were transferred to a 0.45 µm nitrocellulose membrane. Thereafter the membrane was blocked with blocking buffer (10 mM Tris-HCl, 0.15 M NaCl, 5% fat free milk powder (Elite, RSA), 0.02% Thiomersal) for 1 h at 37°C on a shaker (6 rpm) before it was washed three times for 5 min with 20 ml PBS that contained 0.05% (v/v) Tween® 20. The primary antibody used was rabbit anti-OppA anti-serum which was diluted (1:2000) in blocking buffer containing 0.05% (v/v) Tween® 20. The membrane was incubated for 18 h at 4°C on a shaker (6 rpm) followed by a PBS-Tween wash as before.

A goat anti-rabbit HRP conjugate (Santa Cruz, USA) was used as a secondary antibody and was diluted 1:20 000 in blocking buffer containing Tween. The membrane was incubated for 90 min at 37°C on a shaker (6 rpm) and again washed three times with PBS-Tween.

The development of the blot was done in a dark room that was fitted with a red dark room photo lamp (Dr Fischer, Germany) that does not emit UV-light. Within the dark room the substrate, 2 ml of luminol reagent mixed with 2 ml hydrogen peroxide solution (Thermo Scientific, USA), was added, whereafter the membrane was incubated for 1 min. After incubation the membrane was placed in a 18 x 24 cm Cronex® Blot developer (Du Pont, USA) and exposed to X-ray film (Fuji-film) for different time periods ranging from 10 sec to 30 min at room temperature. After exposure, the film was first incubated in a X-ray Developer (Axim, RSA) for 1 min, washed with water and thereafter incubated in a X-ray Fixer (Axim, RSA) for 1 min. This ensured that the film would be unaffected by UV-light afterwards.

Optimisation was done by varying the concentrations and incubation times, as described above, of the primary and secondary antibodies as well as the subsequent X-ray film exposure time.

4.3 Results

4.3.1 Preparation of three DNA vaccine plasmids for transfection

The three plasmids pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* were successfully isolated from the S*almonella* cells whereafter a Nanodrop® reading revealed the concentration to be 95.5 ng/µl, 51.2 ng/µl and 55.1 ng/µl, respectively. The plasmids were subsequently transformed into BL21(DE)pLysS *E. coli* cells and successful transformation confirmed using colony PCR. Figure 4.1 shows the results obtained from the colony PCR. For VR1020 colony 1, 2, 3, 6, 7 and 9 were positive. For Vr1012 all the colonies tested were positive and for pCI-neo only colonies 1, 2, 3, 7 and 9 were positive. Although not all the colonies containing the VR1020_*oppA* and pCI-neo_*oppA* plasmids tested positive, the transformation efficiency was sufficient to allow for the identification of positive colonies for further use.

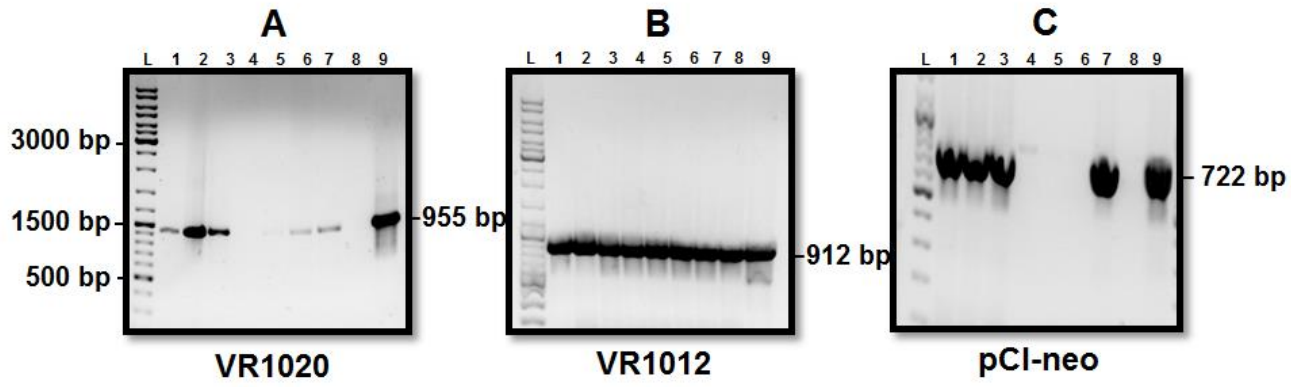

Figure 4.1 A 1% agarose gel electrophoresis image of a colony PCR done with the respective plasmids, pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* within *E. coli* cells. In image (A) lane L: GeneRuler™ (Fermentas), lane 1-9 the different colonies tested. In image (B) lane L represents a Fermentas gene ruler ladder, lane 1-9 the different colonies tested. In image (C) lane L represents a Fermentas gene ruler ladder, lane 1-9 the different colonies tested.

After positive colonies were identified, they were grown to a high density overnight for large scale plasmid isolation. The plasmid DNA were successfully isolated from the cultures and Nanodrop® readings gave concentrations of 816.0 ng/µl, 867.4 ng/µl and 1077 ng/µl for VR1012_*oppA*, VR1020_*oppA* and pCI-neo_*oppA* respectively.

Although in figure 4.2 some of the band sizes appear more intense that others, most of the plasmids were shown to be supercoiled, which is needed for transfection as it is the smallest configuration that will allow for better internalisation. Restriction digestion of the isolated plasmids revealed that the *Acc1* cuts the pCI-neo_*oppA* plasmid once, producing a linear band of about 8000 bp. VR1012_*oppA* was digested with *Sal1* and *Not1* and produced 2 bands, one corresponding to the size of the *oppA* gene of 2828 bp and another that corresponded to the size of the plasmid of 4900 bp. VR1020_*oppA* was digested with *BamH1* and resulted in the plasmid being cut twice, producing 2 bands, one representing the size of the gene and the other the size of the VR1020 plasmid (5000 bp). The restriction digestion revealed the appropriately sized bands for all the plasmids.

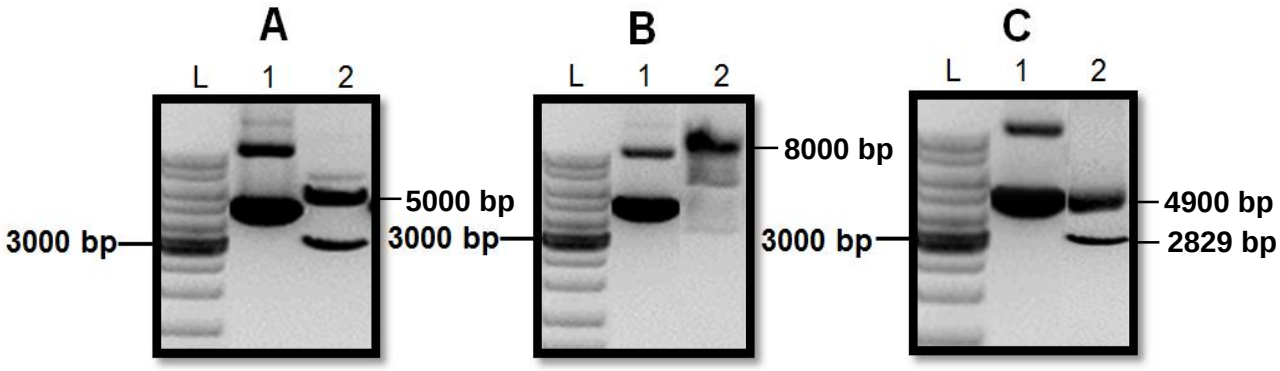

Figure 4.2 A 1% agarose gel electrophoresis image of the enzyme digestion of the respective plasmids. (**A**) Lane L: GeneRuler™ (Fermentas). Lane 1: Undigested supercoiled VR1020_*oppA*. Lane 2: VR1020_*oppA* digested with *BamH1.* (**B**) Lane L: Fermentas gene ruler ladder. Lane 1: Undigested supercoiled pCI-neo_*oppA* Lane 2: pCI-neo_*oppA* digested with *Acc1.* (**C**) Lane L: Fermentas gene ruler ladder. Lane 1: Undigested supercoiled VR1012_*oppA*. Lane 2: VR1012_*oppA* digested with *Sal1* and *Not1.*

4.3.2 Transfection and lysis of COS-1 cells

COS-1 cells prepared for all transfections had a viability rate of about 90% and live cell counts of 6.5 x10^5, although the final transfection was only done on cells with a 72% viability that contained 2.0 x10^5 live cells. According to the manufacturer, the optimum transfection ratio for using the X-tremeGENE HP DNA transfection reagent is 3 µl transfection reagent to 1 ng DNA. Different plasmid concentrations in combination with different ratios were tested using the pCI-neo plasmid, but no distinct difference could be observed during SDS-PAGE and western blot analysis. A final plasmid concentration of 0.01 µg/µl with a plasmid to transfection reagent ratio of 3:1, as suggested by the manufacturer, was therefore used for all plasmids and subsequent transfections.

After COS-1 cells were transfected with the respective plasmids the cells were harvested successfully from the wells using both RTB and RIPA buffer. Harvesting with RTB, however, resulted in a very viscous solution that was probably due to the release of genomic DNA (gDNA) during lysis. This caused difficulty in pipetting the sample into the well of the SDS-PAGE gel. Although RIPA buffer also results in the release of gDNA, the gDNA forms a pellet in the solution and thereby reduces the viscosity of the lysis solution.

4.3.3 Analysis of gene transcription

After harvesting the cell samples from the plate, the newly transcribed mRNA was isolated from the cell solution using both the Trizol® reagent and a Nucleospin RNA midi kit. A Nanodrop reading for Trizol® isolated RNA revealed concentrations for the transcription product (total RNA) of pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* to

be 57.2 ng/µl, 49.2 ng/µl and 47.7 ng/µl respectively. RNA isolation from 60 µl VR1020 using the RNA-Isolation Nucleospin RNA midi kit, yielded 42.3 ng/µl, which was comparable to that of Trizol®.

The *oppA* gene expression results as detected by RT-PCR are shown in Figure 4.3. From the figure a band at approximately 531 bp could be identified for all the samples of the cell cultures that were transfected with *oppA* gene containing plasmids (lane 1-3). The RT-PCR product was already visible at 24 h and continued up to 72 h after transfection with a slight increase in band intensity over time. The samples that were transfected with plasmid without the *oppA* insert as well as un transfected samples did not contain any bands indicating the absence of the *oppA* gene from these plasmids.

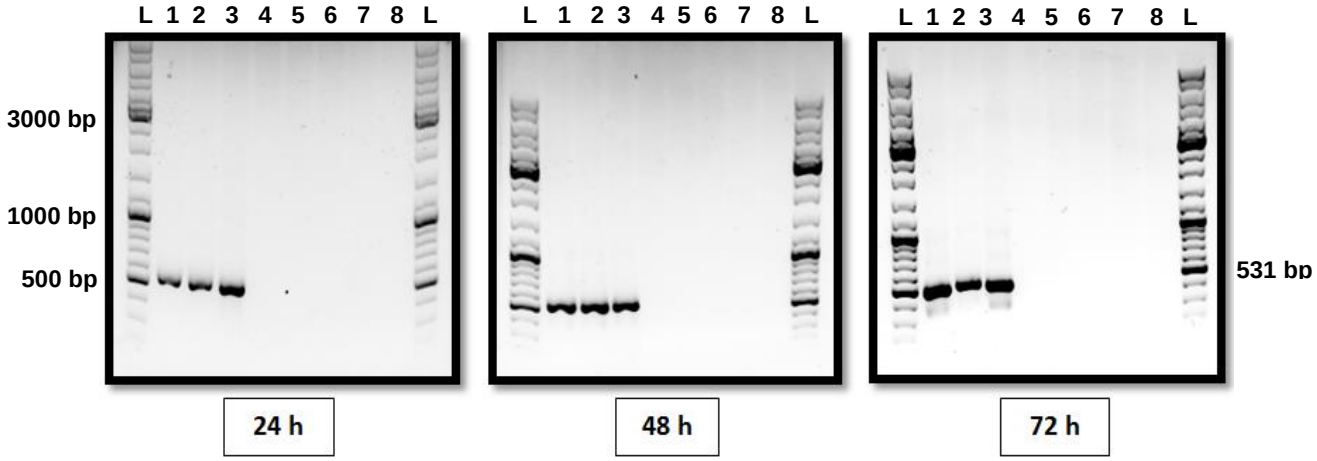

Figure 4.3 An 1% agarose gel electrophoresis image displaying the RT-PCR results of the three different plasmids pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* at three different time points 24 h, 48 h and 72 h. Lane L: GeneRuler™ (Fermentas). Lane 1: pCI-neo with the *oppA* insert. Lane 2: VR1012 with *oppA* insert. Lane 3: VR1020 with *oppA* insert. Lane 4: pCI-neo without *oppA* insert. Lane 5: VR1012 without *oppA* insert. Lane 6: VR1020 without *oppA* insert. Lane 7-8: un-transfected COS-1 cells.

4.3.4 Analysis of protein translation

SDS-PAGE analysis was performed to determine if the OppA protein was expressed after transfection with each of the different *oppA* gene containing plasmids. From Figure 4.4 (A) it can be seen that the 12-well cell samples, lysed with RTB, contained a large amount of other cell proteins making it difficult to identify the 100 kDa OppA protein as being present in the transfected samples, but not the untransfected samples. The positive control contained the OppA-GST protein expressed by the prokaryotic pGEX-4T (Chapter 3), which resulted in a fusion protein, of 126 kDa. The GST dissociates during storage causing visible bands at 126 kDa (OppA + GST), 100 kDa (OppA without tag) and 26

kDa (dissociated tag). Results were not influenced by performing the analysis directly after lysis or only after storage at -20°C.

When RIPA buffer was used for lysis of 12-well samples transfected with the VR1020_*oppA*, cell proteins were also observed upon SDS-PAGE analysis with no distinct band at 100 kDa for the transfected samples. Results did not improve upon analysis directly after lysis. The SDS-PAGE gels from cells harvested with RIPA resulted in a smear on the gel (results not shown). This could be due to protein degradation despite the addition of a protease inhibitor.

Six-well culture plates were subsequently used for transfection of all of the plasmids as this would result in more cells being transfected thereby increasing the concentration of expressed protein and possible detection by SDS-PAGE. RTB and RIPA buffers were used for these samples. Due to the increased transfected cell volume and therefore increased viscosity the RIPA buffer used for lysis was easier to pipette during downstream analysis. The increased transfected cell volume (6-well) had no noticeable effect on the concentration of expressed OppA protein during SDS-PAGE analysis as a band was still not visible at the appropriate size.

However, after combining and concentrating the content of a 6-well plate (lysed with RIPA) a band was identified at the appropriate size of 100 kDa in the VR1020_*oppA* transfected sample after 48 h which was absent in the untransfected sample (Figure 4.4 B, lane 3, indicated by arrow). The same band could also be seen when transfecting cells for 72 h. Since the 48 h and 72 h samples were analysed on a single gel, the 48 h sample was stored at -20°C for 24 h before analysis, but the 72 h sample analysed directly after lysis. This result, however, proved difficult to reproduce even when using the same sample, indicating that the protein was degraded when stored at -20°C, despite the use of RIPA buffer and the addition of a protease inhibitor. In an attempt to repeat protein expression, longer transfection times were used up to 96 h, but did not results in any positive band for the cells transfected with plasmid that was absent in un transfected cells. Analysis was repeated using RTB for lysis, but no positive band was identified for the cells transfected with plasmid that was absent in un transfected cells. The reason for this was probably due to the fact that the COS-1 cells used for transfection only had a confluency of 72 % compared to the previous attempt using RIPA buffer where the confluency was calculated as 94 %. The lower viability was a result of the slower growth rate of the COS-1 cells during the different passage steps in preparation for transfection.

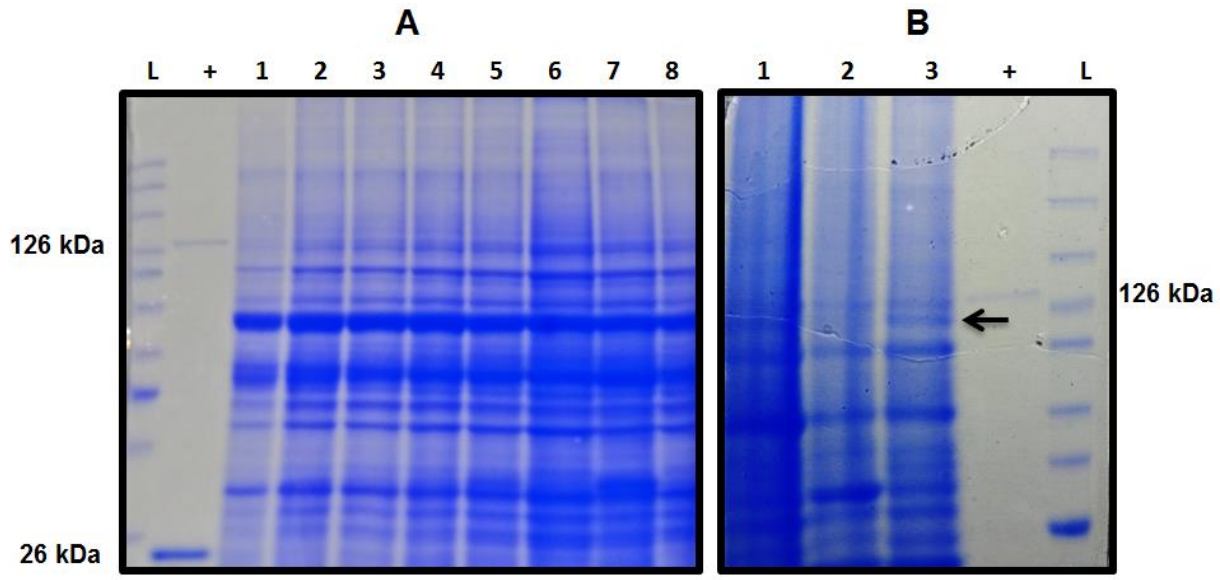

Figure 4.4 An image of two SDS-PAGE gels of harvested COS-1 cells transfected with the VR1020_*oppA* DNA vaccine.
Gel A represents COS-1 cells from 12-well plates lysed with reducing treatment buffer. Lane L: Pageruler™ unstained protein ladder (Thermo Scientific). Lane +: positive control, expressed OppA-GST protein (126 kDa). Lane 1-3: transfected 48 h. Lane 4: untransfected 48 h. Lane 5-8: transfected 72 h.
Gel B represents COS-1 cells from 6-well plates of which the content of three wells were combined and lysed using RIPA. Lane 1: transfected 72 h. Lane 2: untransfected 48 h. lane 3: transfected 48 h indicating 100 kDa band (arrow). Lane +: positive control, expressed OppA-GST protein (126 kDa). Lane L: Pageruler unstained protein ladder.

To confirm the presence of OppA, the proteins on the SDS gel (Figure 4.4 B) were transferred to a nitrocellulose membrane for western blot analysis using the rabbit anti-OppA-GST antibody and a colorimetric detection method. The results of samples harvested from 12-well plates using RTB are shown in Figure 4.5. Two bands were visible for the positive control at 126 and 100 kDa. No bands were visible on the membrane at the appropriate size for either transfected or un transfected samples. The colorimetric western blot was therefore unsuccessful.

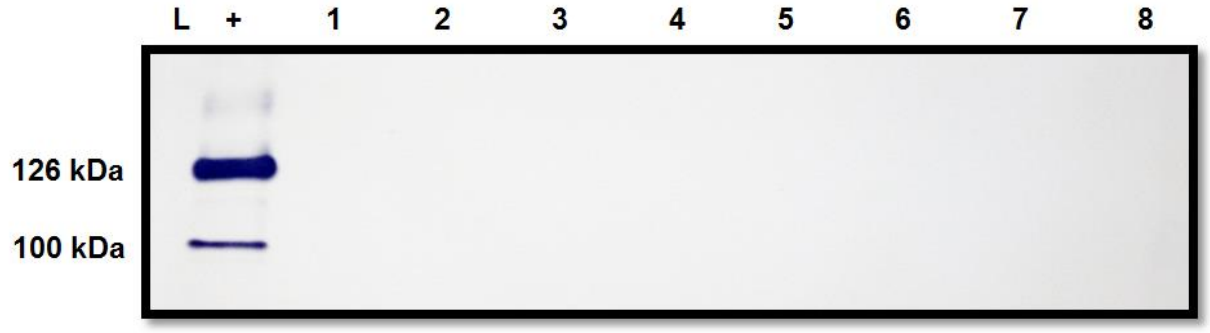

Figure 4.5 The image of a 0.45 µm nitrocellulose membrane displaying the results from a western blot analysis using a colorimetric detection method. Samples were from transfected cells in 12-well plates harvested using RTB and separated on an 8% SDS-PAGE gel. Lane L: Full range rainbow marker (Amersham). Lane +: expressed OppA-GST positive control (OppA + GST at 126 kDa as well as OppA at 100 kDa can be seen). Lane 1-8: different concentrations of pCI-neo_*oppA* plasmid used to transfect COS-1 cells after 24 h, 48 h and 72 h.

The western blot was repeated using chemiluminescence as detection method. Figure 4.6 shows the X-ray film exposed at 30 sec and 1 min to the light emitting membrane. Lane 2 (pCI-neo_*oppA*), 3 (VR1020_*oppA*) and 5 (VR1012_*oppA*) where all lysed with RTB from a 12-well plate. The positive control reveals a band at approximately 100 kDa. A band was visible at 100 kDa across all the samples, but this band was more intense for VR1020_*oppA* than the others. A 100 kDa band was not visible in the un transfected samples after 48 h (lane 1), indicating that the darker, more intense band produced by VR1020_*oppA* (lane 3) represents the expressed OppA protein. Results could not be reproduced with success using a larger culture volume (6-well plates), analysing results directly after lysis, longer incubation periods, RIPA buffer or additional attempts to optimize antibody concentrations and their relevant incubation times as well as exposure time.

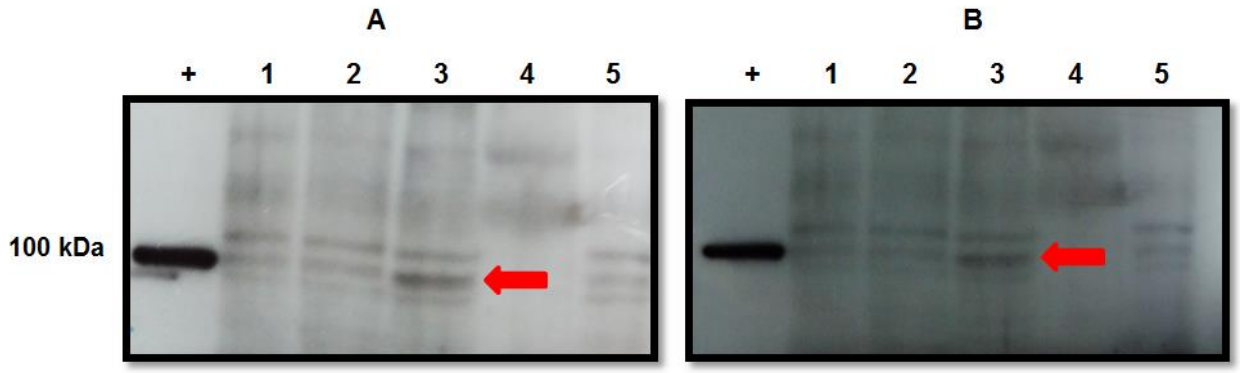

Figure 4.6 The X-ray film image of a western blot using chemiluminescence as detection method. COS-1 cells were transfected with either pCI-neo_*oppA*, VR1012_*oppA* or VR1020_*oppA*. Samples were harvested from a 12-well plate, using RTB and separated on an 8% SDS-PAGE gel and transferred to a 0.45 µm nitrocellulose membrane which was illuminated onto Fujifilm X-ray film for 30 sec (A) and 1 min (B). Lane +: positive control, expressed OppA protein without attached GST (100 kDa). Lane 1: untransfected COS-1 cells after 48h. Lane 2: pCI-neo_*oppA* after 24h, Lane 3: VR1020_*oppA* after 48h. Lane 4: medium sample taken from VR1020_*oppA* cells after 48h. Lane 5: VR1012_*oppA* after 24h.

4.4 Discussion

During this study the three DNA vaccines pCI-neo_*oppA*, VR1012_*oppA* and VR1020_*oppA* were used to transfect a COS-1 mammalian cell line in order to determine transcription and subsequent translation of the OppA protein *in vitro*. The topology of the plasmid DNA (pDNA) is found to be critical in gene expression in mammalian cells and thus influences the transfection efficiency (Maucksch *et al.,* 2009). Supercoiled DNA has shown to have up to 100 fold higher transfection rates when compared to other pDNA topologies such as linearized or open circular pDNA (Weintraub *et al.*, 1986; Maucksch *et al*., 2009). The plasmids used in this study were shown to be predominantly supercoiled, which increased the chance of plasmids entering the COS-1 cells during transfection.

Transcription of the *oppA* gene after transfection was determined using RT-PCR analysis. RNA was isolated from transfected and control cells using both a RNA isolation kit from Macherey-Nagel and the Trizol® reagent from Sigma. Both RNA isolation methods were successful in isolating RNA, but the Trizol® method proved to be more efficient compared to the RNA isolation kit, due to the fact that it was more cost effective. RT-PCR showed that transfection with all three DNA vaccine vectors leads to expression of the *oppA* gene at 24 h after transfection which increases slightly by 72 h after transfection. All three

plasmids were therefore entering the cells and the *oppA* gene was being transcribed into mRNA.

Translation of the *oppA* mRNA into the OppA protein could not be confirmed by SDS-PAGE analysis when using cells harvested from 12-well plates and lysed with RTB or RIPA. Results could not be improved by using a larger number of cells. This indicated that the concentration of translated protein was not sufficient for detection during SDS-PAGE analysis. Expressed protein could however, be detected when combining and concentrating the content of 6-well plates lysed with RIPA buffer. Analysis of these samples after storage for longer than 24 h at -20°C indicated that the OppA protein was being degraded despite the use of RIPA buffer for lysis and the addition of a protease inhibitor. RIPA buffer is known for its stability and reduces non-specific protein-binding. Only VR1020_*oppA* was however analysed using this method, due to the fact that this plasmid produced the most promising results after western blot analysis.

Western blot analysis using a colorimetric method of detection was unable to detect expressed protein. From this it could be concluded that the protein, if being translated, was expressed at a too low concentration to be detected or that the method was not sensitive enough. Chemiluminescence was subsequently used as a more sensitive detection method for western blot analysis as it provides greater sensitivity as well as convenience for the detection of proteins on a film (Alegria-Schaffer *et al.*, 2009). When a 12-well plate, lysed with RTB was used for western blot analysis with chemiluminescence, a band was identified in the VR1020_*oppA* transfected cells that appeared to be more intense when compared to that of pCI-neo_*oppA* and VR1012_*oppA* transfected cells. Although pCI-neo was after 24 h, this was due to insufficient sample, the 24 h time point was included to provide a relative comparison to the others. This was after the sample had been stored at -20°C for about 1 month indicating that the RTB was probably a better alternative for storage of samples at -20°C compared to RIPA buffer. This might be due to the fact that RTB contains β-mercaptoethanol that can act as a biological antioxidant. No results were obtained using samples from a 6-well plate or by combining and concentrating the content of a 6 well plate. The latter was probably due to the use of COS-1 cells with viability below 90% for transfection. This highlights the importance of cell viability in the outcome of transfection results.

There are many contributing factors that influence expression levels of plasmids such as the promoter, presence of enhancing elements, presence of introns and polyadenylation sites. All of the plasmids used in this study, contain a polyadenylation signal that increases mRNA stability with that of VR1012 and VR1020 being from bovine growth hormone (BGH) and that of pCI-neo containing a SV40 late polyadenylation signal. A study by Pfarr *et al.* (1986) revealed that the use of a BGH poly(A) region produced three times higher protein expression when compared to the SV40 late polyadenylation region. Although the study was performed using R1610 hamster mammalian cells, this might also apply to COS-1 cells, resulting in a protein product being detected when the VR1020_*oppA* was used for transfection and not when the pCI-neo_*oppA* was used.

Despite having the BGH polyadenylation signal, a protein product was not detected when VR1012_*oppA* was used for transfection. As with the other plasmids VR1012 also contains a strong CMV promoter, but the VR1012 promoter is optimised for the use in mice (Suarez *et al.*, 2015). This can influence transcription levels and therefore translation of protein expressed by the VR1012 plasmid demonstrating the importance of all elements of the plasmid functioning together to increase expression of a gene.

Although the production of *oppA* mRNA was shown, it is possible that the mRNA is not translated into protein at levels high enough for detection using SDS-PAGE and western blot analysis (Struhl, 1999; Elbashir *et al.,* 2001). A band representing the OppA protein was, however, visible in the VR1020_*oppA* transfected cells on SDS-PAGE and upon western blot analysis using chemiluminescence as detection method.

All of the COS-1 cell cultures transfected with the *oppA* containing DNA vaccine plasmids showed transcription of the *oppA* gene, but translation could possibly only be indicated in COS-1 cell cultures transfected with the VR1020_*oppA* DNA vaccine plasmid. This therefore indicates that the VR1020_*oppA* DNA vaccine plasmid might have the potential of acting as a DNA vaccine and the ability of this vaccine to elicit an immune response was next evaluated in a field trial in ostriches.

5 Vaccination trial

5.1 Introduction

DNA vaccines were used in preliminary vaccination trials in ostriches in which the Ms03 *oppA* gene cloned into the VR1012, VR1020 and pCI-neo plasmids were used to determine their ability to elicit an anti-OppA immune response in ostriches (Wium, 2015). This resulted in an immune response against Ms03 VR1020_*oppA* and Ms03 pCI-neo_*oppA* but no response was observed after vaccination with the Ms03 VR1012_*oppA* plasmid. In this study the cell culture analysis indicated that the Ms01 pCI-neo_*oppA* and Ms01 VR1012_*oppA* did not lead to protein expression but the Ms01 VR1020_*oppA* did lead to OppA protein production. It was therefore concluded that the Ms01 VR1020_*oppA* warrants further consideration as a DNA vaccine in ostriches. However, it was decided to include the Ms01 pCI-neo_*oppA* vaccine in this trial because of the plasmid differences between Ms01 pCI-neo_*oppA* and Ms03 pCI-neo_*oppA*.

For administration, DNA vaccines are routinely dissolved in a saline solution and administered at specific plasmid doses. According to Dunham (2002) mice can receive an intramuscular (i.m.) DNA vaccine dose in the range of 10-100 µg, small animals 100-300 µg and large animals 500-2500 µg. The dose typically administered to birds ranges from 0.5-1500 µg when injected i.m. (Kodihalli *et al.*, 1997) and they also typically receive a boosting dose after about 2-3 weeks (Vanrompay *et al.*, 1999; Fang Yan *et al.*, 2013; Chen *et al.*, 2013). In previous trials vaccination with the Ms01 VR1020_*oppA,* Ms01 pCI-neo_*oppA* and Ms01 VR1012_*oppA* plasmids, a single dose (100 µg/ml) was used for vaccination, but these trials were jeopardised by avian influenza outbreaks and the birds were exposed to stress due to weekly sampling (Brandt, 2012, van Tonder, 2013).

The objectives for this part of the study were therefore to determine the ability of different doses of the Ms01 *oppA* containing DNA vaccines (pCI-neo and VR1020), in combination with a booster immunisation, to elicit an anti-OppA immune response in ostriches. As the detection and quantification of the ostrich cellular immune response elicited by these vaccines is technically complex, only the humoral immune response was evaluated by measuring antibody production.

The two DNA vaccines were firstly produced in different amounts for vaccination purposes. In order to determine if the isolated plasmids were in the supercoiled configuration the plasmids were digested with restriction enzymes and analysed by gel electrophoresis. These plasmids were injected into the ostriches at three different doses

followed by a booster vaccination. To assess mycoplasma infections of the ostriches during the trial, trachea swab samples were taken for PCR analysis. In order to determine the anti-OppA antibody immune response elicited by the ostriches, blood samples were taken during each sampling and analysed using ELISA.

5.2 Materials and methods

5.2.1 Ethical clearance and approval for vaccine trial

Before the start of the trial, ethical clearance was obtained from the Stellenbosch University Animal Ethics Committee (SU-ACUM13-00019). Approval was also obtained from the Department of Agriculture, Forestry & Fisheries (DAFF), through the submission of a section 20 application (Reference: 12/11/1/1/3). This included permission from the National Executive Officer in terms of the Meat Safety Act, 2000 (Act No 40 of 2000) and its regulations to allow the animals to enter the human food chain after slaughter.

5.2.2 Preparation of DNA vaccines

The *oppA* gene of which ten mycoplasmal TGA codons had been modified to the universal TGG codon, was previously sub-cloned into the pCI-neo (Promega, USA) and VR1020 (Vical Inc., USA) DNA vaccine plasmids by Pretorius (2009).

Escherichia coli (*E. coli*) cultures containing the different plasmids were used to prepare overnight cultures by inoculating a 14 ml Falcon® tube containing 5 ml LB-medium and 1 µl/ml ampicillin (100 µg/ml) for pCI-neo_*oppA* or 4 µl/ml kanamycin (100 µg/ml) for VR1020_*oppA*, with 10 µl of freezer culture and incubating them for 16 h at 37°C on an orbital shaker (150 rpm). A starter culture was prepared for both plasmids in a 50 ml Erlenmeyer flask containing 10 ml LB medium and 10 µl of ampicillin (100 µg/ml) for pCI-neo_*oppA* or 40 µl of kanamycin (100 µg/ml) for VR1020_*oppA* and was inoculated with 1 ml of the respective plasmid overnight cultures. Cultures were left to incubate at 37°C while shaking at 200 rpm until an OD_{600} of 0.6 was reached after 8 h. The respective starter culture mixtures were next added to a 1 L Erlenmeyer flask containing 200 ml LB-medium and 1 µl/ml ampicillin (pCI-neo_*oppA*) or 4 µl/ml kanamycin (VR1020_*oppA*) and left to incubate for 16 h at 37°C while shaking at 200 rpm. Subsequent to that the plasmids were purified with an Endotoxin-free plasmid DNA purification kit (NucleoBond® Xtra Maxi plus EF, Macherey-Nagel, Germany). Yields were determined using a Nanodrop® ND 1000 spectrophotometer (Novell®, USA) and the integrity of the plasmids confirmed by electrophoresis using a 1% agarose gel containing 0.5 µg/ml EtBr for visualisation of PCR products under UV-light.

To ensure that the plasmid to be used for vaccination was supercoiled, each plasmid was digested with restriction enzymes. The pCI-neo_*oppA* plasmid was digested with *Acc1* (Promega, USA) and VR1020_*oppA* with *BamH1* Fast-digest (Fermentas, USA) as described in section 4.2.1. All the enzyme digested products were electrophoresed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide (EtBr) for visualisation under UV-light.

The day before vaccination, the two DNA vaccines were diluted to three final concentrations of 100 µg/ml, 300 µg/ml and 600 µg/ml in 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) and stored at 4°C until use. Dilutions were made under sterile conditions in 100 ml serum bottles after which they were sealed using 20 mm silicone rubber stoppers (Sigma Aldrich, USA) covered by a 20 mm Aluminium crimp seal with a tear away centre cap (Sigma Aldrich, USA).

5.2.3 Trial animals

The vaccination trial was conducted in Fraserburg (Northern Cape Province) and Oudtshoorn (Western Cape Province) using 3-4 month old ostriches housed on commercial ostrich farms. The ostrich chicks were raised in Fraserburg until they reached 40 kg (at week 4 of the trial), after which they were transported to Oudtshoorn. This is standard practice in ostrich production in order to limit exposure of ostrich chicks to pathogens. In Fraserburg, the trial ostriches were kept under intensive farming conditions as a separate group and as part of standard farming practice, each received a coloured tag for identification. Each tag contained a number that was unique for each ostrich.

Upon arrival in Oudtshoorn, all ostriches including trial birds were first kept in quarantine for 29 days. The quarantine birds were then tested for avian influenza. This is currently standard practise to ensure that avian influenza is not introduced into the rest of the farm. In Oudtshoorn, the trial ostriches were kept under semi-intensive farming conditions and were therefore grouped with other ostriches not forming part of this trial. They were however, grouped with ostriches of the same age and weight that arrived from Fraserburg at the same time and from the same production farm in Fraserburg.

The ostriches used in the trial were at all times kept under standard farming conditions in open air camps and were not caged. They received regular nutrition as usually given on the farm for production purposes and had access to water at all times. Trial ostriches were at all times only handled by farm personnel that were trained and had experience

in the handling of ostriches. Ostriches were weighed at week 4, 7 and 10 during the trial to monitor weight gain.

5.2.4 Vaccination trial

The trial consisted of 140 ostriches. Group one (60 ostriches) was vaccinated with pCI-neo_*oppA*, group two (60 ostriches) with VR1020_*oppA* and group three (20 ostriches) acted as control and did not receive any vaccine. Each of the vaccinated groups was further subdivided into three groups (20 ostriches per group) with each group receiving a different vaccine dose. The dose per group is outlined in Table 5.1.

The DNA vaccines were injected i.m. into the upper thigh of the ostriches at week 0 and 7 using a single dose (1 ml) with one of the three different plasmid concentrations i.e. 100 µg/ml, 300 µg/ml or 600 µg/ml. Blood (3 ml) was drawn from the jugular vein using Vacuette® Z serum sep clot activator tubes with 18G x 1" needles (Vacuette, UK) at week 0 and 4 in Fraserburg and week 7 and 10 in Oudtshoorn. Serum was separated by low speed centrifugation for 10 min before the serum was transferred to 1.5 ml Eppendorf tubes for storage at -20°C.

Table 5.1 Vaccine trial groups and dose administered

DNA vaccine	Route	Dose µg/ml*	Ostrich number
pCI-neo_*oppA*	Intramuscular	100	20
		300	20
		600	20
VR1020_*oppA*	Intramuscular	100	20
		300	20
		600	20
Control	No vaccination	-	20
Total			**140**

* All vaccination were done using a total volume of 1 ml.

5.2.5 Analysis of trachea swabs by PCR

When blood samples were taken at week 0, 4, 7, 10 and 18 of the trial, ostriches were tested for the presence of existing mycoplasma infections by means of trachea swabs.

At week 18 only 66 ostriches were sampled as the rest had already reached their slaughter weight of 90 kg and were slaughtered before the week 18 sampling point.

Trachea swabs were taken using dry, sterile transport swabs with a plastic applicator and inert, non-toxic cotton tip (Copan, ITA). Each swab was contained in a sterile polypropylene tube for transport purposes. Before PCR analysis each swab was rinsed in an Eppendorf tube containing 200 µl sterile PBS buffer.

Samples were first tested by PCR using a generic primer pair specific for the genus *Mycoplasma* (Table 5.2). Samples that tested positive were further analysed using species-specific primer pairs targeting the *16S rRNA* gene for each of the ostrich-infecting mycoplasmas, i.e. *M. struthionis* sp. nov. str. Ms01, *Mycoplasma sp*. Ms02 and *M. nasistruthionis* sp. nov. str. Ms03 (Botes *et al.*, 2005). The sequences of the different primer pairs used are shown in Table 5.2. Each PCR contained 2 µl of a 10x reaction buffer, 2.4 µl of a 25 mM $MgCl_2$, 0.4 µl of each primer (20 pmol/µl), 0.8 µl of a 5 mM dNTP mix (Kapa Biosystems, RSA), 0.05 µl of a 5 U/µl Supertherm Taq DNA polymerase (JMR Holdings, USA), 9.95 µl Millli-Q water and 4 µl of the PBS trachea swab sample. The thermal cycler (Veriti Applied Biosystems) was programed for 30 cycles of 94°C (30 sec), 55°C (15 sec) and 68°C (1 min) followed by a single cycle of 6 min at 68°C. The same PCR and cycler conditions were used for all primer sets. PCR products were separated on a 1% agarose gel containing 0.5 µg/ml EtBr for visualisation of PCR products under UV-light.

Table 5.2 Primer pairs for the identification of ostrich-infecting mycoplasmas

Target	Primer name	Sequence (5'-3')	Product size (bp)
Genus *Mycoplasma*	*GPO3F (F)	TGGGGAGCAAACAGGATTAGATACC	272
	*MGSO (R)	TGCACCATCTGTCACTCTGTTAACCTC	
Ms01	Ms01Z (F)	AACATTAGTTAATGCCGGATACG	499
	Ms01D (R)	GCCAGTATCCAAAGCGAGCC	
Ms02	Ms02H (F)	AATATAAAAGGAGCGTTTGC	287
	Ms02A (R)	AAGGCAATAGCATTTCCTCTACT	
Ms03	Ms03A (F)	AGTGCTAATGCCGGATACTTATA	521
	Ms03C (R)	CGTTAACCTCTATACAATTCTAGCG	

(F) Forward Primer; (R) Reverse Primer.

*Obtained from Botes *et al.* (2005).

5.2.6 Weight measurements during trial

The weight of each ostrich was measured at week 4, 7 and 10. This was to assess the well-being of the ostriches as well as the effect of vaccination on the ostriches, if any. Weight measurement also allowed dose vs weight relationships to be determined. No scale was available for weighing at week 0.

5.2.7 Biotinylation of rabbit anti-ostrich antibodies

For subsequent ELISA purposes, rabbit anti-ostrich antibodies were first biotinylated. Anti-ostrich immunoglobulin (Ig) antibodies were previously developed in rabbits and provided by Dr. A. Botes, Department of Biochemistry, Stellenbosch University. For isolation of the rabbit anti-ostrich Ig antibodies, 0.5 ml immunised rabbit serum was diluted in 1 ml PBS (pH 7.2) before 1.5 ml saturated ammonium sulfate was added and incubated for 20 min at 4°C for precipitation to occur. The precipitate was thereafter centrifuged at 15 000 x g for 20 min using a Beckman Coulter Avanti J-E centrifuge. The pellet was dissolved in 1 ml PBS (pH 7.2) and 1.5 ml saturated ammonium sulfate again added followed by incubation (20 min, 4°C) and centrifugation (15 000 x g, 20 min). The pellet was dissolved in 0.5 ml PBS (pH 7.2) and dialysed overnight at 4°C against 1800 ml carbonate buffer (0.1 M $NaHCO_3$, pH 8.3). The buffer was changed once after 6 h. The concentration of the antibodies in the dialysate was measured at 280 nm using a

Nanodrop® ND 1000 spectrophotometer (Novell®, USA) and diluted to 5 µg/ml of Ig in carbonate buffer (0.1 M $NaHCO_3$, pH 8.3).

Biotinylated anti-ostrich antibodies were required for use as secondary antibody in the ELISA to determine the immune response elicited by each of the DNA vaccines. The biotinylation of 3 ml diluted (5 µg/ml) Ig was performed by slowly adding 1 ml of biotinylation reagent (2 mg/ml, biotinamidocaproate N-hydroxysuccinimide ester in *N,N* dimethylformamide) and incubating for 2 h at room temperature while stirring. This was followed by dialysis against PBS (pH 7.2) overnight at 4°C with a single buffer change. An equal volume of glycerol was subsequently added to the biotinylated antibodies, mixed and stored at -20°C for no longer than 10 months.

5.2.8 Optimization of ELISA for analysis of anti-OppA immune response

Boinformatic analysis using ProtParam (http://web.expasy.org/cgi-bin/protparam/protparam) was done on the OppA protein to reveal the hydrophobicity index of the protein. Based on this, two different Nunc, 96-well microtitre plates were used during optimization of the ELISA, namely Maxisorp and Medisorp (Nunc, Denmark). Both plates' surfaces are made from polystyrene, with Maxisorp having a high affinity for polar groups and Medisorp binding molecules of an intermediate hydrophobic/hydrophilic nature.

The OppA-GST protein was purified as described in section 3.2.2. The purified OppA-GST protein was diluted with carbonate buffer (50 mM $NaHCO_3$, pH 9.6), and used for coating columns of microtitre plates at 3 different concentrations i.e. 1 µg/ml, 5 µg/ml and 10 µg/ml protein respectively. Rows A and B received 1 µg/ml protein in each well, C and D 5.0 µg/ml in each well, and E and F 10.0 µg/ml protein in each well. The protein was left to incubate for 18 h at 4°C. The OppA solution was removed and 300 µl/well casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.02% Thiomersal and 0.5% casein, pH 7.6) was added to all the wells and left to incubate at 37°C for 1 h to block non-specific binding. Afterwards all the wells were washed 5-10 times with PBS-Tween (PBS buffer (pH 7.2) containing 0.1% (v/v) Tween® 20). To further reduce background finding, the PBS-Tween wash was combined with a 3 times distilled water (dH_2O) wash. Week 0 and corresponding week 3 sera drawn from the VR1020_*oppA* 600 µg/ml immunised ostriches were diluted 1:20 with casein-Tween (casein buffer containing 0.1% (v/v) Tween® 20) and subsequently used as primary antibody. A dilution series of the sera was made starting at 1:20 (100 µl/well) in column 2 up to 1:40 960 in column 12. The antibody

was left to incubate for 1 h at 37°C before it was washed 5-10 times with PBS-Tween and 3 times with distilled water. The secondary antibody (biotinylated rabbit anti-ostrich Ig) was diluted 1:100 in casein-Tween and 100 µl/well added followed by incubation for 1 h at 37°C. The plate was subsequently washed 10 times with PBS-Tween and 3 times with distilled water. Thereafter 100 µl/well of HRP-conjugate (Invitrogen) (2 ml streptavidin HRP, 3 ml 0.5% casein buffer, 40 ml glycerol) was added and left to incubate for 1 h, 37°C and washed 10 times with PBS-Tween and 3 times with distilled water. Finally 100 µl/well ABTS substrate solution, 0.015% H_2O_2 in 0.1 M citrate buffer, pH 5) was added to each well. Absorbance was measured at 405 nm after 30 minutes of incubation at 37°C on a Titertek Multicsan spectrophotometer (Labsystems, Finland).

5.2.9 ELISA analysis of vaccination trial

After determining the optimal coating concentration as 10 µg/ml on Maxisorp (Nunc, Denmark) plates, the ELISA was used to determine the antibody levels in sera of the vaccinated ostriches. Thereafter, Maxisorp (Nunc, Denmark) 96-well plates, coated with 10.0 µg/ml/well purified OppA-GST protein, were used for all analysis. Week 0 and corresponding week 4, 7 and 10 sera drawn from immunised ostriches were diluted 1:100 with casein-Tween and tested in triplicate. The procedure followed was described in 5.2.8.

There were two instances in which blood and swab samples could not be collected from all ostriches. Firstly, due to the loss of tags from some ostriches during the course of the trial and secondly ostriches not reaching 40 kg at week 4 of the vaccination trial causing them not to be transported to Oudtshoorn with the rest of the trial ostriches. Only sera of ostriches from which all 4 blood samples (week 0, 4, 7 and 10) could be collected were used for ELISA analysis. All trial results are given in Addendum A.

5.2.9.1 Statistical analysis of the ELISA results

The ELISA results and weight data obtained during the vaccination trial were used for an analysis of variance (ANOVA) using the General Linear Modal (GLM) procedure using the Agrobase Generation II® (Agronomix Software Inc.) software and the least significant difference (LSD) value was calculated. A significance level of $P < 0.05$ was chosen for analysis. Analyses were only performed on data for ostriches for which blood samples could be obtained throughout the trial period. The input data is given in Addendum A.

5.3 Results

5.3.1 Preparation of DNA vaccines

The two DNA-vaccines were successfully isolated and a total of 46954.83 µg pCI-neo_*oppA* was obtained from 13 plasmid isolations and 51396.30 µg VR1020_*oppA* from 9 plasmid isolations. From Figure 5.1 it can be seen that the majority of the plasmids were in the supercoiled configuration after isolation with a low level of plasmid in the nicked configuration. There is also some nicked plasmid seen. The configuration was further confirmed by enzyme digestion of each vaccine vector (Figure 5.2). On the gel image shown in Figure 5.2 (lane 1) undigested supercoiled VR1020_*oppA* appears at approximately 5000 bp, compared to lane 2 where the plasmid was digested with *BamH1*. Digestion resulted in two bands, one corresponding to the linear VR1020 plasmid (5044 bp) and the other to the *oppA* insert (2829 bp). Similarly undigested supercoiled pCI-neo_*oppA* (lane 3) produced a band size of about 5000 bp. When pCI-neo_*oppA* was digested with *Acc1* it resulted in one linear band at 8300 bp, representing the 5472 bp of the pCI-neo plasmid and the 2829 bp of the *oppA* insert (linear DNA). The digestion was, however, incomplete as other bands were also visible (lane 4).

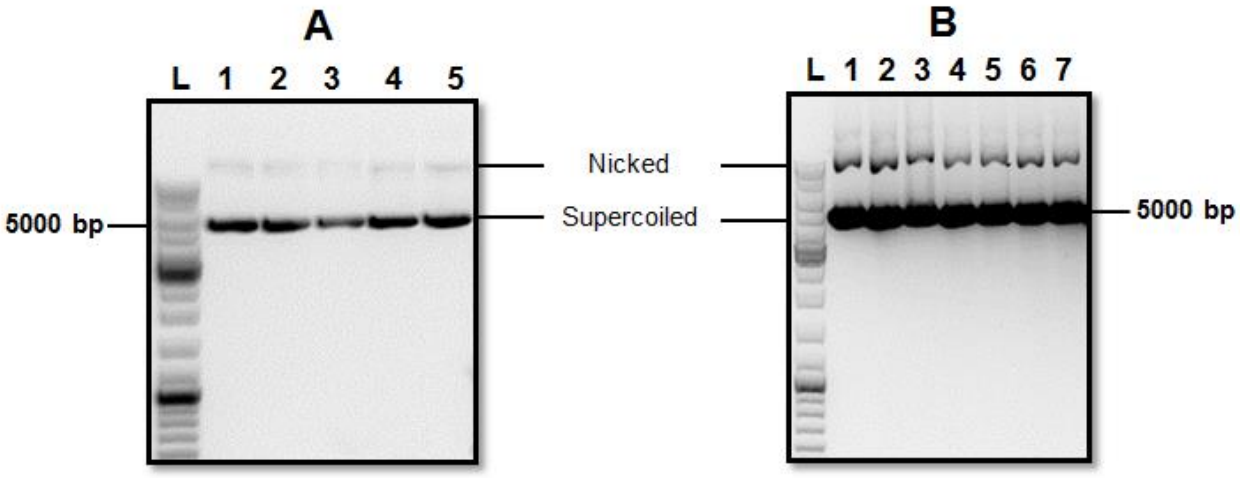

Figure 5.1 A 1% agarose gel electrophoresis image of the two DNA vaccines pCI-neo_*oppA* (A) and VR1020_*oppA* (B) isolated using a Endotoxin-free plasmid DNA purification kit. Lane L: GeneRuler™ (Fermentas). Lane 1-5 (A): Isolated pCI-neo_*oppA* plasmid diluted 1:100 in Milli-Q H_2O. Lane 1-7 (B): Isolated VR1020_*oppA* plasmid diluted 1:100.

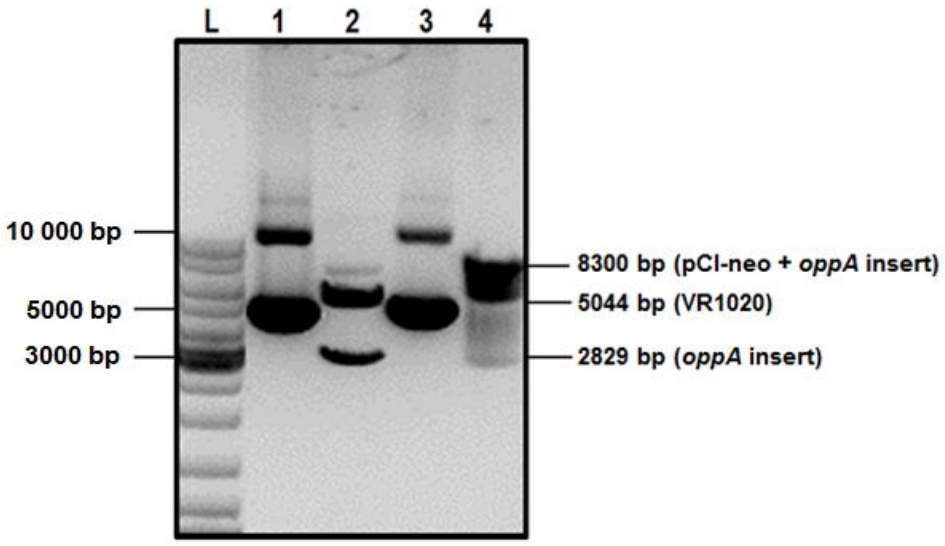

Figure 5.2 Enzyme digestions of the two DNA vaccine vectors. Lane L: GeneRuler™ (Fermentas). Lane 1: VR1020_*oppA* plasmid diluted 1:100. Lane 2: VR1020_*oppA* digested with *BamH1.* Lane 3: pCI-neo_*oppA* plasmid diluted 1:100. Lane 4: pCI-neo_*oppA* plasmid digested with *Acc1.*

5.3.2 Analysis of trachea swabs by PCR

The different mycoplasma infections detected amongst the trial ostriches are shown in Table 5.3. Only the results from ostriches for which serum samples could be obtained throughout the trial are shown. The PCR test indicated the presence of ostrich-infecting mycoplasmas during the trial. Band sizes of 500, 290 and 520 indicated the presence of the positive controls Ms01, Ms02 and Ms03 in samples as indicated by Figure 5.3. Some of the bands are more intense than others and could be an indication of the level of mycoplasma infection present in the sample.

During week 0-4, when the ostriches were kept in Fraserburg, less mycoplasma infections were detected when compared to week 7, 10 and 18, when they were kept in Oudtshoorn. At the week 7 sampling point, ostriches had already been in Oudtshoorn for about 21 days. Results indicated that Ms02 and Ms03 were the more dominant mycoplasma infections throughout the trial and were detected amongst ostriches in all trial groups. The highest number of infections (Ms02 and Ms03) detected was at week 10. Overall at week 10, less Ms02 and Ms03 co-infections were detected in the VR1020_*oppA* vaccinated groups, compared to the pCI-neo_*oppA* vaccinated groups, but infections were much less for both groups, compared to the control group at week 10. On average 98.2% of birds tested at week 7 had no infections (Ms01, MS02 and Ms03) compared to only 23.2% at week 10 and 51.5% at week 18. All ostriches were however

not tested at week 18 which could have caused the increase in the percentage of negative birds.

An Ms01 infection was only detected once during week 10 in an ostrich from the pCI-neo_*oppA* 300 µg/ml group. This ostrich was not tested again at week 18 as it had reached its slaughter weight before this date and was therefore already slaughtered.

Amongst the mycoplasma positive ostriches that received the pCI-neo_*oppA* vaccine the Ms02 infections increased dramatically amongst the averages of the vaccinated groups from week 7 (5%) to week 10 (73.7%). This became less during week 18 (41.2%) but the fact that not all ostriches from the pCI-neo_*oppA* vaccinated groups were tested at week 18 could have influenced the number of detected infections. The ostriches that received the VR1020_*oppA* vaccine had 5% Ms02 infections at week 7, which increased to 52% at week 10. Infections again decreased by week 18 to only 34,3%, but once again not all birds were tested, which could have influenced the number of infections detected. The control group had 25% Ms02 infections during week 0 that decreased to 5% at week 7 before it increased to 84% at week 10. This became less in week 18 (55%). During week 10 both the pCI-neo_*oppA* 600 µg/ml and VR1020_*oppA* 600 µg/ml groups had the lowest amount of Ms02 infections followed by the groups that received a dose of 100 µg/ml and 300 µg/ml. Although not all the ostriches were tested at week 18, the pCI-neo_*oppA* 100 µg/ml group had the least amount of infections followed by the 300 µg/ml and 600 µg/ml groups, while the VR1020_*oppA* 100 µg/ml and 600 µg/ml groups had the least amount of Ms02 infections followed by the 300 µg/ml.

The pCI-neo_*oppA* vaccinated groups showed no Ms03 infections at week 7 but at week 10, 47.3% of the ostriches tested positive for Ms03. Infections dropped slightly to 42.2% by week 18 but not all ostriches were tested. The VR1020_*oppA* vaccinated groups also had no Ms03 infections detected at week 7, but infections increased to 52% during week 10. Infections again decreased to 20% at week 18, but again not all the birds were tested, which could have influenced the number of infections detected. The control group had only one ostrich positive for Ms03 during week one. This increased dramatically during week 10 to 84% before it decreased to 22% by week 18. During week 10 the pCI-neo_*oppA* and VR1020_*oppA* 600 µg/ml groups had the lowest amount of Ms03 infections followed by 100 µg/ml and then 300 µg/ml groups. During week 18 the pCI-neo_*oppA* 100 µg/ml group had no Ms03 infections followed by the 600 µg/ml and 100

µg/ml groups witch did have infections. While the VR1020_*oppA* 100 µg/ml and 600 µg/ml groups had the least amount of Ms03 infections followed by the 300 µg/ml.

Table 5.3 Results of PCR analysis of trachea swabs.

	Infections detected			
	Ms01	**Ms02**	**Ms03**	**Experimental group in which individuals tested positive**
Week 0	-	5/20* (25%)	1/20 (5%)	Control group
Week 4	-	-	-	
Week 7	-	1/20 (5%)	-	pCI-neo_*oppA* 100 µg/ml
	-	1/20 (5%)	-	VR1020_*oppA* 100 µg/ml
Week 10	-	11/13 (84%)	11/13 (84%)	Control group
	-	8/10 (80%)	5/10 (50%)	pCI-neo_*oppA* 100 µg/ml
	1/13	11/13 (85%)	8/13 (62%)	pCI-neo_*oppA* 300 µg/ml
	-	9/15 (60%)	5/15 (33%)	pCI-neo_*oppA* 600 µg/ml
	-	8/15 (53%)	7/15 (47%)	VR1020_*oppA* 100 µg/ml
	-	11/17 (65%)	12/17 (71%)	VR1020_*oppA* 300 µg/ml
	-	6/16 (38%)	6/16 (38%)	VR1020_*oppA* 600 µg/ml
Week 18	-	5/9 (55%)	2/9 (22%)	Control group
	-	-	-	pCI-neo_*oppA* 100 µg/ml
	-	2/7 (29%)	3/7 (43%)	pCI-neo_*oppA* 300 µg/ml
	-	5/10 (50%)	4/10 (40%)	pCI-neo_*oppA* 600 µg/ml
	-	3/10 (30%)	1/10 (10%)	VR1020_*oppA* 100 µg/ml
	-	6/15 (40%)	5/15 (33%)	VR1020_*oppA* 300 µg/ml
	-	3/10 (30%)	1/10 (10%)	VR1020_*oppA* 600 µg/ml

*Number of ostriches that tested positive per total of birds tested in the relevant experimental group. Group sizes decreased over the duration of the trial.

In week 0-4 the ostriches were housed in Fraserburg and from week 7 and onwards in Oudtshoorn.

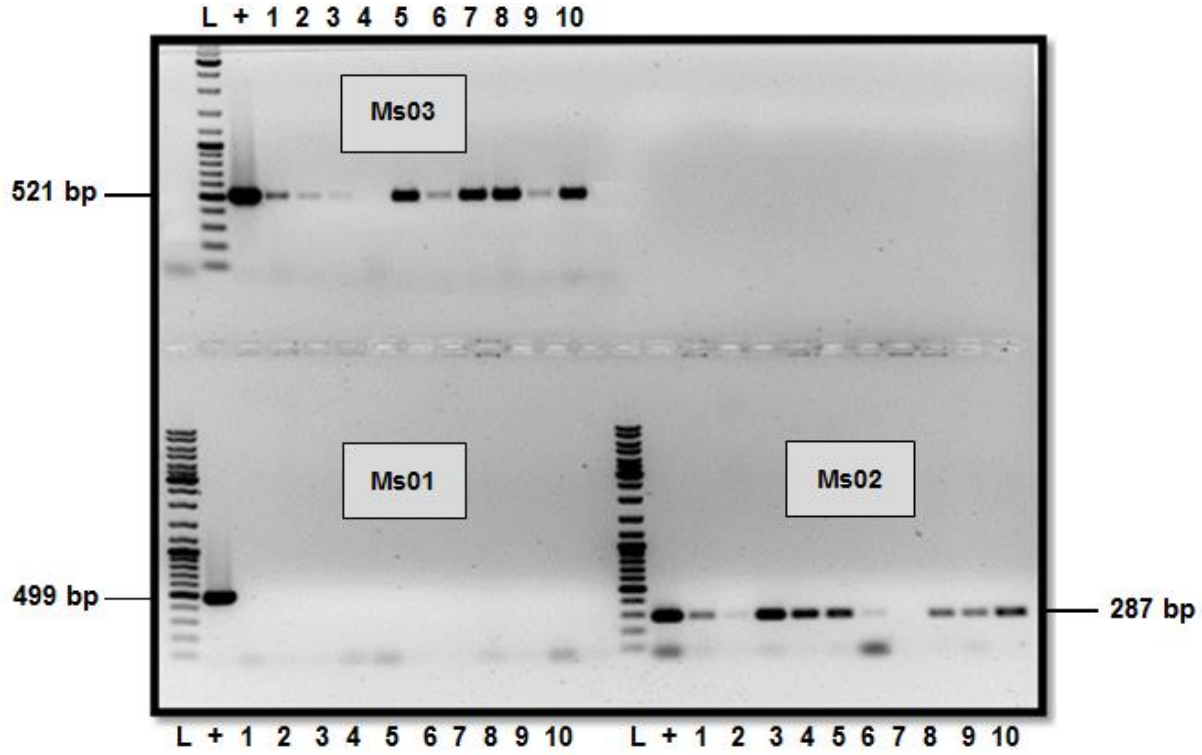

Figure 5.3 An image of the PCR products obtained when analysing the swab samples of the ostriches that were electrophoresed on a 1% agarose gel. Ten random samples were tested for Ms01, Ms02 and Ms03. Lane L: GeneRuler™ (Fermentas). The respective positive controls are indicated in Lane +. Lane 1-10: Ten randomly selected samples.

5.3.3 Ostrich weights during the trial

The weight of the ostriches was measured at each sampling point except at week one and 18. The results of the average weight gained over time of the different experimental groups are shown in Table 5.4. There was a consistent increase in the average weight throughout the trial and the weight increase of vaccinated ostriches did not differ significantly from the control group as shown by an ANOVA analysis (see Addendum A). Vaccination thus had no effect on the growth of the ostriches nor did the transfer of the ostriches between Fraserburg and Oudtshoorn. There were 6 ostriches that did not reach 40 kg at week 4, and were not transported to Oudtshoorn with the rest of the trial ostriches. These include one ostrich that received pCI-neo_*oppA* (100 µg/ml), one receiving pCI-neo_*oppA* (300 µg/ml), one receiving pCI-neo_*oppA* (600 µg/ml) and three ostriches that received VR1020_*oppA* (100 µg/ml). These birds were excluded from the rest of the trial.

Table 5.4 The average weight of the ostriches in the different experimental groups as measured at the different sampling times during the vaccination trial.

	Control	100 µg/ml pCI-neo	300 µg/ml pCI-neo	600 µg/ml pCI-neo	100 µg/ml VR1020	300 µg/ml VR1020	600 µg/ml VR1020
Week 0	No weight measurements taken.						
Week 4	60.1	57.7	55.3	53.1	49.5	57.0	56.4
Week 7	65.5	63.5	61.3	59.4	56.5	61.5	61.6
Week 10	73.3	69.6	68.7	68.7	65.6	69.6	69.5

5.3.4 Optimization of ELISA for analysis of anti-OppA immune response

The OppA-GST protein was purified and based on Bradford analysis, the highest concentration that could be achieved was 0.837 mg/ml. To ensure that all ELISA analyses could be done from a single batch of isolated OppA-GST protein, the yield was increased by incubating the protein solution on the glutathione agarose column for three hours instead of one hour before washing and eluting the protein.

According to a ProtParam analysis the OppA protein has a grand average hydrophobicity (GRAVY) score of -0.499, which indicates an overall hydrophilic structure (polar). An increasing positive score indicates greater hydrophobicity (Kyte *et al.*, 1982). Despite this, no significant difference was observed in terms of antibody titre or ELISA background values between the Maxisorp and Medisorp microtitre plates. The Maxisorp microtitre plates were therefore chosen for further analysis as they are more commonly used according to the literature (Hopfe and Henrich, 2004; Medi *et al.*, 2005; Kennedy *et al.*, 2006; Lassaux *et al.*, 2013).

ELISA optimization revealed that the biotinylated anti-ostrich antibody was successful in identifying the antibodies produced by the ostriches in response to vaccination. A graphical representation of the results for serial dilutions of the serum using different coating concentrations on Maxisorp microtitre plates is shown in Figure 5.4. From these results a final coating concentration of 10 µg/ml was chosen as it gave the greatest difference in absorbance values when week 0 sera were compared to week 3 sera. A final dilution of 1:100 (indicated with a vertical line on graph) was chosen as it gave a better resolution between week 0 sera and week 3 sera and was therefore the dilution factor that was used in previous field trials. The background was reduced from an average

absorbance (A_{405}) of 0.8 to 0.3 by incorporating a 10x PBS-Tween wash, followed by a 3x dH_2O wash between the different incubation steps.

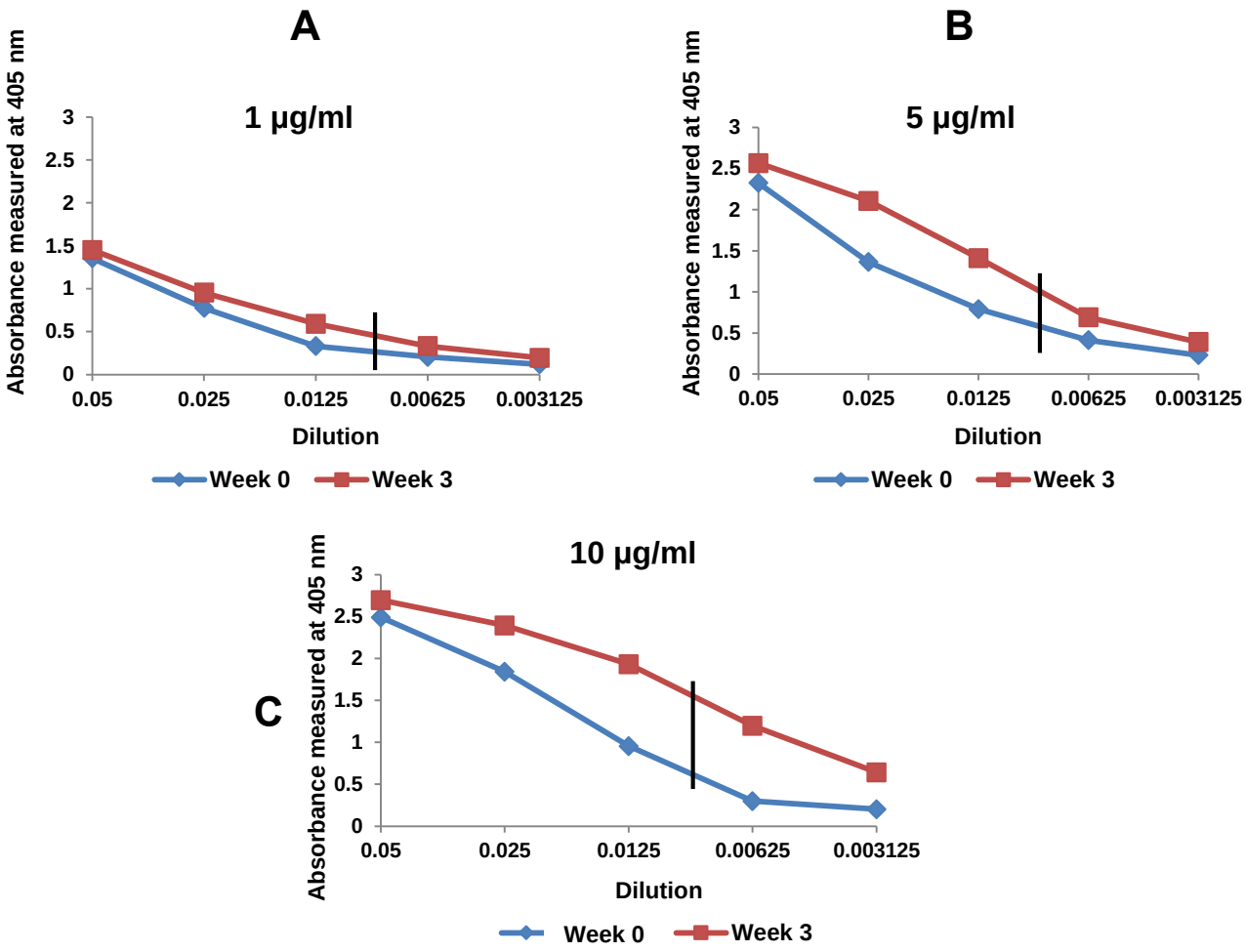

Figure 5.4 ELISA optimization for the three different protein coating concentrations. VR1020_*oppA* 600 µg/ml serum collected during week 0 and week 3 was used for optimization. Plates were coated with 1 µg/ml (A), 5 µg/ml (B) and 10 µg/ml (C) of purified OppA-GST protein. The 1:100 dilution factor is indicated on each graph with a vertical line.

5.3.5 ELISA analysis of vaccination trial

From the graphical results of pCI-neo_*oppA* (Figure 5.5) it could be seen that the control group, which received no vaccine, revealed a slight decrease in average titre values up to week 4, followed by an increase from week 7-10, but on average the titre values were in the same range over the period of 10 weeks. The overall ELISA results indicated much higher titre values for the control group when compared to the pCI-neo_*oppA* vaccinated groups. The pCI-neo_*oppA* 100 µg/ml dose showed a slight increase in average titre values through week 4 to 7 with a slight decrease towards week 10. The pCI-neo_*oppA*

300 µg/ml and 600 µg/ml doses gave similar responses with an increase in average titre values towards week 7, followed by a decrease towards week 10. The average titre values did not increase after the booster injection at week 7.

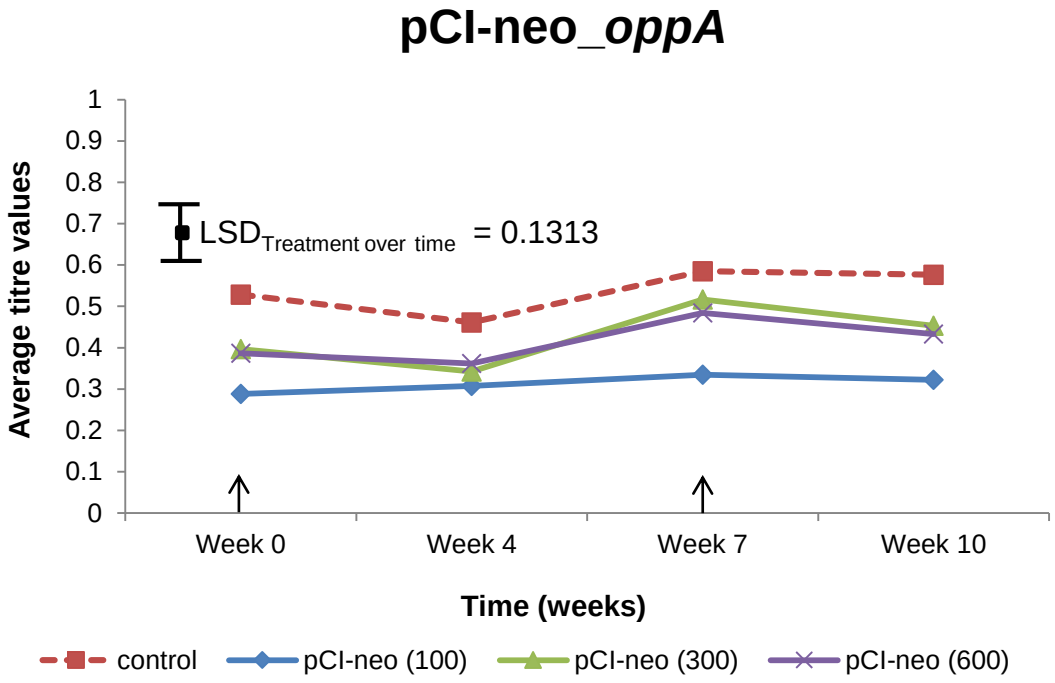

Figure 5.5 Immune response elicited by each of the different concentrations of the pCI-neo_*oppA* vaccine as determined by an ELISA using 10 µg/ml purified OppA-GST protein for coating. Three different ostriches groups were vaccinated with 1 ml of pCI-neo_*oppA* 100 µg/ml, pCI-neo_*oppA* 300 µg/ml and pCI-neo_*oppA* 600 µg/ml, respectively during week 0 and a booster administered during week 7 indicated with arrows. The control group received no immunisations. Blood sampling was done during week 0, 4, 7 and 10.

Compared to the titre values of the VR1020_*oppA* vaccination groups, the control group also had the highest average titre value at week 0 (Figure 5.6). The VR1020_*oppA* 100 µg/ml vaccine dose resulted in a consistent increase in titre values from week 4 through to week 7. After receiving a booster injection at week 7 a further increase in average titre values was observed at week 10. However, these values did not differ significantly from the control group.

In the 300 µg/ml dose group there was one ostrich sample that gave a high titre value (1.078) relative to the rest of the group at week 0, which increased the average titre from 0.440 to 0.482. Despite this high value, the average of the 300 µg/ml dose group was still below the control group. After the first immunisation with VR1020_*oppA* 300 µg/ml there was an increase in average titre value at week 4 which increased further at week 7. At

week 10 there was a slight decrease in titre values compared to week 7 in spite of the booster injection being given at week 7.

Vaccination with VR1020_*oppA* at 600 µg/ml resulted in a similar increase in average titre values after the first vaccination with the average titre reaching a maximum value at week 7. After administering a booster vaccination at week 7, there was a slight decrease in average titre values towards week 10.

The ANOVA analysis of the VR1020_*oppA* vaccination results indicated no significant treatment x time interaction (P = 0.1074) for the data as a whole. The calculated LSD (0.1759) was subsequently used as basis for a pairwise comparison between the immune responses of each of the vaccinated groups and the control, in order to determine statistically significant differences. Only the VR1020_oppA 300 µg/ml and 600 µg/ml treatment groups differed significantly from the control and this applied for all time points, except week 0.

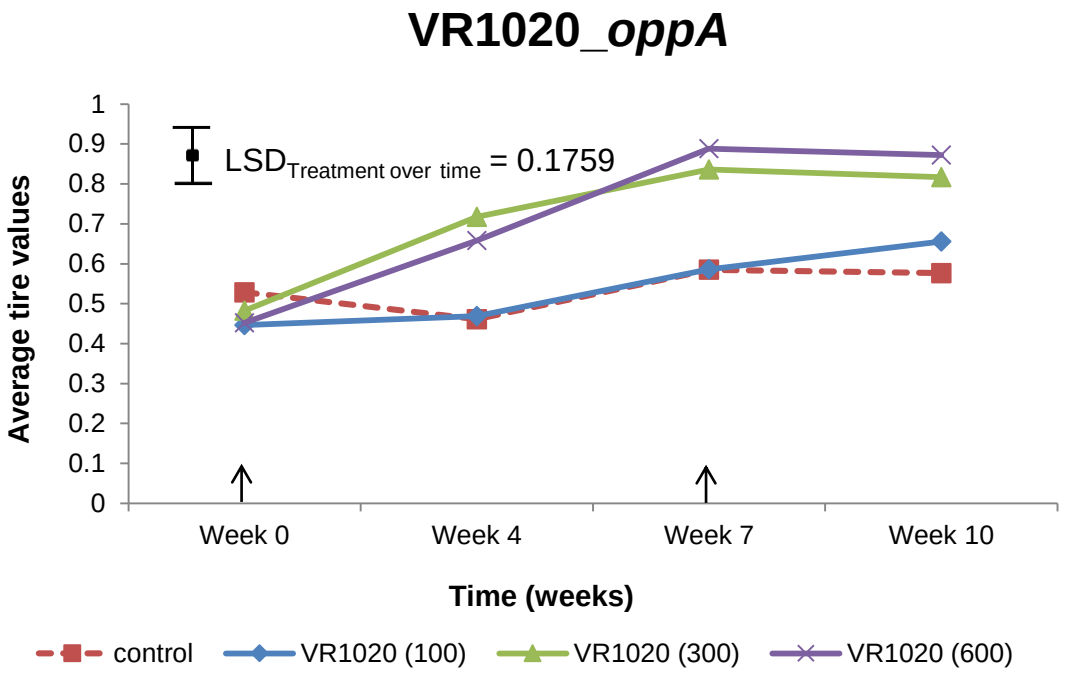

Figure 5.6 Immune response elicited by each of the different concentrations of the VR1020_*oppA* vaccine as determined by an ELISA using 10 µg/ml purified OppA-GST protein for coating. Three different ostriches groups were vaccinated with 1 ml of VR1020_*oppA* 100 µg/ml, VR1020_*oppA* 300 µg/ml and VR1020_*oppA* 600 µg/ml respectively during week 0 and a booster administered during week 7 indicated with arrows. The control group received no immunisations. Blood sampling was done during week 0, 4, 7 and 10.

5.4 Discussion

Mycoplasma infections are a commonly encountered problem in the Oudtshoorn district. Day old ostrich chicks are raised in remote areas such as Fraserburg up to about 3 months of age after which they are transported back to Oudtshoorn. In Fraserburg there are fewer ostrich diseases and neighbouring farms are far apart from one another, limiting the spread of infection. This could be seen from the PCR results where during their time in Fraserburg, only a few cases of mycoplasma infections were detected. When the Ostriches reached 40 kg they were moved back to Oudtshoorn where they were first kept in quarantine for 29 days before they were moved to a semi-intensive feeding system. Within a semi-intensive feeding system about 300 ostriches are kept in a 2-5 hectare environment. This is the feeding system preferred by farmers as the availability and cost of land are limiting factors in the Klein Karoo (Shanawany and Dingle, 1999; Wheeler *et al.*, 2015). Within a camp there are 5-7 feeding bowls, which are washed out with Virkon®S disinfectant on a weekly basis in an attempt to limit bacterial infections (personal communication with farmer). Despite this, once they were introduced into these feeding systems the mycoplasma infections increased dramatically. This was probably due to the close proximity in which they housed aiding the spread of infection (Nicholas and Ayling, 2003; Fox *et al.*, 2005). Except for one of the ostriches there were none that tested positive for Ms01 during the course of the trial. The control group, however, also did not have any Ms01 infections and therefore it cannot be concluded that the lack of Ms01 infections was due to the vaccination with the Ms01 VR1020_*oppA* vaccine. There are no cross reactions between the anti-Ms01 OppA antibodies and the Ms02 and Ms03 OppA proteins (Chapter 3). This may explain the presence of Ms02 and Ms03 throughout the trial as the DNA vaccines developed against Ms01 would have no effect against Ms02 and Ms03. To combat all of the mycoplasma infections encountered simultaneously one could develop a DNA vaccine containing a gene that will lead to the production of antibodies that shows higher cross reactivity between Ms01, Ms02 and Ms03.

The ostriches were tagged on the neck with coloured tags that resulted in them pecking at each other's tags due to the colour of the tags drawing their attention. Loss of tags in some birds resulted in birds being lost from the trial. In future DNA vaccine trials tagging the ostriches underneath their wings might prevent the loss of tags and subsequent loss of data.

Two DNA vaccines, pCI-neo and VR1020, were previously developed that each contain the *oppA* gene of Ms01. It has been reported that DNA vaccines can lead to long-term

protection of animals against pathogens when delivered at the appropriate concentration (Davis *et al*., 2001; Khan, *et al.*, 2010). This led to the decision of administering the DNA vaccines at three different concentrations at week one followed by a booster at week 7. For an immune response to be elicited by these vaccines after i.m. injection, the plasmid has to be internalised into the muscle cells. To ensure internalisation, both plasmids were produced in the supercoiled configuration since supercoiled plasmid is the desired configuration for ensuring better internalisation of the plasmid (Maucksch *et al.*, 2009; Norregaard *et al.*, 2013).

Once the DNA vaccine is internalised into the muscle cell, the plasmid uses the host's cellular machinery in order to reach the nucleus, or to transfect local cells, where the antigen is expressed. This would trigger the B-cells of the immune system to produce antibodies against the expressed antigen (Oshop *et al.*, 2002; Kutzler and Weiner, 2008; Faurez *et al.*, 2010). If a first vaccination results in a primary immune response with memory cells being produced, the secondary immune response (after the booster vaccination) should increase to a greater extent compared to the primary immune response (Klinman *et al.*, 1998; Njongmeta *et al.*, 2012).

Anti-OppA antibodies produced in response to the pCI-neo_*oppA* and VR1020_*oppA* plasmids were evaluated using an ELISA developed for this purpose. No significant average titre increases could be seen when the different dose groups vaccinated with pCI-neo_*oppA* when compared to the control group. Thus, it appears that the different pCI-neo_*oppA* doses in combination with a booster vaccination ultimately failed to induce an anti-OppA immune response. The inability of the vaccine to elicit an immune response could indicate that it was either not expressed or it was expressed at such low levels that it did not evoke an immune response. In the cell culture investigation in this study, the pCI-neo_*oppA* DNA vaccine also did not lead to OppA protein production, supporting this conclusion.

Ostriches vaccinated with the VR1020_*oppA* vaccine were able to produce an anti-OppA immune response. The VR1020_*oppA* 100 µg/ml vaccine, on average, failed to induce an immune response of significant increased value when compared to the control group. Vaccination with VR1020_*oppA* at 300 µg/ml and 600 µg/ml resulted in a statistically significant immune response after the initial i.m. injection when compared to the control group. VR1020 contains a tPa-signal sequence upstream from the *oppA* gene, which assists with the excretion of newly synthesised proteins and could be the reason for the

significant titre increases as a result of vaccination with the VR1020_*oppA* plasmid. After receiving the booster at week 7, the response did not increase further but rather decreased during week 10 although this decrease was not significant. Also, the average titre value was still statistically significant higher if compared to the control.

It may be that the concentration of the booster injected at this point was not high enough, due to the average heavier weight of the ostriches at week 7 (61.3 kg) when compared to week 0 (not measured but estimated to be approximately 46.5 based on average weight gain). Thus an increase in DNA vaccine concentration might be needed after the ostriches reach a weight of 60 kg. If an immune response was produced, several factors could have influenced the observed lack of a secondary immune response after administering the booster vaccination. Firstly, the booster vaccination could have been administered too early. Ostriches typically respond maximally to vaccination within 2-3 weeks after vaccination (Pretorius, 2009). However, in such instance the immune system is directly confronted with the antigen. In the case of a DNA vaccine the antigen still needs to be produced which could add time to the production of an immune response. Secondly, blood samples were only collected up to week 10. A further sampling point at week 13 and week 16 could have led to a better understanding of the immune response encountered after the booster was administered. Memory may have been induced, but due to sampling that only included week 10, it cannot be confirmed.

No significant impact was seen throughout the trial on the average weight of the different experimental groups when compared to the control group. Therefore neither the administered vaccine nor the transport of the ostriches during the trial had a negative affect on the growth or health of the ostriches. Thus significant progress has been made in this study in the development of a DNA vaccine against Ms01, but future trials are still required to optimize booster vaccinations with regard to timing and dosage.

6 Concluding remarks and future perspectives

During this study the aim was to re-evaluate three previously developed DNA vaccines against *Mycoplasma struthionis* sp. nov. str. Ms01, firstly by *in vitro* expression in a mammalian cell culture based system and secondly in a vaccination field trial to determine the ability of these vaccines to elicit immune responses in ostriches. This required the preparation of the Ms01 OppA protein as well as the production of rabbit anti-OppA antibodies. OppA was successfully produced by recombinant expression and purified using affinity chromatography and produced in sufficient quantity for subsequent rabbit anti-OppA antibody production.

Supercoiled DNA vaccine plasmids were used to transfect a COS-1 cell line and lysed with either Trizol® or Trypsin for RNA expression analysis and RTB or RIPA for protein translation analysis. Both the RNA isolation methods proved equally efficient and could be used to show that the *oppA* gene was being transcribed from all the DNA vaccine plasmids. Detecting translation of the *oppA* gene to the OppA protein by SDS-PAGE analysis proved difficult and was only detected after transfection with the VR1020_*oppA* vaccine and only after combining and concentrating the samples of a 6 well plate and analysing them 24 h after lysis with RIPA. This might be due to the low levels of translation or that the protein is degraded soon after production even after the addition of a protease inhibitor to the RIPA buffer. The protein could also be detected with the rabbit anti-OppA antibodies in western blot analysis using a highly sensitive chemiluminescence detection method, but not when using a colorimetric method of detection. This was only observed for the VR1020_*oppA* DNA vaccine and not for the pCI-neo_*oppA* and VR1012_*oppA.* This difference could be due to the presence of the tPa-signal sequence in the VR1020 plasmid causing the expressed protein to be exported from the cell. The OppA protein was however not detected in the cell culture medium, possibly due to low concentrations of protein produced. In future by evaluating the expression of the DNA vaccines in primary ostrich cells, a better indication of whether these DNA vaccines would lead to OppA protein expression once they are injected into ostriches, may be obtained.

The isolated Ms01 OppA protein proved to be efficient as coating antigen in the ELISA analysis of the immune responses elicited by the DNA vaccines. Producing a sufficient concentration of the OppA protein was problematic and several batches of the protein had to be prepared during the course of the ELISA analysis. In future studies the purified OppA-GST protein yield from the glutathione column could be increased by increasing

the incubation time of the protein on the column for up to 3 hours. A higher yield would ensure that all ELISA's are done using the same isolated fraction, ensuring increased consistency and accuracy.

During the DNA vaccine trial on ostriches, the presence of mycoplasmas was evaluated at each sampling date by means of PCR. Raising the ostrich chicks in Fraserburg proved beneficial to their well-being as less mycoplasma infections were encountered during their time in Fraserburg.

The pCI-neo_*oppA* vaccine administered at three different concentrations was unsuccessful in eliciting an immune response. The VR1020_*oppA* vaccine administered at three different concentrations, gave better immune responses when compared to pCI-neo_*oppA*. The VR1020_*oppA* 300 µg/ml and 600 µg/ml doses elicited statistically significant immune responses in comparison to the control. The titre values, however, did not increase after a booster was administered. It might be that a secondary immune response could have occurred, but due to sampling that ended at week 10, it was not observed. These results obtained for the two DNA vaccines were similar as observed during Chapter 4 and again emphasises the importance of the tPa-signal peptide which results in extracellular protein expression.

In future including serum samples at week 13 as well as week 15 of the vaccine trials would give a better indication of whether the booster immunisation would in fact be able to produce a secondary immune response and therefore induce immune memory. Including a serum sample well beyond week 15 could also determine the long-term response of the antibodies circulating the blood.

Although there were still high levels of mycoplasma infections uncounted across all the groups, the focus of the thesis was to prove that the vaccinated animals show an antibody response towards the vaccine. This was however proven, but the efficacy of these developed DNA vaccines was also not evaluated and further studies are required to determine the optimal dose for eliciting a protective immune response against Ms01.

7 References

Abbas, A.K., and Lichtman, A.H. (2011) *Basic Immunology: Functions and Disorders of the Immune*. 3rd ed., Elsevier Health Sciences.

Abbas, A.K., Lichtman, A.H.H., and Pillai, S. (2012) *Basic Immunology*. 4th ed., Elsevier Health Sciences.

Adamu, J.Y., Wawegama, N.K., Browning, G.F., and Markham, P.F. (2013) Membrane proteins of Mycoplasma bovis and their role in pathogenesis. *Res Vet Sci* **95**: 321–325.

Alegria-Schaffer, A., Lodge, A., and Vattem, K. (2009) Performing and optimizing Western blots with an emphasis on chemiluminescent detection. *Methods Enzymol* 463: 573-599.

Allen, H.K., Levine, U.Y., Looft, T., Bandrick, M., and Casey, T.A. (2013) Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol* **21**: 114–119.

Alonso, M., and Leong, J.-A.C. (2013) Licensed DNA Vaccines against Infectious Hematopoietic Necrosis Virus (IHNV). *Recent Pat DNA Gene Seq* **7**: 62–65.

Alvarez, R.A., Blaylock, M.W., and Baseman, J.B. (2003) Surface localized glyceraldehyde-3-phosphate dehydrogenase of Mycoplasma genitalium binds mucin. *Mol Microbiol* **48**: 1417–1425.

Anon. (2002) GST gene fusion system handboek. *Amersham Bioscience.*

Aruffo, A. (1998) Transient Expression of Proteins using COS-1 cells. *Curr Protoc Mol Biol* 1–7.

Babiuk, L.A., Pontarollo, R., Babiuk, S., Loehr, B., and Drunen Littel-van den Hurk, S. van (2003) Induction of immune responses by DNA vaccines in large animals. *Vaccine* **21**: 649–658.

Baldi, L., Hacker, D.L., Adam, M., and Wurm, F.M. (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol Lett* **29**: 677–684.

Balog Ai, and Paz, A. (2007) Ostrich (Struthio camellus) Carcass Yield and Meat Quility Parameters. *Brazilian J Poult Sci* **9**: 215–220.

Bayatzadeh, M.A., Pourbakhsh, S.A., Ashtari, A., and Reza, A. (2014) Molecular typing of Iranian field isolates Mycoplasma synoviae and their differentiation from the live commercial vaccine strain MS-H using vlhA gene. *Br Poult Sci* **55**: 148–156.

Bellstedt, D.U., Human, P.A., Rowland, G.F., and, Van der Merwe K.J. (1987) Acid-treated, naked bacteria as immune carriers for protein antigens. *J Immunol Methods* **98**: 249–55.

Bengtsson-Palme, J., Larsson, D. G. J. (2015) Antibiotic resistance genes in the environment: prioritizing risks. *Nature Rev Microbiol* **13:** 116-123.

Bergman, P.J., Mcknight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., *et al.* (2003) Long-Term Survival of Dogs with Advanced Malignant Melanoma after DNA Vaccination with Xenogeneic Human Tyrosinase: A Phase I Trial. *Clin Cancer Res* **9**: 1284–1290.

Berman, J.W., and Basch, R.S. (1980) Amplification of the Biotin-Avidin immunofluorescence technique. *J Immunol Methods* **36**: 335–338.

Blum, S.A.E., Lorenz, M.G., and Wackernagel, W. (1997) Mechanism of Retarded DNA Degradation and Prokaryotic Origin of DNases in Nonsterile Soils. *Syst Appl Microbiol* **20**: 513–521.

Borrego, B., Argilaguet, J.M., Pérez-Martín, E., Dominguez, J., Pérez-Filgueira, M., Escribano, J.M., *et al.* (2011) A DNA vaccine encoding foot-and-mouth disease virus B and T-cell epitopes targeted to class II swine leukocyte antigens protects pigs against viral challenge. *Antiviral Res* **92**: 359–363.

Botes, A., Peyrot, B.M., Olivier, A.J., Burger, W.P., and Bellstedt, D.U. (2005) Identification of three novel mycoplasma species from ostriches in South Africa. *Vet Microbiol* **111**: 159–169.

Bradbury, J.M. (2005) Gordon Memorial Lecture. Poultry mycoplasmas: sophisticated pathogens in simple guise. *Br Poult Sci* **46**: 125–136.

Brandt, S. (2012) Preliminary investigations into the use of DNA Vaccines to elicit protective immune responses against the ostrich mycoplasma Ms01. MSc dissertation. Stellenbosch University, RSA.

Browning, G.F., Marenda, M.S., Noormohammadi, A.H., and Markham, P.F. (2011) The central role of lipoproteins in the pathogenesis of mycoplasmoses. *Vet Microbiol* **153**: 44–50.

Butler, M. (2004) *Animal Cell culture Technology*.2nd ed. *BIOS Scientific Publishers*.

Butler, M. (2005) Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl Microbiol Biotechnol* **68**: 283–291.

Butt, A.M., Tahir, S., Nasrullah, I., Idrees, M., Lu, J., and Tong, Y. (2012) Mycoplasma genitalium: a comparative genomics study of metabolic pathways for the identification of drug and vaccine targets. *Infect Genet Evol* **12**: 53–62.

Caponi, L., and Migliorini, P. (1999) Antibody Usage in the Lab. *Springer*, Germany

Capozzo, A. V, Wilda, M., Bucafusco, D., los Ángeles Lavoria, M. de, Franco-Mahecha, O.L., Mansilla, F.C., *et al.* (2011) Vesicular Stomatitis Virus glycoprotein G carrying a tandem dimer of Foot and Mouth Disease Virus antigenic site A can be used as DNA and peptide vaccine for cattle. *Antiviral Res* **92**: 219–227.

Chaiet, L., and Wolf, F.J. (1964) The Properties strepavidin, a Biotin-Binding Protein Produced by Streptomycetes. *Arch Biochem Biophys* **106**: 1–5.

Chen, Y.-N., Wu, C.C., Yeo, Y., Xu, P., and Lin, T.L. (2013) A DNA prime-protein boost vaccination strategy targeting turkey coronavirus spike protein fragment containing neutralizing epitope against infectious challenge. *Vet Immunol Immunopathol* **152**: 359–369.

Christensen, N.H., Yavari, C.A., Mcbain, A.J., and Bradbury, J.M. (1994) Investigations into the survival of Mycoplasma gallisepticum, Mycoplasma synoviae and Mycoplasma iowae on materials found in the poultry house environment. *avain Pathol* **23**: 127–143.

Clark, M.F., Lister, R.M., and Bar-Joseph, M. (1986) ELISA techniques. *Immunochem Enzymol* **118**: 742–766.

Corr, B.M., Lee, D.J., Carson, D.A., and Tighe, H. (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J experimantal Med* **184**: 1555–1560.

Cupillard, L., Juillard, V., Latour, S., Colombet, G., Cachet, N., Richard, S., *et al.* (2005) Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats. *Vaccine* **23**: 1910–1916.

DAFF (2012) A Profile of the South African Ostrich Market Value Chain. RSA.

Davis, B.S., Chang, G.J., Cropp, B., John, T., Martin, D.A., Mitchell, C.J., *et al.* (2001) West Nile Virus Recombinant DNA Vaccine Protects Mouse and Horse from Virus Challenge and Expresses In Vitro a Noninfectious Recombinant Antigen That Can Be Used in Enzyme-Linked Immunosorbent Assays. *J Virol* **75**: 4040–4047.

Dhama, K., Mahendran, M., Gupta, P.K., and Rai, A. (2008) DNA vaccines and their applications in veterinary practice: current perspectives. *Vet Res Commun* **32**: 341–356.

de Vries, J., Heine, M., Harms, K., and Wackernagel, W. (2003) Spread of Recombinant DNA by Roots and Pollen of Transgenic Potato Plants, Identified by Highly Specific Biomonitoring Using Natural Transformation of an Acinetobacter sp. *Appl Environ Microbiol* **69**: 4455–4462.

Donnelly, J., Berry, K., and Ulmer, J.B. (2003) Technical and regulatory hurdles for DNA vaccines. *Int J Parasitol* **33**: 457–467.

Dudler, R., Schmidhauser, C., Parishl, R.W., Wettenhall, R.E.H., Schmidt, T., Pflanzenbiologie, I., and Zurich, U. (1988) A mycoplasma high-affinity transport system and the in vitro invasiveness of mouse sarcoma cells. *EMBO J* **7**: 3963–3970.

Dufour, V. (2001) DNA vaccines: new applications for veterinary medicine. *Vet Sci tomorrow* 1–26.

Dunham, S.P. (2002) The application of nucleic acid vaccines in veterinary medicine. *Res Vet Sci* **73**: 9–16.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21 - nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Lett to Nat* **411**: 494–498.

Engvall, E., and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**: 871–874.

Evensen, Ø., and Leong, J.-A.C. (2013) DNA vaccines against viral diseases of farmed fish. *Fish Shellfish Immunol* **35**: 1–8.

Fang Yan, Zhao, Y., Hu, Y., Qiu, J., Lei, W., Ji, W., *et al.* (2013) Protection of chickens against infectious bronchitis virus with a multivalent DNA vaccine and boosting with an inactivated vaccine. *Vet Sci* **14**: 53–60.

Faurez, F., Dory, D., Moigne, V. Le, Gravier, R., and Jestin, A. (2010) Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection. *Vaccine* **28**: 3888–3895.

Ferraro, B., Morrow, M.P., Hutnick, N.A., Shin, T.H., Lucke, C.E., and Weiner, D.B. (2011) Clinical applications of DNA vaccines: current progress. *Vaccines* **53**: 296–302.

Fischer, L., Minke, J., Dufay, N., Baudu, P., and Audonnet, J.-C. (2003) Rabies DNA vaccine in the horse: strategies to improve serological responses. *Vaccine* **21**: 4593–4596.

Fox, L.K., Kirk, J.H., and Britten, A. (2005) Mycoplasma Mastitis: A Review of Transmission and Control. *J Vet Med* **160**: 153–160.

Gao, J., Roston, R.L., Murcha, M.W., Whelan, J., and Benning, C. (2012) TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *J Biol Chem* **287**: 21406–21415.

Giese, M. (2012) *Approved veterinary vaccines: Paving the way to products for human health. In Gene Vaccines*. Springer, Vienna.

Glass, J.I., Assad-garcia, N., Alperovich, N., Yooseph, S., Lewis, M.R., Maruf, M., *et al.* (2006) Essential genes of a minimal bacterium. *PNAS* **103**: 425–430.

Glatz, C.P., Ru, Y.J., Miao, Z.H., Wyatt, S.K., and Rodda, B.J. (2005) Integrating Poultry a Crop and Pasture Farming Systems. *Int J Poult Sci* **4**: 187–191.

Glenting, J., and Wessels, S. (2005) Ensuring safety of DNA vaccines. *Microb Cell Fact* **4**: 1–5.

Gong, Q., Qu, N., Niu, M., Qin, C., Cheng, M., Sun, X., and Zhang, A. (2013) Immune responses and protective efficacy of a novel DNA vaccine encoding outer membrane protein of avian Pasteurella multocida. *Vet Immunol Immunopathol* **152**: 317–324.

Gorres, J.P., Lager, K.M., Kong, W.-P., Royals, M., Todd, J.-P., Vincent, A.L., *et al.* (2011) DNA vaccination elicits protective immune responses against pandemic and classic swine influenza viruses in pigs. *Clin vaccine Immunol* **18**: 1987–1995.

Gouws, J., Personal communication, Laboratory manager, Onderstepoort Veterinary Institute, Department of Veterinary Tropical Diseases, Pretoria.

Gurunathan, S., Klinman, D.M., and Seder, R.A. (2000) DNA vaccines: immunology, application, and optimization. *Annu Rev Immunol* **18**: 927–974.

Hames, C., Halbedel, S., Hoppert, M., Frey, J., and Stülke, J. (2009) Glycerol metabolism is important for cytotoxicity of Mycoplasma pneumoniae. *J Bacteriol* **191**: 747–753.

Harding, H.P., Zhang, Y., and Ron, D. (1999) Protein translation and folding are coupled by an resident kinase. *Nature* **397**: 271–274.

Harper, S., and Speicher, D.W. (2011) Purification of proteins fused to glutathione S-transferase. *Methods Mol Biol* **681**: 1–15.

Harrison, R.G. (1912) The cultivation of tissues in extraneous media as a method of morpho-genetic study. *Anat Rec* **6**: 181–193.

Hay, F.C., and Westwood, O.M.R. (2003) *Practical Immunology*. 4^{ed} . Blackwell Science.

Henrich, B., Hopfe, M., and Kitzerow, A. (1999) The Adherence-Associated Lipoprotein P100, Encoded by an opp Operon Structure, Functions as the Oligopeptide-Binding Domain OppA of a Putative Oligopeptide Transport System in Mycoplasma hominis. *J Bacteriol* **181**: 4873–4878.

Higgins, C.F. (2001) ABC transporters: physiology, structure and mechanism-an overview. *Res Microbiol* **152**: 205–210.

Hiszczyńska-Sawicka, E., Olędzka, G., Holec-Gąsior, L., Li, H., Xu, J.B., Sedcole, R., *et al.* (2011) Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of Toxoplasma gondii. *Vet Parasitol* **177**: 281–289.

Hopfe, M., and Henrich, B. (2004) OppA, the Substrate-Binding Subunit of the Oligopeptide Permease, Is the Major Ecto-ATPase of Mycoplasma hominis. *J Bacteriol* **186**: 1021–1028.

Hopfe, M., and Henrich, B. (2008) OppA, the ecto-ATPase of Mycoplasma hominis induces ATP release and cell death in HeLa cells. *BMC Microbiol* **8**: 1–10.

Jacob, R., Branton, S.L., Evans, J.D., Leigh, S.A., Peebles, E.D., and Al, J.E.T. (2014) Effects of different vaccine combinations against Mycoplasma gallisepticum. *Poult Sci* **1**: 1–6.

Kennedy, N.J., Spithill, T.W., Tennent, J., Wood, P.R., and Piedrafita, D. (2006) DNA vaccines in sheep: CTLA-4 mediated targeting and CpG motifs enhance immunogenicity in a DNA prime/protein boost strategy. *Vaccine* **24**: 970–979.

Khan, A.S., Bodles-Brakhop, A.M., Fiorotto, M.L., and Draghia-Akli, R. (2010) Effects of maternal plasmid GHRH treatment on offspring growth. *Vaccine* **28**: 1905–1910.

Kim, B., Lee, D., Choi, J., Kim, C., Son, M., Suh, Y., *et al.* (2003) In Vivo Kinetics and Biodistribution of a HIV-1 DNA Vaccine after Administration in Mice. *Arch Pharm Res* **26**: 493–498.

Kleven, S.H. (1998) Mycoplasmas in the etiology of multifactorial respiratory disease. *Poult Sci* **77**: 1146–1149.

Kleven, S.H. (2008) Control of Avian Mycoplasma Infections in Commercial Poultry. *avian Dis* **52**: 367–374.

Klinman, D.M., Sechler, J.M.G., Conover, J., Gu, M., and Rosenberg, A.S. (1998) Contribution of Cells at the Site of DNA Vaccination to the Generation of Antigen-Specific Immunity and Memory 1. *J Immunol* **160**: 2388–2392.

Kodihalli, S., Haynes, J.R., Robinson, H.L., and Webster, R.G. (1997) Cross-Protection among Lethal H5N2 Influenza Viruses Induced by DNA Vaccine to the Hemagglutinin. *J Virol* **71**: 3391–3396.

Kretzmer, G. (2002) Industrial processes with animal cells. *Appl Microbiol Biotechnol* **59**: 135–142.

Kutzler, M.A., and Weiner, D.B. (2008) DNA vaccines: ready for prime time? *Nature* **9**: 776–788.

Kyte, J., Doolittle, R.F., Diego, S., and Jolla, L. (1982) A Simple Method for Displaying the Hydropathic Character of a Protein. *J Mol Biol* **157**: 105–132.

Langer, S. (2009) Mycoplasma struthionis sp. nov. and Mycoplasma nasistruthionis sp. nov. from ostrich (Struthio camelus) with respiratory tract diseases. University of Vetinary medicine. Vienna, Austria.

Lassaux, P., Peri, C., Ferrer-Navarro, M., Gourlay, L.J., Gori, A., Conchillo-Solé, O., *et al.* (2013) A structure-based strategy for epitope discovery in Burkholderia pseudomallei OppA antigen. *Cell* **21**: 167–175.

Le, T.P., Coonan, K.M., Hedstrom, R.C., Charoenvit, Y., Sedegah, M., Epstein, J.E., *et al.* (2000) Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* **18**: 1893–1901.

Ledgerwood, J.E., Pierson, T.C., Hubka, S.A., Desai, N., Rucker, S., Gordon, I.J., *et al.* (2011) A West Nile virus DNA vaccine utilizing a modified promoter induces neutralizing antibody in younger and older healthy adults in a phase I clinical trial. *J Infect Dis* **203**: 1396–1404.

Lei, T., Lu, H., Zhang, D., Wang, K., Tian, M., Liu, C., *et al.* (2011) Efficacy of seasonal pandemic influenza hemagglutinin DNA vaccines delivered by electroporation against a seasonal H1N1 virus challenge in mice. *Life Sci* **54**: 293–299.

Le Roux, M.C., and Hoosen, A.A. (2010) Mycoplasma genitalium: a brief review. *South African J Epidermal Infect* **25**: 7–10.

Levy, M.Y., Barron, L.G., Meyer, K.B., and C., S.F. (1996) Characterization of plasmid DNA transfer into mouse skeletal muscle: evaluation of uptake mechanism, expression and secretion of gene products into blood. *Gene Ther* **3**: 201–211.

Lin, M.T.S., Pulkkinen, L., Uitto, J., and Yoon, K. (2000) The gene gun: current applications in cutaneous gene therapy. *Int J Dermatol* **39**: 161–170.

Linton, K.J. (2007) Structure and function of ABC transporters. *Physiology* **22**: 122–130.

Liu, M. (2011) DNA vaccines: an historical perspective and view to the future. *Immunol Rev* **239**: 62–84.

Lobo, C.A., Dhar, R., Kumar, N., and Lobo, C.A. (1999) Immunization of Mice with DNA-Based Pfs25 Elicits Potent Malaria Transmission-Blocking Antibodies. *Infect Immun* **67**: 1688–1693.

Locher, K.P. (2009) Structure and mechanism of ATP-binding cassette transporters. *Philos Trans R Soc Biol Sci* **364**: 239–245.

Loots, K., Vleugels, B., Ons, E., Vanrompay, D., and Goddeeris, B.M. (2006) Evaluation of the persistence and gene expression of an anti-Chlamydophila psittaci DNA vaccine in turkey muscle. *BMC Vet Res* **2**: 1–6.

Luckay, A., Sidhu, M.K., Kjeken, R., Megati, S., Chong, S.-Y., Roopchand, V., *et al.* (2007) Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol* **81**: 5257–5269.

Luke, C.J., Carner, K., Liang, X., and Barbour, A. (1997) An OspA-based DNA vaccine protects mice against infection with Borrelia burgdorferi. *J Infect Dis* **175**: 91–97.

Majewska, D., Jakubowska, M., Ligocki, M., Tarasewicz, Z., Szczerbińska, D., Karamucki, T., and Sales, J. (2009) Physicochemical characteristics, proximate analysis and mineral composition of ostrich meat as influenced by muscle. *Food Chem* **117**: 207–211.

Makela, H. (2000) Vaccines, coming of age after 200 years. *Microbiology* **24**: 9–20.

Manhart, L.E., Broad, J.M., and Golden, M.R. (2011) Mycoplasma genitalium: should we treat and how? *Clin Infect Dis* **53**: 129–142.

Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H.L.E.E., Meek, J., Marquet, M.A.G.D.A., *et al.* (1996) An Improved Plasmid DNA Expression Vector for Direct Injection into Skeletal Muscle. *Hum Gene Ther* **7**: 1205–1217.

Matasci, M., Hacker, D.L., Baldi, L., and Wurm, F.M. (2008) Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. *Drug Discov today Technol* **5**: 37–42.

Mattson, D.L., and Bellehumeur, T.G. (1996) Comparison of three chemiluminescent horseradish peroxidase substrates for immunoblotting. *Anal Biochem* **240**: 306–308.

Maucksch, C., Bohla, A., Hoffmann, F., Schleef, M., Elfinger, M., Hartl, D., and Rudolph, C. (2009) Transgene expression of transfected supercoiled plasmid DNA concatemers in mammalian cells. *J Gene Med* **11**: 444–453.

Medi, B.M., Hoselton, S., Marepalli, R.B., and Singh, J. (2005) Skin targeted DNA vaccine delivery using electroporation in rabbits. I: efficacy. *Int J Pharm* **294**: 53–63.

Mir, R.A., and Kamili, N.M. (2012) DNA vaccines compare to other vaccines. *North-East Vet* **12**: 30–32.

Mora, M., Veggi, D., Santini, L., Pizza, M., and Rappuoli, R. (2003) Reverse vaccinology. *Drug Discov Today* **8**: 459–464.

Morozumi, M., Takahashi, T., and Ubukata, K. (2010) Macrolide-resistant Mycoplasma pneumoniae: characteristics of isolates and clinical aspects of community-acquired pneumonia. *J Infect Chemother* **16**: 78–86.

Moura, L., Dohms, J., Almeida, J.M., Ferreira, P.S., Biffi, C.P., and Backes, R.G. (2012) Development and evaluation of a novel subunit vaccine for Mycoplasma gallisepticum. *Arq Bras Med Veterinária e Zootec* **64**: 1569–1576.

Murphy, K.M., Travers, P., and Walport, M. (2008) *Janeway's immuno biology*. 7th editio., Garland Science, .

Muthumani, K., Lankaraman, K.M., Laddy, D.J., Sundaram, S.G., Chung, C.W., Sako, E., *et al.* (2008) Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. *Vaccine* **26**: 5128–5134.

Nascimento, N.C., Santos, P., Chu, Y., Guimaraes, A.M.S., Pagliaro, A., and Messick, B. (2013) Genome Sequence of Mycoplasma parvum (Formerly Eperythrozoon parvum), a Diminutive Hemoplasma of the Pig. *Genome Announc* **1**: 1–2.

Nepomuceno, R.S.L., Tavares, M.B., Lemos, J.A., Griswold, A.R., Ribeiro, J.L., Balan, A., *et al.* (2007) The oligopeptide (opp) gene cluster of Streptococcus mutans: identification, prevalence, and characterization. *Oral Microbiol Immunol* **22**: 277–284.

Nerurkar, L.S., Namba, M., Brashears, G., Jacob, A.J., Lee, Y.J., and Sever, J.L. (1984) Rapid Detection of Herpes Simplex Virus in Clinical Specimens by Use of a Capture Biotin-Streptavidin Enzyme-Linked Immunosorbent Assay. *J Clin Microbiol* **20**: 109–114.

Nicholas, R.A.J., and Ayling, R.D. (2003) Mycoplasma bovis: disease, diagnosis, and control. *Res Vet Sci* **74**: 105–112.

Nicholas, R.A.J., Ayling, R.D., and McAuliffe, L. (2009) Vaccines for Mycoplasma diseases in animals and man. *J Comp Pathol* **140**: 85–96.

Nicolás, M.F., Barcellos, F.G., Hess, P.N., and Hungria, M. (2007) ABC transporters in Mycoplasma hyopneumoniae and Mycoplasma synoviae : Insights into evolution and pathogenicity. *Genet Mol Biol* **211**: 202–211.

Njongmeta, L.M., Bray, J., Davies, C.J., Davis, W.C., Howard, C.J., Hope, J.C., *et al.* (2012) CD205 antigen targeting combined with dendritic cell recruitment factors and antigen-linked CD40L activation primes and expands significant antigen-specific antibody and CD4(+) T cell responses following DNA vaccination of outbred animals. *Vaccine* **30**: 1624–1635.

Norregaard, K., Andersson, M., Sneppen, K., Nielsen, P.E., Brown, S., and Oddershede, L.B. (2013) DNA supercoiling enhances cooperativity and efficiency of an epigenetic switch. *PNAS* **110**: 17386–17391.

Okamba, F.R., Moreau, E., Cheikh Saad Bouh, K., Gagnon, C.A., Massie, B., and Arella, M. (2007) Immune responses induced by replication-defective adenovirus expressing the C-terminal portion of the Mycoplasma hyopneumoniae P97 adhesin. *Clin vaccine Immunol* **14**: 767–774.

Okuda, K., Wada, Y., and Shimada, M. (2014) Recent Developments in Preclinical DNA Vaccination. *Vaccines* **2**: 89–106.

Olivier. A., personal communication, Technical advisor of the SAOBC.

Oshop, G.L., Elankumaran, S., and Heckert, R.A. (2002) DNA vaccination in the avian. *Vet Immunol Immunopathol* **89**: 1–12.

Osorio, J.E., Tomlinson, C.C., Frank, R.S., Haanes, E.J., Rushlow, K., Haynes, J.R., and Stinchcomb, D.T. (1999) Immunization of dogs and cats with a DNA vaccine against rabies virus. *Vaccine* **17**: 1109–1116.

Pardoll, D.M., and Beckerleg, A.M. (1995) Exposing the Immunology of Naked DNA Vaccines. *Immunity* **3**: 165–169.

Pfarr, D., Rieser, L., Woychik, R., Rottman, F., Rosenberg, M., and Reff, M. (1986) Differential effects of polyadenylation regions on gene expression in mammalian cells. *DNA* **5**: 115–122.

Pham, P.L., Kamen, A., and Durocher, Y. (2006) Large-Scale Transfection of Mammalian Cells for the Fast Production of Recombinant Protein. *Mol Biotechnol* **34**: 225–236.

Plotkin, S.A. (2005) Vaccines: past, present and future Early successes. *Nat Med Suppl* **11**: 5–11.

Pretorius, B. (2009) The ostrich mycoplasma Ms01: The identification, isolation, and modification of the P100 vaccine candidate gene and immunity elicited by poultry mycoplasma vaccines. MSc dissertation. Stellenbosch University, RSA.

Raviprakash, K., Apt, D., Brinkman, A., Skinner, C., Yang, S., Dawes, G., *et al.* (2006) A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. *Virology* **353**: 166–173.

Razin, S. (2010) Time-line of significant contributions to mycoplasmology. *Biologicals* **38**: 191–192.

Razin, S., and Hayflick, L. (2010) Highlights of mycoplasma research - An historical perspective. *Biologicals* **38**: 183–190.

Razin, S., Yogev, D., and Naot, Y. (1998) Molecular Biology and Pathogenicity of Mycoplasmas Molecular Biology and Pathogenicity of Mycoplasmas. *Microbiol Mol Biol Rev* **62**: 1094–1156.

Rios-Esteves, J., and Resh, M.D. (2013) Stearoyl CoA desaturase is required to produce active, lipid-modified Wnt proteins. *Cell Rep* **4**: 1072–1081.

Robinson, H.L., and Torres, C.A.T. (1997) DNA vaccines. *Semin Immunol* **9**: 271–283.

Rosati, S., Robino, P., Fadda, M., Animali, P., and Ecologia, E. (2000) Expression and antigenic characterization of recombinant Mycoplasma agalactiae P48 major surface protein. *Vet Microbiol* **71**: 201–210.

Rottem, S. (2003) Interaction of mycoplasmas with host cells. *Physiol Rev* **83**: 417–432.

Sardesai, N., Khan, A., McCoy, J., Lin, F., Mendoza, J., Yang, M., *et al.* (2012) Minimally invasive and surface electroporation delivery of DNA vaccines for the induction of robust humoral immune responses against HIV antigens. *Retrovirology* **9**: 1–9.

Schadeck, E.B., Sidhu, M., Egan, M.A., Chong, S.-Y., Piacente, P., Masood, A., *et al.* (2006) A dose sparing effect by plasmid encoded IL-12 adjuvant on a SIVgag-plasmid DNA vaccine in rhesus macaques. *Vaccine* **24**: 4677–4687.

Schalk, J., FR, M., GA, B., LA, van A., H, O., and TG, K. (2006) Preclinical and Clinical Safety Studies on DNA Vaccines. *Hum Vaccin* 45–53.

Scheerlinck, J.-P.Y., Karlis, J., Tjelle, T.E., Presidente, P.J.A., Mathiesen, I., and Newton, S.E. (2004) In vivo electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* **22**: 1820–1825.

Shanawany, M.M., and Dingle, J. (1999) *Ostrich productive systems*. Food and Agricultural Orginization of the United Nations, Rome.

Shil, P.K., Kanci, A., Browning, G.F., and Markham, P.F. (2011) Development and immunogenicity of recombinant GapA(+) Mycoplasma gallisepticum vaccine strain ts-11 expressing infectious bronchitis virus-S1 glycoprotein and chicken interleukin-6. *Vaccine* **29**: 3197–3205.

Simionatto, S., Marchioro, S.B., Galli, V., Luerce, T.D., Hartwig, D.D., Moreira, Â.N., and Dellagostin, O.A. (2009) Efficient site-directed mutagenesis using an overlap extension-PCR method for expressing Mycoplasma hyopneumoniae genes in Escherichia coli. *J Microbiol Methods* **79**: 101–105.

Skinner, M.A., Buddle, B.M., Wedlock, D.N., Keen, D., Lisle, G.W. De, Tascon, R.E., *et al.* (2003) A DNA Prime-Mycobacterium bovis BCG Boost Vaccination Strategy for Cattle Induces Protection against Bovine Tuberculosis A DNA Prime- Mycobacterium bovis BCG Boost Vaccination Strategy for Cattle Induces Protection against Bovine Tuberculosis. *Infect Immun* **71**: 4901–4907.

Smooker, P.M., Steeper, K.R., Drew, D.R., Strugnell, R.A., and Spithill, T.W. (1999) Humoral responses in mice following vaccination with DNA encoding glutathione S-transferase of Fasciola hepatica: effects of mode of vaccination and the cellular compartment of antigen expression. *Parasite Immunol* **21**: 357–364.

Sneath, A., and Subcommittee, T. (1979) Proposal of Minimal Standards for Descriptions of New Species of the Class Mollicutes. *Int J Syst Backteriology* **29**: 172–180.

Snyder, D.B., Marquardt, W.W., Yancey, F.S., and Savage, P.K. (2014) An Enzyme-Linked Immunosorbent Assay for the Detection of Antibody Against Avian Influenza Virus. *avian Dis* **29**: 136–144.

Song, H., Yan, R., Xu, L., Song, X., Shah, M.A.A., Zhu, H., and Li, X. (2010) Efficacy of DNA vaccines carrying Eimeria acervulina lactate dehydrogenase antigen gene against coccidiosis. *Exp Parasitol* **126**: 224–231.

Steenmans, S. (2010) The identification, contiguous sequence annotation, cloning and site-directed mutagenesis of the P100 vaccine candidate gene of the ostrich mycoplasma. MSc dissertation. Stellenboch University, RSA

Strayer, D.S. (1996) SV40 As an Effective Gene Transfer Vector in Vivo SV40 As an Effective Gene Transfer Vector in Vivo. *J Biol Chem* **196**: 24741–24746.

Struhl, K. (1999) Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell* **98**: 1–4.

Suarez, D.L., Schultz-cherry, S., Suarez, D.L., and Schultz-cherry, S. (2015) The Effect of Euikaryotic Expression Vectors and Adjuvants on DNA Vaccines in Chickens Using an Avian Influenza Model. *Am Assoc Avain Pathol* **44**: 861–868.

Taylor-Robinson, D., and Jensen, J.S. (2011) Mycoplasma genitalium: from Chrysalis to multicolored butterfly. *Clin Microbiol Rev* **24**: 498–514.

Thompson, C.C., Vieira, N.M., Vicente, A.C.P., and Thompson, F.L. (2011) Towards a genome based taxonomy of Mycoplasmas. *Infect Genet Evol* **11**: 1798–1804.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. (1998) Protective Efficacy of DNA Vaccines against Duck Hepatitis B Virus Infection Protective. *J Virol* **72**: 84–94.

Tully, J.G., Cole, R.M., Taylor-robinson, D., and Rose, D.L. (1981) A NEWLY DISCOVERED MYCOPLASMA IN THE HUMAN UROGENITAL TRACT. *Lancet* 1288–1291.

Uzzau, S., Marogna, G., Leori, G.S., Iii, C., Schianchi, G., Stocker, B.A.D., *et al.* (2005) Virulence Attenuation and Live Vaccine Potential of aroA, crp cdt cya, and Plasmid-Cured Mutants of Salmonella enterica Serovar Abortusovis in Mice and Sheep. *Infect Immun* **73**: 4302–4308.

van Drunen Littel-van den Hurk, S., Loehr, B.I., and Babiuk, L.A. (2001) Immunization of livestock with DNA vaccines: current studies and future prospects. *Vaccine* **19**: 2474–2479.

Vanrompay, D., Cox, E., and Volckaert, G. (1999) Turkeys are protected from infection with Chlamydia psittaci by plasmid DNA vaccination against the major outer membrane protein. *Clin Exp Immunol* **118**: 49–55.

van Tonder, A. (2013) Development of an oral vaccine against the ostrich-specific mycoplasma, Mycoplasma struthionis. MSC dissertation. Stellenbosch University, RSA

Voller, A., Bartlett, A., and Bidwell, D.E. (1978) Enzyme immunoassays with special reference to ELISA techniques. *J Clin Pathol* **31**: 507–520.

Weintraub, H., Cheng, P.F., and Conrad, K. (1986) Expression of transfected DNA depends on DNA topology. *Cell* **46**: 115–122.

Wheeler, A., Knight, A.T., and Vetter, S. (2015) Examining the evidence for ecologically sustainable ostrich breeding practices on natural veld in the Little Karoo, South Africa. *African J Range Forage Sci* 1–9.

Whithear, K.. (1996) Control of avian mycoplasmoses by vaccination. *revenue Sci Tech Rev Off Int des Epizoot* **15**: 1527–1553.

Wium, M., Botes, A., and Bellstedt, D.U. (2015) The identification of oppA gene homologues as part of the oligopeptide transport system in mycoplasmas. *Gene* **558**: 31–40.

Wolff J. A. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **60**: 1–6.

Wurm, F.M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* **22**: 1393–1398.

Yamao, F., Muto, A., Kawauchi, Y., Iwami, M., Iwagami, S., Azumi, Y., and Osawa, S. (1985) UGA is read as tryptophan in Mycoplasma capricolum. *Proc Natl Acad Sci U S A* **82**: 2306–2309.

Yao, Q., Fischer, K.P., Li, L., Agrawal, B., Berhane, Y., Tyrrell, D.L., *et al.* (2010) Immunogenicity and protective efficacy of a DNA vaccine encoding a chimeric protein of avian influenza hemagglutinin subtype H5 fused to CD154 (CD40L) in Pekin ducks. *Vaccine* **28**: 8147–8156.

Zhang, H.-Y., Sun, S.-H., Guo, Y.-J., Chen, Z.-H., Huang, L., Gao, Y.-J., *et al.* (2005) Tissue distribution of a plasmid DNA containing epitopes of foot-and-mouth disease virus in mice. *Vaccine* **23**: 5632–5640.

Zhou, W., Parent, L.J., Wills, J.W., and Reshl, M.D. (1994) Identification of a Membrane-Binding Domain within the Amino-Terminal Region of Human Immunodeficiency Virus Type 1 Gag Protein Which Interacts with Acidic Phospholipids. *J Virol* **68**: 2556–2569.

8 Addendum A

Statistical analysis data of an ostrich vaccination trial using Agrobase Generation II®

The anti-OppA humoral immune response elicited by the DNA vaccines

The humoral immune response elicited by the ostriches that were vaccinated with the VR1020_*oppA* and pCI-neo_*oppA* vaccines was analysed using ELISA as described in section 5.2.9. The data obtained was subsequently used for an analysis of variance (ANOVA) and subsequent least significant difference (LSD) calculations using the Agrobase Generation II® (Agronomix Software Inc.) software.

ANALYSIS OF VARIENCE

Bertie, VR1020, 2015

Variable: Response to DNA vaccine VR1020_*oppA*

Source	df	SS	MS	F-value	Pr>F
Total	243	18.921			
FAC_A	3	1.389	.463	7.62	0.0010
FAC_B	3	2.784	0.928	15.27	0.0000
FAC_A by FAC_B	9	0.892	0.099	1.63	0.1074
Residual	228	13.856	0.061		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares;
F- value =

Grand mean = 0.646	R-squared = 0.2677	C.V = 38.16%

C.V. = coefficient of variation

LSD for FAC_A = 0.0880	S.E.D = 0.0446	r = 61.0
t (2-sided a=0.050, 228 df) = 1.9704		MSE = 0.06077

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

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
3	0.72	49.5	1 VR1020 (600)
2	0.71	36.7	2 VR1020 (300)
4	0.61	42.8	3 Control
1	0.54	31.7	4 VR1020 (100)

LSD for FAC_B = 0.0880	S.E.D = 0.0446	r = 61.0
t (2-sided a=0.050, 228 df) = 1.9704		MSE = 0.06077

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

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
6	0.75	40.3	1 Week 7
9	0.74	41.1	2 Week 10
3	0.60	38.3	3 Week 4
0	0.49	33.5	4

LSD for FAC_A*FAC_B = 0.1759	S.E.D = 0.0893	r = 15.3
t (2-sided a=0.050, 228 df) = 1.9704		MSE = 0.06077

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

Two-way table for FAC_A*FAC_B, n=15									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.470	0.000	0.000	0.586	0.000	0.000	0.656
2	0.000	0.000	0.718	0.000	0.000	0.838	0.000	0.000	0.816
3	0.000	0.000	0.659	0.000	0.000	0.889	0.000	0.000	0.873
4	0.000	0.000	0.542	0.000	0.000	0.690	0.000	0.000	0.628

ANALYSIS OF VARIENCE

VR1020, 2015 (Fraserburg-Oudtshoorn)

Variable: Weight

Source	df	SS	MS	F-value	Pr>F
Total	182	15920.277			
FAC_A	3	1298.027	432.676	7.71	0.0001
FAC_B	2	5004.695	2502.348	44.57	0.0000
FAC_A by FAC_B	6	16.488	2.748	0.05	0.9995
Residual	171	9601.066	56.147		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares;
F- value =

Grand mean = 62.587	R-squared = 0.3969	C.V = 11.97%

C.V. = coefficient of variation

LSD for FAC_A = 3.0925	S.E.D = 1.5797	r = 45.0
t (2-sided a=0.050, 171 df) = 1.9739		MSE = 56.14659

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

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
4	66.10	13.1	1 control
2	63.13	13.9	2 VR1020 (300)
3	62.75	16.0	3 VR1020 (600)
1	14.7	14.7	4 VR1020 (100)

LSD for FAC_B = 2.6782	S.E.D = 1.3568	r = 61.0
t (2-sided a=0.050, 228 df) = 1.9739		MSE = 56.14659

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

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	69.42	11.2	1 Week 10
6	61.7	12.5	2 Week 7
3	56.64	13.9	3 Week 4

LSD for FAC_A*FAC_B = 5.3564	S.E.D = 2.7136	r = 15.3
t (2-sided a=0.050, 171 df) = 1.9739		MSE = 56.1459

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

Two-way table for FAC_A*FAC_B, n=15									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	52.000	0.000	0.000	57.467	0.000	0.000	65.633
2	0.000	0.000	57.353	0.000	0.000	62.471	0.000	0.000	69.559
3	0.000	0.000	56.969	0.000	0.000	62.063	0.000	0.000	69.219
4	0.000	0.000	60.231	0.000	0.000	64.808	0.000	0.000	73.269

The trial input data follows for the VR1020_*oppA* vaccine. On each page the data is arranged in two columns, each containing the ostrich number, treatment received (trt), time (in weeks), response gained using ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right.

*Ostrich	TRT	TIME	RESP	WEIGHT
81	1	0	0.498	-9.00
83	1	0	0.368	-9.00
84	1	0	0.461	-9.00
85	1	0	0.488	-9.00
86	1	0	0.455	-9.00
87	1	0	0.495	-9.00
88	1	0	0.356	-9.00
90	1	0	0.408	-9.00
91	1	0	0.424	-9.00
92	1	0	0.449	-9.00
95	1	0	0.508	-9.00
96	1	0	0.491	-9.00
97	1	0	0.427	-9.00
99	1	0	0.431	-9.00
100	1	0	0.442	-9.00
101	2	0	0.553	-9.00
103	2	0	0.484	-9.00
104	2	0	0.285	-9.00
106	2	0	1.078	-9.00
107	2	0	0.391	-9.00
108	2	0	0.32	-9.00
109	2	0	0.346	-9.00
110	2	0	0.44	-9.00
111	2	0	0.474	-9.00
112	2	0	0.357	-9.00
113	2	0	0.385	-9.00

*Ostrich	TRT	TIME	RESP	WEIGHT
114	2	0	0.49	-9.00
115	2	0	0.424	-9.00
116	2	0	0.776	-9.00
117	2	0	0.523	-9.00
118	2	0	0.562	-9.00
119	2	0	0.392	-9.00
122	3	0	0.649	-9.00
123	3	0	0.365	-9.00
124	3	0	0.416	-9.00
126	3	0	0.293	-9.00
127	3	0	0.525	-9.00
128	3	0	0.204	-9.00
129	3	0	0.666	-9.00
130	3	0	0.407	-9.00
131	3	0	0.382	-9.00
133	3	0	0.442	-9.00
135	3	0	0.411	-9.00
136	3	0	0.472	-9.00
137	3	0	0.496	-9.00
138	3	0	0.573	-9.00
139	3	0	0.435	-9.00
140	3	0	0.496	-9.00
1	4	0	0.681	-9.00
2	4	0	0.664	-9.00
4	4	0	1.099	-9.00
7	4	0	0.521	-9.00

*Ostrich	TRT	TIME	RESP	WEIGHT
8	4	0	0.725	-9.00
9	4	0	0.24	-9.00
10	4	0	0.405	-9.00
15	4	0	0.442	-9.00
16	4	0	0.687	-9.00
17	4	0	0.44	-9.00
18	4	0	0.83	-9.00
19	4	0	0.403	-9.00
20	4	0	0.461	-9.00
81	1	3	0.537	45.50
83	1	3	0.241	46.00
84	1	3	0.455	38.00
85	1	3	0.354	57.50
86	1	3	0.828	57.00
87	1	3	0.428	56.50
88	1	3	0.471	52.00
90	1	3	0.543	61.00
91	1	3	0.746	49.00
92	1	3	0.558	54.50
95	1	3	0.28	55.00
96	1	3	0.386	50.00
97	1	3	0.468	46.00
99	1	3	0.435	55.00
100	1	3	0.303	57.00
101	2	3	0.78	66.00
103	2	3	0.668	55.00
104	2	3	0.752	57.00
106	2	3	0.542	57.00
107	2	3	0.389	65.50
108	2	3	0.555	60.00
109	2	3	0.361	65.00
110	2	3	0.574	63.00
111	2	3	0.835	63.50
112	2	3	0.624	54.50
113	2	3	0.783	42.50
114	2	3	0.729	51.00
115	2	3	0.754	41
116	2	3	1.33	49
117	2	3	0.676	60.50
118	2	3	0.848	65.00
119	2	3	1.005	59.50
122	3	3	1.047	54.00
123	3	3	0.769	78.00
124	3	3	0.728	54.50
126	3	3	0.424	61.00
127	3	3	0.986	53.00

*Ostrich	TRT	TIME	RESP	WEIGHT
128	3	3	0.468	62.50
129	3	3	0.81	45.00
130	3	3	0.533	61.00
131	3	3	0.745	71.00
133	3	3	0.671	50.50
135	3	3	0.414	52.00
136	3	3	0.422	48.00
137	3	3	0.788	50.00
138	3	3	0.583	58.50
139	3	3	0.389	60.00
140	3	3	0.758	53
1	4	3	0.468	48.00
2	4	3	0.637	59
4	4	3	1.215	58
7	4	3	0.474	50.50
8	4	3	0.547	60.50
9	4	3	0.254	66.00
10	4	3	0.544	68.00
15	4	3	0.304	52.00
16	4	3	0.762	59.50
17	4	3	0.66	59.50
18	4	3	0.383	74.00
19	4	3	0.515	64.50
20	4	3	0.287	64.00
81	1	6	0.575	49.50
83	1	6	0.403	66.00
84	1	6	0.455	44.50
85	1	6	0.495	57.50
86	1	6	0.494	62.00
87	1	6	1.003	58.50
88	1	6	0.594	60.00
90	1	6	0.552	68.50
91	1	6	0.743	53.00
92	1	6	0.755	56.50
95	1	6	0.442	57.50
96	1	6	0.625	53.50
97	1	6	0.573	50.50
99	1	6	0.548	59.50
100	1	6	0.533	65.00
101	2	6	0.749	71.50
103	2	6	0.935	60.50
104	2	6	0.695	59.00
106	2	6	0.645	64.00
107	2	6	0.587	69.50
108	2	6	0.802	64.50
109	2	6	0.499	67.50

*Ostrich	TRT	TIME	RESP	WEIGHT
110	2	6	0.792	70.50
111	2	6	1.065	66.50
112	2	6	1.238	62.50
113	2	6	0.929	49.00
114	2	6	1.102	57.50
115	2	6	0.471	46.00
116	2	6	1.263	57.50
117	2	6	0.866	63.50
118	2	6	0.496	68.00
119	2	6	1.092	64.50
122	3	6	0.956	62.50
123	3	6	1.133	82.50
124	3	6	0.752	60.00
126	3	6	0.566	67.00
127	3	6	0.873	54.00
128	3	6	0.55	64.50
129	3	6	0.929	49.00
130	3	6	0.823	67.50
131	3	6	0.745	77.00
133	3	6	2.254	56.00
135	3	6	0.855	55.50
136	3	6	0.695	54.00
137	3	6	0.569	56.00
138	3	6	0.992	62.50
139	3	6	0.754	66.50
140	3	6	0.769	58.50
1	4	6	0.747	53.50
2	4	6	0.774	65.50
4	4	6	1.311	59.00
7	4	6	0.573	58.00
8	4	6	0.566	64.50
9	4	6	0.429	70.00
10	4	6	0.593	70.00
15	4	6	0.643	56.50
16	4	6	1.225	62.00
17	4	6	0.546	67.50
18	4	6	0.554	80.00
19	4	6	0.707	68.50
20	4	6	0.304	67.50
81	1	9	0.77	57.00
83	1	9	0.645	74.00
84	1	9	0.533	51.50
85	1	9	0.822	65.50
86	1	9	0.496	73.00
87	1	9	0.561	60.00
88	1	9	0.575	72.50

*Ostrich	TRT	TIME	RESP	WEIGHT
90	1	9	0.458	76.00
91	1	9	1.068	62.00
92	1	9	0.652	67.50
95	1	9	0.634	65.50
96	1	9	0.409	62.00
97	1	9	0.548	59.50
99	1	9	0.574	69.50
100	1	9	1.088	69.00
101	2	9	0.837	75.00
103	2	9	0.772	68.00
104	2	9	0.44	66.50
106	2	9	0.544	74.00
107	2	9	0.722	75.00
108	2	9	0.473	72.50
109	2	9	0.733	74.00
110	2	9	0.626	77.00
111	2	9	1.098	80.00
112	2	9	0.73	67.50
113	2	9	1.131	57.00
114	2	9	0.735	62.00
115	2	9	1.176	55.00
116	2	9	0.856	62.50
117	2	9	1.111	71.00
118	2	9	0.949	74.00
119	2	9	0.933	71.50
122	3	9	1.113	67.00
123	3	9	1.185	92.00
124	3	9	0.534	66.00
126	3	9	0.73	77.00
127	3	9	0.683	66.00
128	3	9	0.545	75.00
129	3	9	0.951	53.00
130	3	9	0.673	76.50
131	3	9	1.095	80.00
133	3	9	2.257	61.50
135	3	9	0.642	66.50
136	3	9	0.614	63.00
137	3	9	0.542	63.00
138	3	9	0.84	65.00
139	3	9	0.72	72.50
140	3	9	0.835	63.50
1	4	9	0.4	65.00
2	4	9	1.166	74.00
4	4	9	0.932	64.50
7	4	9	0.51	71.50
8	4	9	0.806	73.00

*Ostrich	TRT	TIME	RESP	WEIGHT
9	4	9	0.488	76.00
10	4	9	0.705	77.00
15	4	9	0.42	64.00
16	4	9	0.876	72.00

*Ostrich	TRT	TIME	RESP	WEIGHT
17	4	9	0.484	75.00
18	4	9	0.377	87.00
19	4	9	0.824	77.00
20	4	9	0.161	76.50

* Entry – ostrich name; TRT – Treatment received; TIME – time in weeks; RESP – Titre response; WEIGHT – weight of ostrich at relevant week.

ANALYSIS OF VARIENCE

Bertie, pCI-neo, 2015

Variable: Response to DNA vaccine pCI-neo_*oppA*

Source	**df**	**SS**	**MS**	**F-value**	**Pr>F**
Total	199	7.774			
FAC_A	3	2.229	0.743	26.83	0.0000
FAC_B	3	0.375	0.125	4.51	0.0044
FAC_A by FAC_B	9	0.073	0.008	0.29	0.9759
Residual	184	5.096	0.028		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares;
F- value =

Grand mean = 0.444	R-squared = 0.3444	C.V = 37.48%

C.V. = coefficient of variation

LSD for FAC_A = 0.0657	S.E.D = 0.0333	r = 50.0
t (2-sided a=0.050, 184 df) = 1.9729		MSE = 0.02770

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

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
4	0.62	44.0	1 Control
2	0.43	23.9	2 VR1020 300
3	0.42	30.9	3 VR1020 600
1	0.32	30.9	4 VR1020 100

LSD for FAC_B = 0.0657	S.E.D = 0.0333	r = 50.0
t (2-sided a=0.050, 184 df) = 1.9729		MSE = 0.02770

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

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
6	0.51	40.4	1 Week 7
9	0.46	44.0	2 Week 10
0	0.42	43.5	3
3	0.39	44.5	4 Week 4

LSD for FAC_A*FAC_B = 0.1313	S.E.D = 0.0666	r = 12.5
t (2-sided a=0.050, 184 df) = 1.9729		MSE = 0.02770

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

Two-way table for FAC_A*FAC_B, n=15									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	0.308	0.000	0.000	0.333	0.000	0.000	0.323
2	0.000	0.000	0.343	0.000	0.000	0.518	0.000	0.000	0.454
3	0.000	0.000	0.363	0.000	0.000	0.484	0.000	0.000	0.434
4	0.000	0.000	0.542	0.000	0.000	0.690	0.000	0.000	0.628

ANALYSIS OF VARIENCE

pCI-neo, 2015 (Fraserburg-Oudtshoorn)

Variable: Weight

Source	df	SS	MS	F-value	Pr>F
Total	149	11695.766			
FAC_A	3	602.172	200.724	3.98	0.0093
FAC_B	2	4114.389	2057.194	40.82	0.0000
FAC_A by FAC_B	6	24.017	4.003	0.08	0.9980
Residual	138	6955.189	50.400		

df = degrees of freedom; SS = Sum of Squares; MS = Mean of Sum of Squares;
F- value =

Grand mean = 63.065	R-squared = 0.4053	C.V = 11.26%

C.V. = coefficient of variation

LSD for FAC_A = 3.2418	S.E.D = 1.6506	r = 37.0
t (2-sided a=0.050, 138 df) = 1.9773		MSE = 50.39992

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

FAC_A			
Averages			
Level	**Y**	**Cv**	**Rank**
4	66.10	13.1	1 Control
1	63.23	12.4	2 VR1020 100
2	61.96	14.9	3 VR1020 300
3	60.97	15.0	4 VR1020 600

LSD for FAC_B = 2.8075	S.E.D = 1.4199	r = 50.0
t (2-sided a=0.050, 138 df) = 1.9773		MSE = 50.39992

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

FAC_B			
Averages			
Level	**Y**	**Cv**	**Rank**
9	70.03	9.8	1 Week 10
6	61.97	11.5	2 Week 7
3	57.20	13.2	3 Week 4

LSD for FAC_A*FAC_B = 5.6150	S.E.D = 2.8397	r = 12.5
t (2-sided a=0.050, 138 df) = 1.9773		MSE = 50.39992

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

Two-way table for FAC_A*FAC_B, n=15									
	1	2	3	4	5	6	7	8	9
1	0.000	0.000	57.600	0.000	0.000	62.450	0.000	0.000	69.650
2	0.000	0.000	56.208	0.000	0.000	61.208	0.000	0.000	68.458
3	0.000	0.000	54.767	0.000	0.000	59.400	0.000	0.000	68.733
4	0.000	0.000	60.231	0.000	0.000	64.808	0.000	0.000	73.269

The trial input data follows for the pCI-neo_*oppA* vaccine. On each page the data is arranged in two columns, each containing the ostrich number, treatment received (trt), time (in weeks), response gained using ELISA analysis (resp) and weight of the ostriches, read from top to bottom and left to right.

ENTRY	TRT	TIME	RESP	WEIGHT
21	1	0	0.263	-9.00
23	1	0	0.395	-9.00
25	1	0	0.252	-9.00
29	1	0	0.290	-9.00
30	1	0	0.182	-9.00
31	1	0	0.427	-9.00
33	1	0	0.265	-9.00
34	1	0	0.308	-9.00
37	1	0	0.297	-9.00
38	1	0	0.292	-9.00
41	2	0	0.292	-9.00
44	2	0	0.356	-9.00
46	2	0	0.347	-9.00
47	2	0	0.445	-9.00
48	2	0	0.397	-9.00
50	2	0	0.380	-9.00
51	2	0	0.418	-9.00
52	2	0	0.366	-9.00
53	2	0	0.443	-9.00
55	2	0	0.358	-9.00
56	2	0	0.489	-9.00
60	2	0	0.472	-9.00
62	3	0	0.320	-9.00
63	3	0	0.353	-9.00
64	3	0	0.383	-9.00
66	3	0	0.277	-9.00
68	3	0	0.615	-9.00
70	3	0	0.297	-9.00
71	3	0	0.284	-9.00
72	3	0	0.369	-9.00
73	3	0	0.387	-9.00
74	3	0	0.385	-9.00
75	3	0	0.546	-9.00
77	3	0	0.255	-9.00
78	3	0	0.419	-9.00
79	3	0	0.484	-9.00
80	3	0	0.429	-9.00
1	4	0	0.681	-9.00
2	4	0	0.664	-9.00
4	4	0	1.311	-9.00
7	4	0	0.521	-9.00
8	4	0	0.725	-9.00
9	4	0	0.240	-9.00
10	4	0	0.405	-9.00
15	4	0	0.442	-9.00
16	4	0	0.687	-9.00
17	4	0	0.440	-9.00
18	4	0	0.830	-9.00
19	4	0	0.403	-9.00
20	4	0	0.461	-9.00

ENTRY	TRT	TIME	RESP	WEIGHT
21	1	3	0.329	57.00
23	1	3	0.243	49.00
25	1	3	0.266	65.00
29	1	3	0.172	62.50
30	1	3	0.173	59.50
31	1	3	0.426	60.50
33	1	3	0.206	65.00
34	1	3	0.398	54.00
37	1	3	0.430	61.50
38	1	3	0.431	42.00
41	2	3	0.183	55.50
44	2	3	0.336	69.50
46	2	3	0.362	52.50
47	2	3	0.389	46.50
48	2	3	0.422	41.00
50	2	3	0.332	56.50
51	2	3	0.256	56.00
52	2	3	0.408	62.50
53	2	3	0.362	56.00
55	2	3	0.376	63.50
56	2	3	0.326	64.50
60	2	3	0.361	50.50
62	3	3	0.405	57.00
63	3	3	0.276	54.00
64	3	3	0.182	57.00
66	3	3	0.562	58.50
68	3	3	0.435	40.00
70	3	3	0.222	61.00
71	3	3	0.373	58.50
72	3	3	0.219	48.50
73	3	3	0.437	52.00
74	3	3	0.304	46.00
75	3	3	0.388	56.50
77	3	3	0.285	60.00
78	3	3	0.385	62.00
79	3	3	0.480	44.50
80	3	3	0.474	66.00
1	4	3	0.468	48.00
2	4	3	0.637	59
4	4	3	1.215	58
7	4	3	0.474	50.50
8	4	3	0.547	60.50
9	4	3	0.254	66.00
10	4	3	0.544	68.00
15	4	3	0.304	52.00
16	4	3	0.762	59.50
17	4	3	0.660	59.50

ENTRY	TRT	TIME	RESP	WEIGHT
18	4	3	0.383	74.00
19	4	3	0.515	64.50
20	4	3	0.287	64.00
21	1	6	0.343	64.00
23	1	6	0.442	55.50
25	1	6	0.231	69.00
29	1	6	0.273	65.00
30	1	6	0.305	62.50
31	1	6	0.304	61.50
33	1	6	0.201	69.50
34	1	6	0.478	56.00
37	1	6	0.404	64
38	1	6	0.363	57.50
41	2	6	0.609	58.00
44	2	6	0.623	69.50
46	2	6	0.597	58.50
47	2	6	0.510	51.50
48	2	6	0.646	44.50
50	2	6	0.470	65.00
51	2	6	0.368	62.00
52	2	6	0.405	67.50
53	2	6	0.535	62.00
55	2	6	0.469	71.00
56	2	6	0.424	69.50
60	2	6	0.545	55.50
62	3	6	0.390	62.00
63	3	6	0.475	59.50
64	3	6	0.241	54.50
66	3	6	0.383	66.50
68	3	6	0.637	47.00
70	3	6	0.554	65.00
71	3	6	0.440	64.00
72	3	6	0.464	53.50
73	3	6	0.550	52.50
74	3	6	0.280	52.50
75	3	6	0.477	62.50
77	3	6	0.610	64.00
78	3	6	0.695	65.50
79	3	6	0.612	49.50
80	3	6	0.450	72.50
1	4	6	0.747	53.50
2	4	6	0.774	65.50
4	4	6	1.311	59.00
7	4	6	0.573	58.00
8	4	6	0.566	64.50
9	4	6	0.429	70.00

ENTRY	TRT	TIME	RESP	WEIGHT
10	4	6	0.593	70.00
15	4	6	0.643	56.50
16	4	6	1.225	62.00
17	4	6	0.546	67.50
18	4	6	0.554	80.00
19	4	6	0.707	68.50
20	4	6	0.304	67.50
21	1	9	0.453	71.00
23	1	9	0.226	62.00
25	1	9	0.197	75.00
29	1	9	0.242	73.50
30	1	9	0.584	71.00
31	1	9	0.304	75.00
33	1	9	0.228	73.50
34	1	9	0.346	66.00
37	1	9	0.236	72.50
38	1	9	0.406	57.00
41	2	9	0.552	66.00
44	2	9	0.536	76.50
46	2	9	0.378	66.00
47	2	9	0.361	59.00
48	2	9	0.534	54.00
50	2	9	0.335	70.00
51	2	9	0.447	66.50
52	2	9	0.354	74.50
53	2	9	0.666	69.50
55	2	9	0.387	79.50
56	2	9	0.437	76.50
60	2	9	0.451	63.50
62	3	9	0.225	72.50
63	3	9	0.422	62.50
64	3	9	0.208	71.50
66	3	9	0.404	72.00
68	3	9	0.696	57.00
70	3	9	0.433	73.50
71	3	9	0.455	72.00
72	3	9	0.326	63.00
73	3	9	0.399	62.50
74	3	9	0.289	62.50
75	3	9	0.572	69.50
77	3	9	0.475	73.50
78	3	9	0.424	73.50
79	3	9	0.716	62.00
80	3	9	0.453	83.50
1	4	9	0.400	65.00
2	4	9	1.166	74.00

ENTRY	TRT	TIME	RESP	WEIGHT
4	4	9	0.932	64.50
7	4	9	0.510	71.50
8	4	9	0.806	73.00
9	4	9	0.488	76.00
10	4	9	0.705	77.00
15	4	9	0.420	64.00
16	4	9	0.876	72.00
17	4	9	0.484	75.00
18	4	9	0.377	87.00
19	4	9	0.824	77.00
20	4	9	0.161	76.50

* Entry – ostrich name; TRT – Treatment received; TIME – time in weeks; RESP – Titre response; WEIGHT – weight of ostrich at relevant week.