PRELIMINARY INVESTIGATIONS INTO OSTRICH MYCOPLASMAS: IDENTIFICATION OF VACCINE CANDIDATE GENES AND IMMUNITY ELICITED BY POULTRY MYCOPLASMA VACCINES

Elizabeth Frances van der Merwe

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at the University of Stellenbosch.

Study leader: Prof D.U. Bellstedt

December 2006

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: **Date:**

Summary

Ostrich farming is of significant economical importance in South Africa. Three ostrich mycoplasmas, Ms01, Ms02 and Ms03 have been identified previously, and were provisionally named '*Mycoplasma struthiolus*' (Ms) after their host *Struthio camelus*. Ostrich mycoplasmas are the major causative organisms of respiratory diseases, and they cause stock losses, reduced production and hatchability, and downgrading of carcasses and therefore lead to large economic losses to the industry. In order to be pathogenic to their host, they need to attach through an attachment organelle, the so-called tip structure. This structure has been identified in the poultry mycoplasma, *M. gallisepticum*, and is made up of the adhesin GapA and adhesin-related CrmA. Currently, no ostrich mycoplasma vaccine is commercially available and for this reason the need to develop one has arisen. Therefore the first part of this study was dedicated to the identification and isolation of vaccine candidate genes in the three ostrich mycoplasmas. Four primer approaches for polymerase chain reactions (PCR's), cloning and sequencing, were used for the identification of adhesin or adhesin-related genes from Ms01, Ms02 and Ms03. The primer approaches revealed that the target genes could not be identified due to the high diversity of sequences that were generated. Therefore sequences were also compared with those of other mycoplasma species in BLAST searches. Results showed that the most significant hit was with the human pathogen *M. hominis* oppD, which is located in the same operon as the membrane protein P100 involved in adhesion. Other hits were with ABC transporters which may also play a role in cytadhesion.

The second part of this study was aimed at testing whether two poultry mycoplasma vaccines, *M. synoviae* and *M. gallisepticum*, can be used in ostriches to elicit immune responses until an ostrich mycoplasma vaccine has been developed. Ostriches on three farms of different age groups in the Oudsthoorn district were therefore vaccinated with these vaccines in a vaccine trial. The enzyme-linked immunosorbent assay (ELISA) was used to test the level of antibody response. Results showed that both vaccines elicited an immune response in all three age groups. A high percentage of the ostriches reacted positively, which indicates that both vaccines elicit antibody responses and may therefore give protection against ostrich mycoplasma infections.

Pectora roborant cultus recti

Opsomming

Volstruisboerdery is ‘n belangrike ekonomiese sektor in Suid-Afrika. Drie volstruismikoplasmas, Ms01, Ms02 en Ms03, is voorheen geïdentifiseer en voorlopig ‘*Mycoplasma struthiolus*’ (Ms) benaam na aanleiding van hul gasheer, *Struthio camelus*. Volstruismikoplasmas is die grootste oorsaaklike organismes van respiratoriese siektes, kudde verliese en die afgradering van karkasse wat lei tot groot ekonomiese verliese in die volstruisbedryf. Ten einde patogenies vir die gasheer te wees, moet mikoplasmas deur middel van ‘n aanhegtingsmeganisme vasheg – die sogenaamde puntvormige struktuur. Hierdie struktuur is in die pluimvee mikoplasma *M. gallisepticum* geïdentifiseer, en bestaan uit aanhegting proteïen GapA en die aanhegting verwante proteïen CrmA. Tans is geen volstruismikoplasma entstof kommersieel beskikbaar nie, en derhalwe het die behoefte ontstaan om so ‘n entstof te ontwikkel. Die eerste gedeelte van hierdie studie is dus gewy aan die identifisering en isolering van entstof kandidaat gene in al drie volstruismikoplasmas. Vier inleier benaderings vir polimerase ketting reaksies (PKR), klonering asook geenopeenvolging bepalings vir die identifisering van aanhegting of aanhegting verwante gene vanuit Ms01, Ms02 en Ms03 is gebruik. Die inleier benaderings het getoon dat die teikengene nie geïdentifiseer kon word nie as gevolg van hoë variasie in die gegenereerde geenopeenvolgings. Derhalwe is geenopeenvolgings met ander mikoplasma spesies deur middel van BLAST soektogte vergelyk. Resultate het getoon dat die betekenisvolste ooreenstemming dié met die menslike patogeen *M. hominis* oppD was, wat deel vorm van die membraan proteïen P100 operon wat betrokke is by aanhegting. Ander ooreenstemmings sluit ABC transporters in wat moontlik betrokke kan wees by aanhegting.

Die tweede gedeelte van hierdie studie het ten doel gehad om te toets of twee pluimvee mikoplasma entstowwe, *M. synoviae* en *M. gallisepticum*, gebruik kan word in volstruise om immuunresponse te ontlok tot tyd en wyl ‘n volstruismikoplasma entstof ontwikkel is. Volstruise vanaf drie plase in verskillende ouderdomsgroepe in die Oudtshoorn distrik was ingeënt met hierdie entstowwe in ‘n entstof proefneming. Die ensiem-afhanklike immuno-absorpsie essaï (ELISA) was gebruik om antiliggaam response te toets. Die resultate het getoon dat beide entstowwe immuunresponse ontlok het in al drie ouderdomsgroepe. ‘n Groot persentasie van die volstruise het positief gereageer wat ‘n aanduiding is dat beide entstowwe immuunresponse ontlok het en kan dus beskerming bied teen volstruismikoplasma infeksies.

Acknowlegements

I would like to express my sincere appreciation to the following people:

- Prof. D.U. Bellstedt for his guidance and support as study leader, and also the opportunity to have been part of this research project in his laboratory.
- Dr. A. Botes for sharing her knowledge on mycoplasmas, and working with her on this project.
- Prof. T. McCutchan for broadening our knowledge on how to use the BLAST tools more efficiently.
- Mnr. W. Botes for the statistical analysis of the ELISA results.
- Klein Karoo Group for financial support.
- Very special thanks to Jim, Elsabé & Carel van der Merwe, and Salmien & Chris Symeonidis for their constant love and support.
- Wilhelm Uys for his patience and help with the editing, and his love and support.
- God, for making all things possible.

Table of Contents

Declaration i
Summary .... **Error! Bookmark not defined.**
Opsomming .... **Error! Bookmark not defined.**
Acknowledgements .... **Error! Bookmark not defined.**
List of Figures viii
List of Tables x
Abbreviations xi
1. Introduction 1
1.1 Objectives of the Study 2
2. Avian Mycoplasmas
2.1 Introduction 4
2.2 Early mycoplasma identification and taxonomy 4
2.3 Distribution of mycoplasmas 5
2.4 Evolution and Taxonomy 5
2.5 Phylogenetic Studies Using Mycoplasma Ribosomal Genes 7
2.5.1 rRNA and tRNA genes 7
2.5.2 Use of 16S rRNA as phylogenetic marker 8
2.5.3 Ostrich specific mycoplasmas 9
2.6 Morphology and Biochemistry 11
2.7 Mycoplasmas Affecting Domestic Poultry 13
2.7.1 Epidemiology 13
2.7.1.1 Natural host 13
2.7.1.2 Infection 14
2.7.1.3 Transmission 15
2.7.2 Clinical signs and lesions 16
2.7.3 Diagnosis 18
2.7.3.1 Diagnostic samples 19
2.7.3.2 Identification of a mycoplasma infection 19
2.7.3.3 Serology 21
2.7.4 Treatment with antibiotics 22
2.7.5 Prevention and control of poultry mycoplasmas 23
2.8 The South African Ostrich 24
2.8.1 Respiratory system and respiration 24
2.8.2 Mycoplasma infections in the ostrich 25
2.8.3 Other respiratory diseases in the ostrich 26
2.9 Poultry Mycoplasma Vaccines 28
2.9.1 Vaccines 30
2.9.1.1 Killed vaccines (bacterins) 30
2.9.1.2 Live vaccines 30
2.9.1.3 *M. gallisepticum* vaccines 31
2.9.1.4 *M. synoviae* vaccines 32

Pectora roborant cultus recti

2.9.1.5 DNA vaccines 32
2.9.2 Administration of vaccine 33
2.9.2.1 Individual vaccination 33
2.9.2.2 Flock vaccination 34
2.9.3 Previous studies with poultry mycoplasma vaccines 35
2.9.4 Antibody response 37
2.10 Pathogenicity of Mycoplasmas 38
2.10.1 Adhesion to host cell 38
2.10.2 Interaction with the host immune system 40
2.10.3 Other possible virulence causal factors 41
2.11 The Mycoplasma Genome 42
2.11.1 General characteristics of the genome 42
2.11.2 The *M. gallisepticum* strain Rlow genome 44
2.11.3 The genes and proteins involved in host cell adhesion 46
3. Genomic Investigations towards Vaccine Candidate Genes against Ostrich Mycoplasmas
3.1 Introduction 52
3.2 Materials and Methods 53
3.2.1 Gene order comparisons of mycoplasma genomes 53
3.2.2 Primer development 54
3.2.2.1 Primer approach 1 54
3.2.2.2 Primer approach 2 56
3.2.2.3 Primer approach 3 58
3.2.2.4 Primer approach 4 60
3.2.3 Isolation of genomic DNA 61
3.2.4 PCR amplification 62
3.2.5 Detection of PCR products 64
3.2.6 Cloning of PCR products 64
3.2.6.1 Ligation of PCR product into pGEM-T Easy Vector 66
3.2.6.2 Transformation of *E. coli* with ligation products 66
3.2.6.3 Diagnostic PCR 67
3.2.6.4 Overnight culture of recombinant colonies 67
3.2.6.5 Isolation of recombinant plasmid DNA 68
3.2.6.6 Insert check PCR 68
3.2.7 Sequencing 68
3.2.7.1 Sequencing of PCR products 68
3.2.7.2 Sequencing of isolated plasmid DNA 69
3.2.8 Analysis of sequences 69
3.2.9 Comparison of mycoplasma sequences using BLAST 70
3.3 Results 71
3.3.1 Gene order comparisons of mycoplasma genomes 71
3.3.2 PCR amplification 72
3.3.2.1 Primer approach 1 73
3.3.2.2 Primer approach 2 75
3.3.2.3 Primer approach 3 77
3.3.2.4 Primer approach 4 78
3.3.3 Cloning of PCR products 80
3.3.4 Alignment of sequences 82
3.3.5 Sequence analysis of cloned DNA fragments using BLAST 83
3.4 Discussion 88

Pectora roborant cultus recti

4. Trials with Poultry Mycoplasma Vaccines in Ostriches

4.1 Introduction 92

4.2 Material and Methods 93
4.2.1 Poultry mycoplasma vaccines used in study 93
4.2.2 Serum from ostriches included in the vaccine trial 93
4.2.3 Enzyme-linked immunosorbent assay 94
4.2.3.1 Isolation and biotinylation of rabbit anti-ostrich Ig 94
4.2.3.2 ELISA for detection of humoral Ig antibodies to *M. synoviae* 95
4.2.4 Statistical analysis 96

4.3 Results 96
4.3.1 Adaptation of ELISA 96
4.3.2 Statistical analysis of ELISA results 97
4.3.3 Immune response of ostrich chicks 98
4.3.3.1 Farm 1: 3 month old ostrich chicks 98
4.3.3.2 Farm 2: 4-5 month old ostrich chicks 100
4.3.3.3 Farm 3: 6-7 month old ostrich chicks 101

4.4 Discussion 102

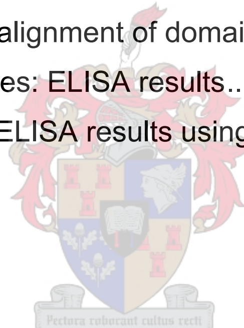
5. Conclusion and Future Perspectives 106

Literature Cited 109

Appendix A Nucleotide sequence alignment of domain B 120

Appendix B Vaccine trial in ostriches: ELISA results 124

Appendix C Statistical analysis of ELISA results using SAS 132



List of Figures

Figure 2.1 Phylogenetic analysis of the 16S rRNA gene of avian mycoplasmas.. 10
Figure 2.2 Complete genome of *M. gallisepticum* strain R_{low} (Papazisi *et al.*, 2003). 45
Figure 2.3 The *gapA* operon of *M. gallisepticum*.. 47
Figure 2.4 Operon of *M. pneumoniae* surface adhesin P1.. 48
Figure 2.5 The MgPa operon of *M. genitalium*.. 49
Figure 2.6 The *M. hominis opp* operon consisting of P100 and OppBCDF downstream of it.. 49
Figure 3.1 Primer approach 1: Primer pairs used for amplification of *M. gallisepticum* GapA and CrmA (Papazisi *et al.,* 2000). 55
Figure 3.2 Amino acid alignment of the domain B region of mycoplasma cytadhesin as well as cytadhesin-related molecules.. 56
Figure 3.3 Primer approach 2: Primers developed from the nucleotide as well as amino acid alignment of mycoplasma cytadhesin and cytadhesin-related sequences.. 57
Figure 3.4 Nucleotide alignment of *M. synoviae* (Synoviae) against *M. gallisepticum* GapA and domain B (GapA and GapADB respectively)... 59
Figure 3.5 Primer approach 3: Primer E2R was developed for the area between EF and DR, but still in *M. gallisepticum* GapA domain B... 60
Figure 3.6 Primer approach 4: Primers E2F and E3R were developed from the alignment of *M. synoviae* with *M. gallisepticum* GapA. 61
Figure 3.7 The pGEM-T Easy Vector circle map used for cloning of PCR products.. 65
Figure 3.8 Comparison of mycoplasma genomes using the Gene plot tool on the NCBI website. . 72
Figure 3.9 Gel electrophoresis of amplification products during optimisation of PCR reactions for primer approach 1.. 74
Figure 3.10 Gel electrophoresis of amplification products during optimisation of PCR reactions for primer approach 2. 76
Figure 3.11 Gel electrophoresis of amplification products for primer approach 3 with DNA from Ms01 and Ms03 using primer combination EF+E2R.. 78
Figure 3.12 Gel electrophoresis of amplification products during optimisation of PCR reactions at 36 ºC for primer approach 4. 79
Figure 3.13 Gel electrophoresis of insert check PCR using primers T7 and SP6. 81
Figure 4.1 ELISA for detection of humoral Ig antibodies to *M. synoviae*... 95
Figure 4.2 Average antibody response to *M. synoviae* of 3 month old ostrich chicks on Farm 1. . 99
Figure 4.3 Average antibody response to *M. synoviae* of 4-5 month old ostrich chicks on Farm 2. 100

Figure 4.4 Average antibody response to *M. synoviae* of 6-7 month old ostrich chicks on Farm 3. 101

List of Tables

Table 2.1 Molecular characteristics and taxonomy of the class Mollicutes. 7
Table 3.1 Primers A – E used in primer approach 1. Base pair positions given are relative to the *M. gallisepticum gapA* and *crmA* genes. 55
Table 3.2 Sequence of the primers used in primer approach 2, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes. 58
Table 3.3 Sequence of primer E2R developed for primer approach 3 and primer EF, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes. 60
Table 3.4 Sequence of primers developed for primer approach 4, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes. 61
Table 3.5 T7 and SP6 promoter primers used for sequencing of cloned inserts. The bp-position is that of the pGEM-T Easy vector. 65
Table 3.6 Summary of master mix for individual primer combinations.. 63
Table 3.7 PCR programs used in DNA amplification reactions for Ms01, Ms02 and Ms03.. 64
Table 3.8 Protocol for the ligation reaction of standard reactions for cloning PCR products into pGEM-T Easy Vector (Promega), as well as positive control and background control.. 66
Table 3.9 Expected amplification products as well as actual amplification products obtained with primers A – E for primer approach 1. 73
Table 3.10 Expected amplification products as well as actual amplification products obtained with primers used in primer approach 2.. 75
Table 3.11 Amplification products expected as well as products obtained from primer combination for primer approach 3.. 77
Table 3.12 Expected amplification products as well as products amplified with primer combinations used in primer approach 4.. 79
Table 3.13 Summary of the PCR products of the four primer approaches that were used for cloning with the pGEM-T Easy Vector System.. 80
Table 3.14 Summary of significant hits of Ms01 with Mycoplasma species with BLASTN search.. 84
Table 3.15 Summary of most significant hits of Ms01 sequences with Mycoplasma species with the TBLASTX search 85
Table 4.1 Summary of the ostriches used in the poultry mycoplasma vaccine trial. 94
Table 4.2 Fraction and percentage of the ostriches on Farm 1 that reacted to vaccination............ 99
Table 4.3 Fraction and percentage of the ostriches on Farm 2 that reacted to vaccination.......... 101
Table 4.4 Fraction and percentage of the ostriches on Farm 3 that reacted to vaccination.......... 102

Abbreviations

A+T	adenine and thymine
ABTS	2,2'-Azino-di(3-ethylbenzthiazoline sulphonic acid-6)
ANOVA	analysis of variance
AVPO	streptavidin peroxidase
Biotin	biotinamidocaproate N-Hydroxysuccinimide ester
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CDS	coding DNA sequences
crm	cytadherence-related molecule
DAPSA	DNA and protein sequence alignment
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DMF	N,N Dimethylformamid
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
ELISA	enzyme-linked immunosorbent assay
EU	European Union
E-value	Expect value
G+C	guanine and cytosine
GLM	general linear model
h	hours
HI	haemagglutination inhibition
HMW	high-molecular-weight protein
HPAI	high-pathogenic avian influenza
HRPO	horseradish peroxidase
Ig	immunoglobulin
IPTG	isopropyl ß-D-thiogalactopyranoside

Pectora roborant cultus recti

kb	kilobase pairs
kDa	kilo Dalton
LB	Luria-Bertani
LSD	least significant difference
MHC	major histocompatibility complex
min	minutes
mol%	molecular percentage
NCBI	National Center for Biotechnology Information
nr	non-redundant
nt	nucleotide
Opp	oligopeptide permease
oriC	origin of replication
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RB	reaction buffer
RNA	ribonucleic acid
rpm	resolutions per minute
rRNA	ribosomal RNA
SAS	Statistical Analysis System
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
SPA	serum plate agglutination
TA	tube agglutination
Tc	cytotoxic T-cells
TCA	tricarboxylic acid
Th	T-helper cells
T_m	melting temperature

Pectora roborant cultus recti

tRNA	transfer RNA
UV	ultra-violet

1. Introduction

In South Africa, ostrich farming is of significant economical importance. The farming of domestic ostriches, *Struthio camelus*, commenced in South Africa in 1857 and is still an important contributor to the agricultural economy. In the twentieth century during World War I, the industry, which then focused mainly on the marketing of feathers, experienced a decrease in demand and almost collapsed. However, after World War II, it slowly recovered again and South Africa has ever since been in control of the world ostrich industry (Van der Vyfer, 1992; Kimminau, 1993; Deeming, 1999). Even though the industry is mainly confined to the Oudsthoorn area in the Klein Karoo, its importance as a foreign currency earner is expanding. With a yearly export income of R1,2 billion, the ostrich production is one of the top twenty agro-based industries in South Africa. Employment for a broad range of employees is also provided, specifically to the unskilled in areas where employment would otherwise be scarce (http://www.saobc.co.za/modules.php?name=News&file=article&sid=19).

Ostriches are not only of importance for the production of feathers. Ostrich leather is presently considered to be a very glamorous product, and the meat is considered healthy since it contains less fat, calories and cholesterol than any other meat (Kimminau, 1993). All these products as well as fertile eggs and live ostriches are exported (Verwoerd, 2000). This export places an expanding demand on the industry regarding product quality and disease control, in particular that the meat does not contain any disease-forming organisms that might infect humans and poultry in the European Union (EU). The recent outbreak of avian influenza in South Africa serves to illustrate this point.

Pectura roborant cultus recti

On 6 August 2004, the South African Department of Agriculture implemented a voluntary ban on the export of ostriches and ostrich products due to the outbreak of avian influenza in the Eastern Cape on two farms. On 11 August 2004, the EU confirmed that the ban was restricted to the import of live ostriches, ostrich meat and ostrich eggs. The resumption of imports was approved by the EU in November 2005 after the voluntary ban on ostrich meat exports was lifted by the Department of Agriculture on 13 September 2005. Exports have been resumed since November 2005. However, losses to the industry ran into millions of rands (Gerber, 2005; http://www.saobc.co.za/modules.php?name=News&file=article&sid=51; http://www.saobc.co.za/modules.php?name=News&file=article&sid=32; http://www.saobc.co.za/modules.php?name=News&file=article&sid=31).

Diseases, especially respiratory diseases, also cause significant losses in ostrich production, not only in South Africa but also in the rest of the world. Mycoplasmas are one of the causative organisms of respiratory diseases (Botes *et al.*, 2005b). They cause high mortalities in ostrich chicks and are responsible for downgrading of carcasses in slaughter ostriches, which has a meaningful effect on the production of ostrich products. Although there are serious concerns about the transmissibility of mycoplasmas via ostrich products, there has been no indication that mycoplasmas spread through the meat (Verwoerd, 2000). In spite of this, serious concerns exist about the transmission of mycoplasmas to other countries via contaminated meat and it is for this reason that meat exports have to be kept under control.

In previous studies in this laboratory, three ostrich specific mycoplasmas have been identified (Botes *et al.*, 2004, 2005a). Mycoplasma infections are seasonal, mostly during winter and when rapid changes in temperature occur, such as from winter to summer. Although vaccines and antibiotics against poultry mycoplasmas are available, currently no registered mycoplasma vaccine specific for use in ostriches exists.

1.1 Objectives of the Study

Pectora roborant cultus recti

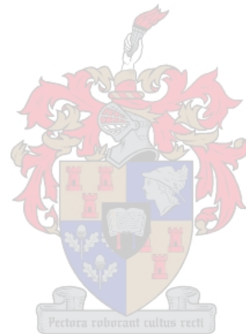
In order to overcome mycoplasma infections the ostrich industry took a decision to investigate vaccine strategies against these organisms. Strategies include, firstly, the development of specific vaccines against the three ostrich mycoplasmas, and, secondly, an investigation into the effectiveness of poultry mycoplasma vaccines against ostrich mycoplasmas.

The objectives of this study, based on the strategies, were therefore:

- the identification of an attachment organelle gene with a possible role in virulence;
- the isolation of the attachment organelle gene once it has been identified with a view to use it as a vaccine candidate gene; and
- testing whether existing poultry mycoplasma vaccines could elicit an immune response in ostriches

In this thesis, a literature review regarding mycoplasmas and the importance of genes related to adhesion, and possibly pathogenicity, with specific focus on poultry mycoplasmas is given in Chapter 2. Chapter 3 deals with a genomic investigation towards finding candidate genes with a possible role in virulence from the three ostrich mycoplasmas, identified by Botes *et al.* (2004, 2005a). Chapter 4 describes a vaccine trial using poultry mycoplasma vaccines in

ostriches in the Oudtshoorn area. A brief summary and future perspectives are given in Chapter 5.



2. Avian Mycoplasmas

2.1 Introduction

In order to understand the biochemical processes that allow mycoplasmas to survive and grow, it is necessary to understand their origin and development. How they evolved, as well as their characteristics and morphology, their distribution in nature and how they attach to their host cell in order to be pathogenic, has been studied extensively.

Mycoplasmas are widespread in nature and infect many vertebrate and invertebrate organisms. In this literature review, general information regarding mycoplasma species will be discussed, including how they evolved. Thereafter the focus will move to avian mycoplasmas and more specifically the four major poultry pathogens. The diseases that they cause as well as available treatments, which include different methods of vaccination, will be outlined. Since this research project focuses on mycoplasmas in the South African ostrich, other respiratory diseases in ostriches will also be discussed briefly. A short discussion on their morphology and characteristics, with special reference to pathogenicity and survival in their hosts, will follow this. Finally, the genes as well as proteins involved in adhesion will be discussed.

2.2 Early mycoplasma identification and taxonomy

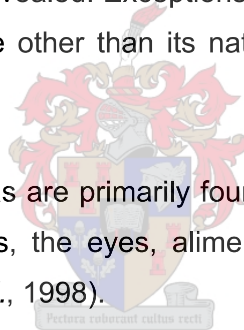
Mycoplasmas are the smallest self-replicating organisms and have been a popular research topic since the 1800's. These fascinating organisms were cultivated successfully for the first time in 1898 by E. Nocard and E.R. Roux at the Pasteur Institute in Paris (Edward *et al*. 1967 as referred to in Razin, 1992). The name "mycoplasma" is derived from the Greek mykes (fungus) and plasma (something molded or formed) (Edward *et al*. 1967 as referred to in Razin, 1992), which is ironic as mycoplasmas are not fungi. At first, mycoplasmas were believed to be viruses because of their small size as they could pass through filters with a pore size of 450 nm. However, when the characteristics of a true virus were clarified in the 1930's, this theory proved to be wrong. Later on it was implied that mycoplasmas were stable L-phase variants of common bacteria, but this relationship was also ruled out in the late 1960's (Razin, 1992; Rottem and Barile, 1993; as referred to in Baum, 2000).

In 1967, the wall-less prokaryotes were divided from the eubacteria into a class of their own, namely the Mollicutes, for which the trivial name mycoplasmas is used (Freundt, 1973; Razin, 1978). The name Mollicutes was derived from the Latin mollis (soft) and cutis (skin) which

implies the absence of a cell wall (Razin *et al.*, 1998). It is now accepted that mycoplasmas are a group of eubacteria that evolved from Gram-positive bacteria and maintain the unique position of being the smallest self-replicating prokaryote lacking a cell wall (Razin, 1992; Rottem and Barile, 1993; Dybvig and Voelker, 1996).

2.3 Distribution of mycoplasmas

Mycoplasmas have a wide variety of hosts which include humans, domestic and wild mammals, birds, plants, reptiles, fish, arthropods and insects (Razin and Freundt, 1984; Razin, 1992; Razin *et al.*, 1998). All mycoplasmas, of which there are over 180 species, are parasites, commensals or saprophytes, and many are pathogenic (Razin and Freundt, 1984; Razin *et al.*, 1998; Rottem, 2002). They cause chronic, generally mild infections, but rarely kill their host which makes them an ideal parasite (Razin, 1999). They are relatively strict host, organ and tissue specific organisms through which their obligate parasitic mode of life and nutritionally exacting nature is revealed. Exceptions are also possible where a mycoplasma is found in a host, organ or tissue other than its natural habitat (Razin, 1992; Coetzer *et al.*, 1994; Razin *et al.*, 1998).



Human and animal mycoplasmas are primarily found to occur in the mucous surfaces of the respiratory and urogenital tracts, the eyes, alimentary canal, mammary glands and joints (Coetzer *et al.*, 1994; Razin *et al.*, 1998).

2.4 Evolution and Taxonomy

One hypothesis was that the mycoplasma genome evolved several times, as early as 590 to 600 million years ago from the *Clostridium* – *Lactobacillus* – *Streptococcal* branch from an organism with a genome size about 2000 kb. Approximately 450 million years ago the mycoplasma phylogenetic tree split into two major branches, possibly from an organism with genome size of 1700 – 2000 kb. Mycoplasma sublines with genome sizes of 1200 – 1700 kb evolved from both branches. Mycoplasma species with small genome sizes of 600 – 1100 kb arose later on independently on several different sublines. However, the smallest genome on each subline is 600 – 800 kb and this seems to be the lower limit for mycoplasma, and probably cell, genome content (Maniloff, 1992, 1996). This hypothesis of multiple origin of the genus proved to be incorrect and a different model was composed by Woese, Maniloff and co-workers. In this they stated that the mycoplasma phylogenetic tree is monophyletic which emerged from a branch of the Gram-positive bacterial phylogenetic tree. Mycoplasma

evolution has been by attrition, identified by rapid evolution and reduced physiological and genetic complexity. This is illustrated to some extent by the fact that species currently included in the genus *Mycoplasma* are polyphyletic (Maniloff, 1992).

In 1956, Edward and Freundt allocated all known mycoplasmas into one family, Mycoplasmataceae, with only one genus, *Mycoplasma*, under the order Mycoplasmatales. At that stage no more than 15 mycoplasma species were recognized. The Mycoplasmatales, which was previously placed as order X of the class Schizomycetes, was separated into a new class in 1967. This new class of microbes was named Mollicutes. In 1969 and 1970 they suggested a second family and genus, Acholeplasmataceae and *Acholeplasma*, for a species which was up until then known under the name of *M. laidlawii*. The main rule to distinguish between these two families was the need versus no need for cholesterol or other sterols as growth factors (Freundt, 1973).

Currently, eight genera of Mollicutes in five families are recognized (Dybvig and Voelker, 1996) as shown in Table 2.1. Some families have certain characteristics which distinguishes them from the other families. Members of Spiroplasmataceae have a helical morphology, rotating motility and chemotaxis, and members of Ureaplasma are capable of hydrolyzing urea (Razin and Freundt, 1984; Razin *et al*., 1998). It is believed that acholeplasmas and anaeroplasmas were the first Mollicutes that evolved from Gram-positive bacteria by reductive evolution. Spiroplasmas evolved from an early split of the acholeplasmal branch, and it is believed that mycoplasmas and ureaplasmas have a spiroplasmal ancestor (Razin *et al*., 1998).

The class Mollicutes is presently the only one in the division *Tenericutes* (wall-less bacteria) which forms one of the four divisions of the kingdom *Procaryotae*. The other three divisions are the Gram-positive bacteria, *Firmicutes*, the Gram-negative bacteria, *Gracilicutes*, and the archaebacteria, *Mendosicutes* (Razin *et al.*, 1998). The current taxonomic scheme for the class Mollicutes is presented in Table 2.1.

Pectora roborant cultus recti

Table 2.1 Molecular characteristics and taxonomy of the class Mollicutes.

Classification	No. of species[1]	Genome size (kb)	Mol% G+C of genome	Host
Order I: *Mycoplasmatales*				
Family I: *Mycoplasmataceae*				
Genus I: *Mycoplasma*	102	580-1350	23-40	Humans, animals
Genus II: *Ureaplasma*	6	760-1170	27-30	Humans, animals
Order II: *Entomoplasmatales*				
Family I: *Entomoplasmataceae*				
Genus I: *Entomoplasma*	5	790-1140	27-29	Insects, plants
Genus II: *Mesoplasma*	12	870-1100	27-30	Insects, plants
Family II: *Spiroplasmataceae*				
Genus I: *Spiroplasma*	33	780-2220	24-31	Insects, plants
Order III: *Acholeplasmatales*				
Family I: *Acholeplasmataceae*				
Genus I: *Acholeplasma*	13	500-1650	26-36	Animals, plants, insects
Order IV: *Anaeroplasmatales*				
Family I: *Anaeroplasmataceae*				
Genus I: *Anaeroplasma*	4	1500-1600	29-34	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1	1500	40	Bovine/ovine rumen
Undefined				
Phytoplasma	ND*	640-1185	23-29	Insects, plants

[1] Number of species recognized currently
* Not defined
Table adapted from Razin *et al.*, 1998

Pectora roborant cultus recti

2.5 Phylogenetic Studies Using Mycoplasma Ribosomal Genes

Phylogenetic studies on mycoplasmas have been made easier by the conserved nature of the rRNA and ribosomal protein genes, especially the 16S rRNA gene. This phylogenetic tool has also been used successfully for the identification of three ostrich mycoplasmas.

2.5.1 rRNA and tRNA genes

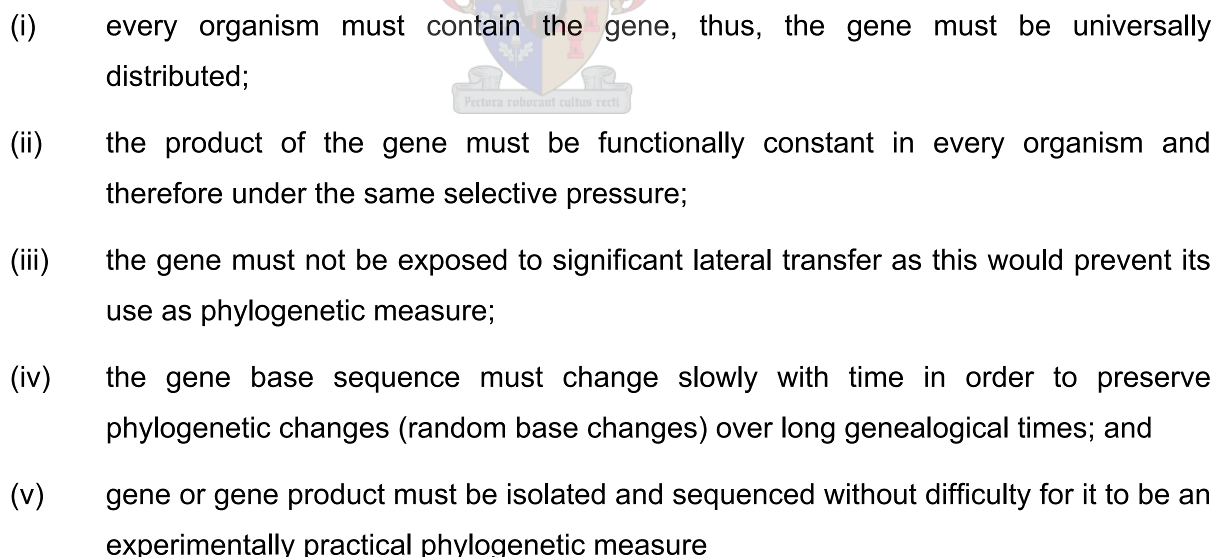
Ribosomes are the only structures, apart from DNA, detected in the cytoplasm. Their genes are possibly the best-characterized mycoplasmal genes (Razin *et al.*, 1998). They resemble typical eubacterial ribosomes in having three rRNA species, namely 5S, 16S and 23S. The genes for rRNA as well as the products are highly conserved throughout prokaryotic organisms. It seems as if there is a correlation between genome size and the number of

rRNA genes since mycoplasmas carry only one or two sets of rRNA genes, but there is no strict relationship. The *Escherichia coli* (Gram-negative) genome can carry seven individual rRNA transcription units for 16S and 23S rRNAs, and its 5S and 16S rRNAs are larger than those of the mycoplasmas. In mycoplasmas, the order of the rRNA genes is similar to that found in prokaryotes, namely 16S-23S-5S, and they function as an operon. The genes are close to each other and take up a chromosomal segment of about 5 kb (Glaser *et al.*, 1992; Bové, 1993; Razin *et al.*, 1998).

The tRNAs are also highly conserved molecules regarding size, composition and function, but their structure might be closer to Gram-positive than Gram-negative bacteria. The low G+C content of the mycoplasma genome is not reflected in the G+C content of the tRNAs (Razin, 1978). Gene duplicates are very rare and the number of genes is kept to a minimum. The number of anticodons in *Mycoplasma pneumoniae* is only 32 compared to the 86 in the *E. coli* K-12 genome (Razin *et al.*, 1998).

2.5.2 Use of 16S rRNA as phylogenetic marker

To qualify as the best candidate gene to be phylogenetically useful, certain criteria has to be met. These include (Maniloff, 1992):



(i) every organism must contain the gene, thus, the gene must be universally distributed;

(ii) the product of the gene must be functionally constant in every organism and therefore under the same selective pressure;

(iii) the gene must not be exposed to significant lateral transfer as this would prevent its use as phylogenetic measure;

(iv) the gene base sequence must change slowly with time in order to preserve phylogenetic changes (random base changes) over long genealogical times; and

(v) gene or gene product must be isolated and sequenced without difficulty for it to be an experimentally practical phylogenetic measure

Since rRNA genes are conserved between mycoplasmas and are ideal to use as probes in mycoplasma detection and identification (Weisburg *et al.*, 1989; Glaser *et al.*, 1992). The 16S rRNA gene is an effective phylogenetic tool since certain parts evolved slowly and thus provides a phylogenetic measure of deep genealogical events. Other parts evolved more

quickly and measure more recent genealogical events. The smaller 5S rRNA gene evolved faster and is therefore not apt as a phylogenetic measure (Maniloff, 1992).

In order to describe a new mycoplasma species, its 16S rDNA sequence has to be included (Razin *et al.*, 1998). Phylogenetically, the Mollicutes and their walled relatives consist of six definite clades: (i) the pneumoniae group, (ii) the hominis group, (iii) the spiroplasma group, (iv) the anaeroplasma group, (v) the asteoleplasma group, and (vi) the walled relatives. Of these groups, the hominis group is the largest within the mycoplasmas (Weisburg *et al.*, 1989; Pettersson *et al.*, 2000). Figure 2.1 illustrates the 16S rRNA gene tree of avian mycoplasmas as determined by Botes *et al.* (2005a). The three hitherto unnamed species identified by Botes *et al.* (2005a) are also included namely Ms01, Ms02 and Ms03. They are ostrich specific mycoplasmas and more detail will be given on them in section 2.5.3.

Although 16S rRNA sequences are viewed to be the most effective tool for phylogeny and taxonomy of bacteria, additional phylogenetic markers have been identified to verify conclusions based on the 16S rRNA data. These include conserved ribosomal protein genes, the heat shock protein gene *hsp70*, the elongation factor EF-Tu (*tuf*) gene, and the 16S/23S rRNA intergenic sequences (Razin *et al.*, 1998). Denaturing gradient gel electrophoresis (DGGE), which theoretically can detect single-base mutations in DNA, has also been used successfully combined with polymerase chain reaction (PCR) amplification of the 16S rRNA gene (McAuliffe *et al.*, 2003, 2005).

Pectora roborant cultus recti

2.5.3 Ostrich specific mycoplasmas

Three ostrich mycoplasmas, Ms01, Ms02 and Ms03, were identified by Botes *et al.* (2004, 2005a) using 16S rRNA gene sequencing. They were provisionally named *'Mycoplasma struthiolus'* (Ms) after their host, *Struthio camelus*, until formally described. Sequence similarity between Ms01 and Ms02 is 88.4%, sequence similarity between Ms01 and Ms03 is 88.7% and sequence similarity between Ms02 and Ms03 is 93.1% respectively as shown by alignment data (Botes 2004; Botes *et al.* 2005a). The 16S rRNA sequences of Ms01, Ms02 as well as Ms03 are available in GenBank under accession numbers DQ223545 for Ms01, DQ223546 for Ms02 and DQ223547 for Ms03 (Botes *et al.*, 2005a).

Phylogenetic analysis (see Figure 2.1) showed Ms02 and Ms03 to fall together in one clade with Ms02 closely related to *M. synoviae* (92.2% sequence similarity) and Ms03 closely related to *M. gallinaceum* (94.6% sequence similarity). Ms01 falls into a separate clade with

M. falconis being its closest relative (97.8% sequence similarity). The diversity of these three ostrich mycoplasmas is revealed by the two different phylogenetic mycoplasma groupings they fall under (Botes *et al.*, 2005a).

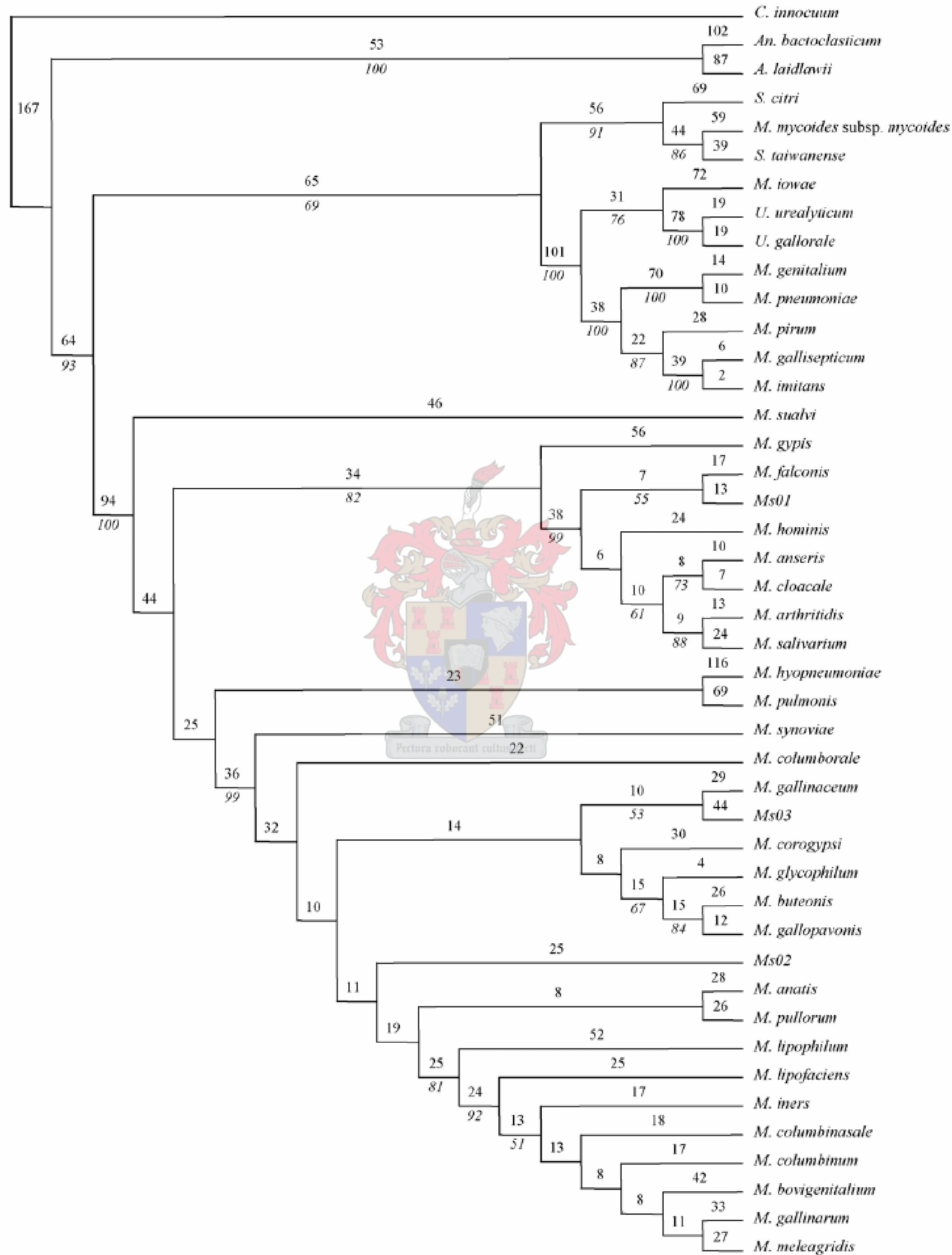


Figure 2.1 Phylogenetic analysis of the 16S rRNA gene of avian mycoplasmas. The three ostrich specific mycoplasmas, Ms01, Ms02 and Ms03 are also indicated (Botes *et al.*, 2005a).

2.6 Morphology and Biochemistry

The most outstanding characteristic of a mycoplasma is the absence of a cell wall. However, this is not the only characteristic that describes its uniqueness. Other aspects include their pleomorphic shape which varies from spherical or pear-shaped cells (0.3 – 0.8 µm in diameter) to branched or helical filaments with a length from a few to 150 µm. Coccoidal and diploform patterns have also been reported (Freundt, 1973; Klainer and Pollack, 1973; Razin and Freundt, 1984; Carson *et al.*, 1992; Rottem and Barile, 1993; Coetzer *et al.*, 1994). Although mycoplasmas evolved from Gram-positive bacteria, they stain negative in the Gram test. Genome replication is not synchronized with cell division, and therefore budding forms and chains of beads as well as typical binary fission is often observed. Cytoplasmic division, which should be synchronized with genome replication for binary fission to occur, may lag behind genome replication in the case of mycoplasmas and multinucleate filaments are a result of this. Thus, cells are either divided by regular binary fission, or elongate first to multinucleate filaments which break into coccoid bodies afterwards (Morowitz and Wallace, 1973; Razin, 1978; Razin and Freundt, 1984; Rottem and Barile, 1993).

Mycoplasmas are dependent on their hosts for many nutrients since they have restricted biosynthetic capabilities due to their small genome size. For growth most species require cholesterol, related sterols and fatty acids as they have lost the ability to synthesise these compounds, and they use either sugars or arginine as energy source (Freundt, 1973; Razin and Freundt, 1984; Rottem and Barile, 1993; Rottem, 2002). Mycoplasmas are the only prokaryotes dependent on cholesterol for growth. It is believed that their inability to regulate membrane fluidity through fatty acid synthesis is compensated through their ability to take up large quantities of cholesterol into their membranes (Rottem, 2002). It seems that the shape of the cell is determined by the growth medium's nutritional qualities, osmotic pressure as well as the growth phase of the culture. Some species are obligate anaerobes and are killed when in contact with low levels of oxygen, however, most species are facultatively anaerobic. When grown on solid media, mycoplasmas tend to penetrate deeply and grow inside the media. Colonies formed are generally much smaller than 1 mm in diameter, and have a characteristic "fried egg" appearance (Freundt, 1973; Razin and Freundt, 1984; Rottem and Barile, 1993). They can be differentiated without difficulty from other bacteria because of their particular colony shape and inability to be scraped off easily from the media surface (Rottem and Barile, 1993). Another feature of mycoplasmas is their resistance to penicillin and lysozyme due to the fact that they lack a cell wall. They are, however, usually susceptible to antibiotics such as

tetracyclines and chloramphenicol that inhibits protein synthesis in prokaryotes (Freundt, 1973; Razin and Freundt, 1984).

The cell membrane of mycoplasmas is a typical prokaryotic plasma membrane, consisting of lipids (phospholipids, glycolipids, lipoglycans and sterols) and proteins. A capsular material or nap covers the cell surface of many mycoplasma species. Through thin sections of mycoplasmas it was observed that the cells are made up of only three vital organelles, namely the cell membrane, the ribosomes and a typical prokaryotic genome (Razin, 1978; Razin and Freundt, 1984). No intracellular membranous structures, such as mesosomes, are indicated (Razin and Freundt, 1984). One structure that has been detected in different species is a specialized cell membrane tip structure. These cell surface tip structures, in the form of short, dense rodlets, play a vital role in attachment of mycoplasmas to host cells as well as in their gliding motility (Razin and Freundt, 1984; Razin and Jacobs, 1992; Trachtenberg, 1998).

Although mycoplasmas lack flagella and are generally nonmotile, a gliding motility has been reported in some species (Razin and Freundt, 1984; Trachtenberg, 1998; Wolgemuth *et al.*, 2003). Mycoplasmas are also capable of performing contractile cell movements (Trachtenberg, 1998). The tip structure which determines the direction of movement is situated at the leading end, which never changes (Razin, 1978; Razin and Freundt, 1984; Trachtenberg, 1998). Mycoplasmas usually move individually and not as a mass, and their moving pattern consists primarily of circles and narrow bends (Razin, 1978). The mycoplasma motility mechanism is still unclear, but it is acceptable to presume that motility plays a role in the penetration of the mucous layer of the host (Razin and Jacobs, 1992; Razin *et al.*, 1998).

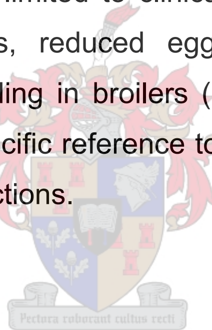
Pectora roborant cultus recti

As mentioned previously, mycoplasmas acquire needed nutrients from their host and environment due to their limited anabolic capabilities. Most species have a glycolytic pathway that supplies energy through glycolysis. It is believed that species lacking this ability, obtain energy via the arginine hydrolase pathway or through urea catabolism. ATP synthesis is most likely substrate-level phosphorylation since cytochromes and quinones are absent in mycoplasmas. Enzymes involved in *de novo* biosynthesis of purines and pyrimidines, and also in the tricarboxylic acid (TCA) cycle are also absent since no genes encode for them (Dybvig and Voelker, 1996; Razin *et al.*, 1998). This means the nucleic acid precursors must be obtained from the medium or the host.

Mycoplasmas developed from the *Clostridium* branch and metabolic pathways were lost due to the attrition of genes necessary for metabolism. Since mycoplasmas have a parasitic lifestyle, they are able to steal the necessary nutrients from their host. Thus the loss of the metabolic pathways has no influence on their survival. The loss of a cell wall is also typical of the parasitic lifestyle.

2.7 Mycoplasmas Affecting Domestic Poultry

Several mycoplasma species are of economical importance in the poultry industry because of their association with disease and reduced production. The implication of mycoplasma infections in diseases in other avian species still needs to be determined (Jordan, 1979). To date, seventeen avian mycoplasmas have been identified of which four are pathogenic to poultry, namely *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae* (Jordan, 1990a, 1996). These four poultry pathogens are mainly responsible for respiratory and locomotory disorders. However, they are not limited to clinical disorders; they are also responsible for reduced hatchability in breeders, reduced egg production in breeders, and reduced production and carcass downgrading in broilers (Bradbury, 2005). An overview of the four pathogenic mycoplasmas with specific reference to their epidemiology, diagnosis and control will be given in the subsequent sections.



2.7.1 Epidemiology

As in the case of many mycoplasma species, the poultry pathogens may have more than one natural host which they infect. Sometimes more than one mycoplasma species is responsible for an infection. Under this section dealing with epidemiology, the four poultry mycoplasmas' natural host and diseases that they cause, target organ or tissue for infection in the host, as well as method of transmission and thus spreading of infection between poultry, will be discussed.

2.7.1.1 Natural host

M. gallisepticum occurs naturally in chickens and turkeys worldwide. It is the causative organism of diseases in the respiratory complex resulting in suboptimal egg production in layers, downgrading of carcasses of broilers and turkeys, and reduced hatchability of chicks and poults (turkey chicks). Sometimes it is associated with encephalopathy in

turkeys and with salpingitis, arthritis and tenosynovitis in chickens (Jordan, 1979; Yoder, 1984; Jordan 1990a, 1996; Ley and Yoder, 1997; Levisohn and Kleven, 2000).

Respiratory diseases in chickens, turkeys, fowl and guinea fowl are also caused by *M. synoviae*. This includes a mild upper respiratory disease or chronic airsacculitis. The chicken is more susceptible to infection than the turkey. *M. synoviae* is also associated with joint lesions and lameness and retarded growth in broilers, pullets and turkeys (Olson, 1984; Jordan, 1990a; Kleven, 1997). Arthritis can also be caused by *M. synoviae* as well as infection of the eyes (Cline *et al.*, 1997; Nicholas *et al.*, 2002).

M. meleagridis is a turkey specific pathogen. It is generally associated with poor growth, airsacculitis, osteodystrophy, crooked necks, reduced hatchability in breeding birds, and abnormalities of the primary wing feathers. This pathogen has not been isolated from any other avian species (Jordan, 1979, 1990a, 1996; Yamamoto and Ghazikhanian, 1997).

The natural host of *M. iowae* is turkeys, but chickens and free-flying birds have also been shown to be infected. Reduced hatchability and embryo mortality is caused by this mycoplasma in turkeys (Jordan, 1990a, 1996; Kleven and Baxter-Jones, 1997).

Pectora roborant cultus recti

2.7.1.2 Infection

Environmental factors influence mycoplasma infections. During the cold winter months, diseases due to mycoplasma infections are of longer duration and often more severe (Yoder, 1984; Simecka *et al.*, 1992).

In the case of *M. gallisepticum* infection, the respiratory tract is the main target. The route of infection, which could be entrance through the host's respiratory tract or via the infected embryo, influences the degree of pathogenicity (Yoder, 1984; Jordan, 1990a). Embryos may be weakened by *M. gallisepticum* infection, resulting in difficulty in hatching or low-quality chicks (Levisohn and Kleven, 2000). An infection may remain dormant until debilitating factors occur. These factors include for example nutritional deficiency, excessive environmental dust and ammonia, limited effects of antibiotic treatment as well as stressing the bird (Jordan, 1979, 1990a; Simecka *et al.*, 1992; Winner *et al.*, 2000). The eyes of the birds may also be infected (Nicholas *et al.*, 2002). Resistance to *M. gallisepticum* increases with age, and some protection is provided by an immune response upon infection (Jordan, 1979, 1990a).

M. synoviae gains entry through the respiratory tract of its host or via the infected embryo and may last for several years. In combination with *M. meleagridis*, it may cause a more severe coryza in turkeys than on its own (Jordan, 1990a, 1996). Diseases caused by *M. synoviae* only are associated with infection in very young chicks or poults (Jordan, 1996). Acute infection occurs in adult chickens from time to time. Chronic infection, which follows the acute phase, may persist for longer than 5 years (Olson, 1984).

M. meleagridis enters its host either congenitally or through the respiratory tract. It may be harboured in the bursa of Fabricius and cloaca of poults, and in the case of mature birds on the phallus, in the oviduct as well as the upper respiratory tract where it may remain dormant for several months. Respiratory diseases due to infection with *M. meleagridis* can be aggrevated by a high concentration of atmospheric dust (Jordan, 1979, 1990a).

In turkey poults, *M. iowae* is harboured in the cloaca and upper respiratory tract. In the case of mature stock, it is harboured in the oviduct, cloaca and the phallus. No diseases are caused in any of these tissues (Jordan, 1990a). The pathogenicity and virulence of the *M. iowae* strains also vary (Kleven and Baxter-Jones, 1997).

Mycoplasma diseases are also subject to the concomitant presence of other respiratory viruses and bacteria. These include the viruses of infectious bronchitis, Newcastle disease, and turkey rhinotracheitis as well as the pathogenic strains of *E. coli* and *Avibacterium* (formerly *Haemophilus*) *paragallinarum*. The presence of these pathogens can also cause secondary complications during mycoplasma infections (Jordan, 1979; Olson, 1984; Jordan, 1990a, 1996; Ley and Yoder, 1997).

2.7.1.3 Transmission

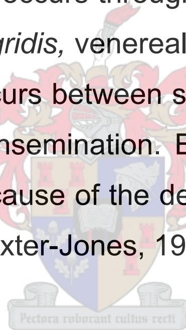
Transmission of *M. gallisepticum* infection may occur horizontally by direct contact from bird to bird, or vertically (*in ovo*) from an infected breeder flock, chicken or turkey, to the progeny (Jordan, 1990a; Ley and Yoder, 1997; Levisohn and Kleven, 2000). It can be spread by droplets, dust and contaminated equipment (Yoder, 1984; Cline *et al.*, 1997). Intercurrent infections may influence egg transmission and airborne spread since it stimulates multiplication of the mycoplasma (Jordan, 1979).

M. synoviae infection spreads through infected eggs or laterally from bird to bird. Contaminated equipment, droplets and dust spreads the infection (Jordan, 1990a; Cline *et*

al., 1997; Kleven, 1997). Transmission through the egg is variable and is most prevalent early after infection of adult stock, it can also occur at a low rate (Jordan, 1979).

The primary route of transmission of *M. meleagridis* is through the egg. The egg probably became infected in the oviduct, which may have been infected from the air sacs or cloaca or from infected semen at insemination. Venereal transmission is very important in sustaining infection of the oviduct which implies that the male is a significant contributor to the spread of infection. Eggs laid early are less likely to be infected, as well as eggs laid late in the laying cycle. Lateral transmission is also an important means of spread and can occur from bird to bird. Airborne transmission usually results in a high infection rate which persists in the sinus and trachea. Indirect spread occurs through human handling of stock at sexing, artificial insemination and vaccination (Jordan, 1979, 1990a; Yamamoto and Ghazikhanian, 1997; Bradbury, 2005).

Transmission of *M. iowae* also occurs through the egg which probably became infected in the oviduct. As with *M. meleagridis,* venereal transmission is of considerable importance, but lateral spread probably occurs between sister hens and stags housed together due to unhygienic conditions during insemination. Eggs laid late in the laying season are less prone to infection probably because of the development of a protective immune response (Jordan, 1990a; Kleven and Baxter-Jones, 1997; Bradbury, 2005).



2.7.2 Clinical signs and lesions

Several clinical signs and gross lesions are associated with *M. gallisepticum* infection of the respiratory tract. At the acute stage of infection, the level of *M. gallisepticum* is at its highest in the trachea even before any serological responses can be observed (Levisohn and Kleven, 2000). Clinical signs include coryza, which is an inflammation of the mucous membrane usually associated with nasal discharge, sneezing, coughing, tracheal rales and breathing through a partially open beak. If only the air sacs are affected no respiratory signs are visible. Reduced feed consumption results in the birds losing weight (Jordan 1979; Yoder, 1984; Jordan 1990a, 1996; Ley and Yoder, 1997). Mild conjunctivitis can be a sign of coryza, which is more severe in turkeys than in chickens, or the early stages of a more severe disease (Jordan, 1990a). Bulging eyes with caseous material under the eyelids, corneal oedema, watery conjunctivitis and sometimes large corneal ulcers are signs of infected eyes (Nicholas *et al*., 2002). Sometimes the eyes close partially or completely as a result of severe sinus swelling (Ley and Yoder, 1997). Ataxia in the turkey and lameness as

well as swelling of the hock in chickens is not seen very often (Jordan, 1990a). Male chickens often have the most pronounced signs (Yoder, 1984; Ley and Yoder, 1997). Intercurrent infections influences morbidity, and when they occur, the signs may be more severe and prolonged (Jordan, 1979, 1990a; Ley and Yoder, 1997).

Gross lesions due to *M. gallisepticum* infection are seen most frequently in the respiratory tract, less often in the oviduct and rarely in the hocks. Lesions of the respiratory tract can be very mild and almost unnoticeable, or consist primarily of excess mucous or catarrhal exudates in the trachea and lungs, nares, and oedema of air sac walls (Jordan, 1979, 1990a; Ley and Yoder, 1997). Sinusitis is normally most common in turkeys, but is also observed in chickens. Some degree of pneumonia has also been observed (Ley and Yoder, 1997). Mortality due to *M. gallisepticum* infection is, however, relatively rare in poultry (Jordan, 1979, 1990a).

When clinical signs occur due to *M. synoviae* infection, they take on an arthritic or respiratory form. In the acute arthritic form there is paleness of the face and comb, marked depression, swelling of the joints and rapid loss of condition. The hock joints and feet are affected in particular and accompanied by lameness. Feathers become ruffled and the comb shrinks as the disease progresses. Other clinical signs include retarded growth, birds becoming listless, dehydrated, emaciated and droppings have a greenish discolouration due to the large amounts of uric acid and urates it contains. Clinical signs for infection of the eyes are the same as for *M. gallisepticum* (Olson, 1984; Jordan, 1990a, 1996; Kleven, 1997). Recovery from the acute signs is very slow, but synovitis may remain for life in the flock. In the chronic form, swelling of the joints occurs without severe systemic disturbance, but with lameness. Lameness is also the most prominent sign in turkeys (Olson, 1984; Jordan, 1990a, 1996; Kleven, 1997). In the respiratory form, mild rales and coryza may occur, as well as swelling of the infraorbital sinuses in turkeys. This may occur independently of joint lesions. Lesions in the respiratory form are similar to those with *M. gallisepticum* infection, but generally none are seen in the upper respiratory tract. Oedema and thickening of periarticular tissues occurs when synoviae and joints are involved, the foot and hock joints are often affected. The spleen of some chickens in an affected flock is enlarged, the liver mottled green or dark red in colour and swollen, the kidneys are also pale or mottled and swollen, and the bursa of Fabricius and thymus are atrophied (Jordan, 1979; Olson, 1984; Jordan, 1990a; Kleven, 1997).

Pectora roborant cultus recti

In the case of chickens with a *M. synoviae* infection, the morbidity varies from 2-75% and mortality is usually low, ranging from less than 1-10%. Morbidity in infected turkey flocks is usually low, 1-20%, but mortality may be significant from trampling and cannibalism (Olson, 1984).

M. meleagridis infections cause no clinical signs in mature birds, but there may be reduced hatchability. Infection in young poults may also occur without clinical signs. In spite of a high rate of airsacculitis in poults, respiratory signs are rarely noticed. Lesions due to airsacculitis are usually not seen after 12-16 weeks of age. The initial infection of the thoracic air sacs spreads to the cervical and abdominal sacs. Skeletal lesions of osteodystrophy are seen and synovitis and oedema have also been reported. Although none of the clinical signs or gross lesions is specific to *M. meleagridis* infection, poor growth and feathering, airsacculitis and leg abnormalities in young poults are indicative of an infection (Jordan, 1990a; Yamamoto and Ghazikhanian, 1997). Even though *M. meleagridis* has a high infectivity, mortality due to this infection is low. *M. meleagridis* thus has an ideal host-parasite relationship (Yamamoto and Ghazikhanian, 1997).

No clinical signs are caused by *M. iowae* infections in mature birds, only reduced hatchability and abnormal feathering are observed (Jordan, 1990a; Kleven and Baxter-Jones, 1997). Gross lesions of affected embryos consist primarily of congestion and stunting, with various degrees of oedema, hepatitis, splenomegaly and sometimes a down abnormality. None of the lesions can be considered as pathognomic. Lesions due to airsacculitis in inoculated turkeys and chickens are normally mild to moderate and similar to those caused by other mycoplasmas. Inoculation of poults with *M. iowae* leads to several lesions, which include stunting, tenosynovitis, poor feathering, and several leg abnormalities such as toe deviations. Experimental chicks show similar leg lesions, but overall their lesions are less severe. Bursal atrophy may be a result of inoculation of turkey poults. Under field conditions, such severe lesions have not been reported, possibly since infected embryos do not hatch. Mortality due to *M. iowae* infections have only been observed in turkey embryos (Kleven and Baxter-Jones, 1997).

2.7.3 Diagnosis

None of the clinical signs or gross or histological lesions are pathognomic for any mycoplasma infection. They are simply an indication of an infection by one of the mycoplasma species (Jordan, 1990a). Samples can be isolated from various places

Pectora roborant cultus recti

depending on the mycoplasma being tested for. Several techniques that are available for testing the mycoplasma isolate will be discussed briefly. It is important that these diagnostic methods are rapid and precise.

2.7.3.1 Diagnostic samples

M. gallisepticum can be isolated from the oropharynx of the embryo or newly hatched bird, or in the case of an older bird from the respiratory tract, infraorbital sinus and cloaca (Jordan, 1990a). When infection occurs in the eye, *M. gallisepticum* can be isolated from the conjunctiva (Nicholas *et al.*, 2002). Fresh carcasses can also be used to take samples from a variety of organs, usually from the reproductive or respiratory tract (Levisohn and Kleven, 2000). The organism has also been isolated from cockerel and turkey semen as well as the oviduct of fowls and turkey hens (Jordan, 1996).

Samples of *M. synoviae* can be isolated from the trachea, joint lesions, and lungs and air sacs (Jordan, 1990a). *M. synoviae* can also be isolated from the transparent membrane covering the eyeball (Nicholas *et al.*, 2002).

In order to identify infection with *M. meleagridis*, isolates are usually taken from the respiratory tract or cloaca in the poults. In breeding birds, *M. meleagridis* can be isolated from the cloaca, oviduct or semen (Jordan, 1990a; Simecka *et al.*, 1992).

Pectora roborant cultus recti

M. iowae can be isolated from the oviduct, cloaca and phallus of mature stock, and in the case of recently hatched stock from the oropharynx, cloaca and air sacs. Only the vent is a suitable site for isolating *M. iowae* from turkeys in the age group between these ages. Because of its widespread nature its effects may pass unrecognized and therefore has to be closely monitored (Jordan, 1990a, 1996; Kleven and Baxter-Jones, 1997).

2.7.3.2 Identification of a mycoplasma infection

After collecting a sample of a possible mycoplasma infection, it can be used to inoculate a suitable solid agar or broth medium of choice (Ley and Yoder, 1997). Several techniques are available for the identification or confirmation of a mycoplasma infection. These techniques are listed below:

- Antibody-based procedure: Antigens are prepared from isolates and tested against known antiserum. This method is rarely satisfactory when testing recently isolated cultures (Ley and Yoder, 1997).
- *In vivo* bioassay: Mycoplasma free poultry is inoculated with the isolate and their serum tested with a known mycoplasma antiserum (Jordan, 1996; Ley and Yoder, 1997).
- Direct or indirect immunofluorescence: Mycoplasma colonies from the surface of agar plates or colony imprints are used, and this is a very effective method for culture identification (Jordan, 1996; Ley and Yoder, 1997).
- Agar gel precipitin test: In this test cultures are identified by using mycoplasma species specific antibodies (Ley and Yoder, 1997).
- Direct immunoperoxidase test: This test, of which the principle is very similar to the immunofluorescence test, is a very effective technique for indicating the presence of as well as identification of *M. gallisepticum* and *M. synoviae* cultures (Ley and Yoder, 1997).
- Compare protein banding patterns: Results from sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are used for comparison (Ley and Yoder, 1997).
- Restriction fragment length polymorphism (RFLP) of DNA: The sensitivity of this technique is greater than that of SDS-PAGE for differentiating strains of the same species from each other (Ley and Yoder, 1997).
- DNA and rRNA gene probes: Although the gene probes are highly sensitive, it is not in widespread use due to insensitivity for many clinical applications (Ley and Yoder, 1997).
- PCR: Specific DNA nucleotide sequences are employed in this rapid and sensitive technique. Clinical swabs can be directly tested (Ley and Yoder, 1997; Levisohn and Kleven, 2000; Nicholas *et al.*, 2002).

Up until now there is no single generic test to identify mycoplasmas to species level. Denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene could distinguish almost all mycoplasmas within a host animal group, but other bacteria will also generate a band on DGGE gel which may give confusing results (McAuliffe *et al.*, 2003). This

disadvantage can be overcome by designing mycoplasma-specific primers (McAuliffe *et al.*, 2003).

2.7.3.3 Serology

Serological tests are used to demonstrate the presence of a specific antibody. They are used to aid in diagnosis and are also useful for flock monitoring in control programs (Yoder, 1984; Ley and Yoder, 1997). These tests are listed below:

- Serum plate agglutination (SPA) test: It is a commercially available, quick, relatively inexpensive and sensitive test. Widely used to indicate infection in a flock rather than an individual infection, and detects IgM (Jordan, 1979; Ley and Yoder, 1997; Levisohn and Kleven, 2000; Butcher, 2002). Non-specific reactions do occur in some flocks that have a *M. gallisepticum* infection, or were recently vaccinated with oil emulsion vaccines or that is of tissue culture origin. Cross-reactions do occur between *M. gallisepticum* and *M. synoviae* which complicates serological detection (Jordan, 1979; Ley and Yoder, 1997; Levisohn and Kleven, 2000).
- Tube agglutination (TA) test: Takes longer to perform than the SPA test and although it is more accurate, it is rarely used anymore (Jordan, 1990a; Ley and Yoder, 1997).
- Haemagglutination-inhibition (HI) test: This test is time consuming, its reagents are not commercially available and it is not very sensitive. The test is highly specific, but it may take up to three or four weeks after infection to detect diagnostically significant titres. The HI test detects IgG levels. It is used routinely to confirm SPA, TA and ELISA tests (Yoder, 1984; Jordan, 1990a; Ley and Yoder, 1997; Levisohn and Kleven, 2000; Butcher, 2002).
- Enzyme-linked immunosorbent assay (ELISA): This test is more sensitive and specific than the SPA and HI test, and can also be used to detect levels of different classes of immunoglobulins. It is used commonly as an initial screening test for flock monitoring and sero-diagnosis, but false positive and negative reactions may occur (Jordan, 1990a; Ley and Yoder, 1997; Levisohn and Kleven, 2000; Butcher, 2002; McAuliffe *et al.*, 2003).

Pectora roborant cultus recti

Serological tests for determining flock status also have some pitfalls, namely (i) antibodies may be transient, (ii) the development of the immune response may be influenced by another flock treatment, and (iii) the onset of a detectable serological response may be delayed by immune suppressive agents (Levisohn and Kleven, 2000). In some cases the symptoms observed in the poultry are not unique to a mycoplasma infection. Both serological and cultural test procedures are then necessary to differentiate between a mycoplasma infection and another infecting agent (Ley and Yoder, 1997).

2.7.4 Treatment with antibiotics

Antibiotic therapy can reduce the severity of mycoplasma diseases and is thus very useful in treatment. However, neither termination of infection nor eradication of colonization is affected by treatment (Ellison *et al.*, 1992). Resistance to antibiotics can also develop as a result of gene mutation, acquisition of new genetic material, or it can be innate to the species, genus or family. Mycoplasmas have shown all three types of resistance to antibiotics (Roberts, 1992).

Mycoplasmas are known to be resistant to penicillin as well as other antibiotics that inhibit cell wall biosynthesis. However, they are susceptible to fluoroquinolones, macrolides, tetracyclines, and other antibiotics (Levisohn and Kleven, 2000). Tetracyclines are effective against almost all the mycoplasma species. It is a broad-spectrum antibiotic, has relatively low toxicity, causes few side effects, and prevents the proper functioning of the ribosomes by binding to them. Fluoroquinolones are active against a broad range of bacteria, and is a potent synthetic agent. Its primary target is DNA gyrase and thus blocks DNA replication (Roberts, 1992). They are known to kill bacteria rapidly, but decreased killing has been observed in mycoplasmas when present at high concentrations. Mycoplasmas are generally not treated with chloramphenicol because of its potential toxicity (Roberts, 1992). Antibiotics that have been used in poultry mycoplasma infections include the following:

- *M. gallisepticum* infection: It is susceptible to streptomycin, erythromycin, lincomycin, oxytetracycline, magnamycin, spectinomycin, chlortetracycline, spiramycin and tylosin. Some isolates, however, are quite resistant to tylosin, streptomycin, spiramycin and erythromycin (Yoder, 1984; Jordan, 1996; Ley and Yoder, 1997).
- *M. synoviae* infection: It is susceptible to chlortetracycline, lincomycin, tetracycline, danofloxacin, oxytetracycline, tiamulin, enrofloxacin, spiromycin, spectinomycin, and

tylosin among others. Isolates do appear to have resistance to erythromycin (Kleven, 1997).

- *M. meleagridis* infection: Hatching eggs are treated effectively with antimicrobials (Jordan, 1990a, 1996).
- *M. iowae* infection: Enrofloxacin has been used for egg treatment. Tiamulin and danofloxacin have also been found to be effective (Jordan, 1996; Kleven and Baxter-Jones, 1997).

Although antibiotics are often used, it is still better to keep the flock free from mycoplasma infection or to use a vaccine if necessary (Perelman, 1999).

2.7.5 Prevention and control of poultry mycoplasmas

Eradication of infection is the most efficient means of control for all four poultry pathogens. It is not always possible or wise to slaughter all the poultry, and therefore better to limit the spread of infection. Control in breeding stocks involves the following:

- minimum contact between the host and the pathogen (Jordan, 1979);
- treatment of hatching eggs to reduce transmission, for example by manual injection into the air sac or dipping them in a solution of a suitable drug (Jordan, 1990a, 1996; Ley and Yoder, 1997);
- keeping progeny flocks in flocks of small numbers and isolated from other flocks (Yoder, 1984; Jordan, 1990a; Ley and Yoder, 1997); and
- monitoring the progeny for infection (Jordan, 1990a)

Several antibiotics and vaccines, both live and killed (bacterins) are available that protect against mild falls in egg production in layers. Methods of vaccination and commercially available vaccines will be discussed in more detail in section 2.9.

Biosecurity is the preferred method of control in poultry to exclude an infection from stock. Immunization or anti-microbial medication may be required in instances where infection cannot be readily excluded by economically sustainable biosecurity (Whithear, 1996; Perelman, 1999).

Pectora roborant cultus recti

Flocks are considered free of infection when serologically negative progeny have been derived from negative parent birds and hatching eggs, and none of the generations have been subjected to antimycoplasma treatment (Jordan, 1996).

2.8 The South African Ostrich

The ostrich, *Struthio camelus*, is a ratite, paleognathic (primitive) bird and also the largest living bird presently in Southern Africa (Huchzermeyer, 1998a; Bezuidenhout, 1999). Ostriches are flightless running birds and their feathers lack the typical interlinked structure of flying birds. They are mainly herbivores, can swim and have two toes. Ostrich eggs weigh 1-1.5 kg, and the chicks hatch after 42 days of incubation. An adult ostrich can weigh between 120 and 160 kg (Huchzermeyer, 1998a).

The main focus of this dissertation is on mycoplasmas as causative agents of respiratory diseases in the ostrich. However, they are not the only organisms causing respiratory diseases in the ostrich. In this section, the respiratory system as target of mycoplasmas will be discussed briefly to give an overview of the areas where the diseases occur. A discussion of mycoplasma infections in the South African ostrich will follow as well as other respiratory diseases with reference to their symptoms and treatment available where possible.

2.8.1 Respiratory system and respiration

The glottis (laryngeal opening) is situated close to the front of the mouth, but the mucous membrane surrounding it as well as the larynx lacks papillae. From the larynx, the trachea extends to the syrinx, which is uncomplicated and consists of the last tracheal rings. The lungs are attached to the rib cage, and so firmly that deep grooves have developed on the lung surfaces. Ostriches have ten air sacs similar to those of other avian species. These include: cervical air sacs, paired lateral clavicular air sacs, paired cranial thoracic air sacs, paired caudal thoracic air sacs and a right and left abdominal air sac (Huchzermeyer, 1998a; Bezuidenhout, 1999).

Ostriches use a costal pump for ventilation rather than a diaphragmatic pump. The series of air sacs connected to each lung forms the basis of three distinctive avian respiratory characteristics. Firstly, the lungs are more efficient than the mammalian lung since air flows through continuously in one direction. Secondly, breathing is slower and deeper due to the large residual volume provided. And thirdly, the large source of air provided can be used for

gaseous exchange as well as transfer of heat by evaporation. The air sac system of the ostrich is well developed, and together with the lungs it can hold a total volume of about 15 litres for an ostrich of 100 kg. As in other birds, air flow during inspiration and expiration is in the same direction with little change in the volume of the lungs. The lungs have thinner walls which permit more efficient gaseous exchange, and the air sacs are responsible for pumping air. An increase in respiration rate is not necessarily related to an increase in the oxygen consumption rate (Skadhauge and Dawson, 1999).

2.8.2 Mycoplasma infections in the ostrich

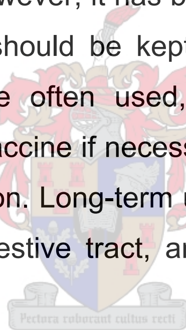
Ostriches in South Africa as well as other countries worldwide have been found to be affected by mycoplasmas. None of these infections were identified as poultry mycoplasmas and knowledge on mycoplasma-associated diseases in ostriches is also very limited (Shivaprasad, 1993; Botes *et al.*, 2005a). However, Cline *et al.* (1997) could induce clinical signs of infection by experimentally infecting ostriches with *M. gallisepticum*, but not by infecting them with *M. synoviae*. For this reason, keeping both chickens and ostriches in close proximity is not recommended, and it was thought that poultry mycoplasmas may be transmitted to the ostriches.

In a study done by Botes *et al.* (2005b), samples from ostriches in South Africa were analysed to evaluate the correlation between disease symptoms and mycoplasma occurrence. As described previously, three ostrich specific mycoplasmas were identified by Botes *et al.* (2005a), namely Ms01, Ms02 and Ms03. In these studies, the samples were divided into two groups, the first consisting of 206 samples that were used for mycoplasma cultivation and the second consisting of 162 samples that were used directly for PCR testing. None of these samples were found to contain poultry mycoplasmas. From the first group, 185 out of the 206 isolates tested ostrich mycoplasma positive, and in some samples a combination of Ms01/03 or Ms02/03 infection was present. Of the 185 mycoplasma positive samples, 184 were isolated from the upper respiratory system, namely trachea, sinus, air sac, choana and eye, and only one was isolated from the caecum. From the second group, 85 out of the 162 samples tested positive for Ms01, Ms02 or Ms03 respectively and only eight had an Ms01/03 infection. Seventy-seven of the 85 samples were isolated from the respiratory tract, namely the trachea, sinus, air sac, choana and eye. Only seven samples were isolated from the alimentary tract, namely the cloaca, and one was from yolk. These results strongly implicate mycoplasmas as one of the most important organisms in respiratory diseases in ostriches.

Pectora roborant cultus recti

Huchzermeyer (1994) found that mycoplasmas in the South African ostriches are associated with respiratory infections in feedlot birds during winter causing rhino-tracheitis, or air-sacculitis as an extension of nasal infections. From the study by Botes *et al.* (2005b), it was observed that the three ostrich mycoplasmas occur throughout the year. However, the highest incidence seems to be at the beginning of the cold winter months and again at the beginning of summer. The ostriches in which respiratory tract mycoplasmas were detected, also exhibited respiratory diseases such as rhinitis, tracheitis, sinusitis and air-sacculitis (Huchzermeyer, 1994; Botes *et al.*, 2005b). Pathological, as well as respiratory lesions characteristic of poultry mycoplasma infections also occurred in many of the sampled ostriches. Since no poultry mycoplasmas were observed, these lesions provide further evidence implicating that Ms01, Ms02, or Ms03 caused the infection (Botes *et al.*, 2005b).

Symptoms of this respiratory disease can be reduced by treatment with the mycoplasma specific antibiotic tylosin. Tylosin can be administered orally via the feed, dosed orally, or injected (Botes *et al.*, 2005b). However, it has been recommended that the use of antibiotics as well as other antibacterials should be kept to the absolute minimum (Huchzermeyer, 1998a). Although antibiotics are often used, it is better to keep the flock free from mycoplasma infection or use a vaccine if necessary. Antibiotics should not be used in young birds for the prevention of infection. Long-term use of antibiotics predisposes birds to fungal infections of the mouth and digestive tract, and therefore should be avoided (Perelman, 1999).



In the case of ostriches, biosecurity is the preferred method of control to exclude an infection from stock. Immunization or anti-microbial medication may be required in instances where infection cannot be readily excluded by economically sustainable biosecurity (Whithear, 1996; Perelman, 1999).

2.8.3 Other respiratory diseases in the ostrich

Ostriches are very sensitive to stress and this creates the ideal environment for an organism to cause disease. No ostrich-specific infectious or contagious disease exists, but the wireworm, *Libyostrongylus*, and the tapeworm, *Houttuynia*, are the only ostrich-specific pathogens (Huchzermeyer, 1998b, 1999, 2002). Diseases that have no respiratory involvement include (i) Newcastle disease, which affects the nervous system; (ii) fading chick syndrome, which is characterised by a halt in growth and loss of weight; (iii) tibiotarsal rotation, which is the outward rotation of the lower tibiotarsus; and (iv) enteritis, which is

characterised by an abnormal intestinal flora of the chicks (Huchzermeyer, 1998b, 1999, 2002).

Infections are usually transmitted by domestic or wild birds, but flies, lice and ticks are also important in transmitting infectious diseases. Humans can also act as passive carriers. As with mycoplasma infections, high dust and ammonia levels, and cold conditions are important factors involved in respiratory disease. The ostrich's immune system can be depressed by these and other stressors, making the birds more sensitive to bacteria, fungi or viral agents (Huchzermeyer, 1994, 1998b, 1999). Symptoms that are generally associated with respiratory diseases are:

- upper respiratory infections that affects the nasal passages (rhinitis), the infraorbital sinuses (sinusitis), the conjunctivae (conjunctivitis), larynx (laryngitis) and trachea (tracheitis). The lungs are constructed in such a way that bacteria and spores that were inhaled move through to the air sacs, which make them affected less frequently (Huchzermeyer, 1998b, 1999); and
- airsacculitis caused by agents, like dust particles, aerosols and fungi, are carried with the air and deposited in the air sacs. This happens because they bypass the gas exchange areas of the lung during inhalation. Aspiration airsacculitis occurs in feedlot ostriches (Huchzermeyer, 1998b)

Respiratory diseases, other than those caused by mycoplasmas are listed below:

- Aspergillosis – mycosis of the air sacs: It is caused by a build-up of contamination in the environment by fungal spores produced by moulds, particularly those of *Aspergillus* spp. Nodular lesions are caused in the trachea, air sacs, lungs, nasal passages as well as on the conjunctivae. Fumigation or aerosol of the room with enilkonazole is a successful method of treatment when the birds are present. Avoidance of mouldy conditions, good ventilation, keeping the birds warm as well as avoiding stress and malnutrition also helps the prevention of aspergillosis (Huchzermeyer, 1994, 1998b, 1999, 2002).
- Bacteria related to respiratory diseases that have been isolated from ostriches include: *Pasteurella haemolytica, Pseudomonas aeruginosa, Bordetella* spp.*, Haemophilus* spp.*, Staphylococcus* spp.*, Streptococcus viridans, Corynebacterium pyogenes, Mycoplasma* spp. and *Chlamydia psittaci* (Huchzermeyer, 1994, 1998b, 1999).

- *Chlamydia psittaci*: Conjunctivitis has been reported in ostriches due to infection with these bacteria. Fibrinopurulent tracheitis, pneumonia, pericarditis and perihepatitis have been reported in the case of a generalized disease. Treatment is available through a prolonged course of tetracyclines (Huchzermeyer, 1994, 1999).
- Avian influenza: Several strains of the virus, namely H7N1 (1991 and 1992), H5N9 (1994) and H9N2 (1995) have been isolated from ostriches in South Africa (Huchzermeyer, 1999, 2002). More recently in 2004, the high-pathogenic avian influenza virus (HPAI) H5N2 was isolated from ostriches in the Eastern Cape (http://www.saobc.co.za/modules.php?name=News&file=article&sid=32). However, not all strains are equally pathogenic, and its severeness depends on the age of the bird as well as complicating secondary respiratory infections. Respiratory signs, ocular discharge, green urine and severe depression are clinical signs of avian influenza. It is important to treat the secondary infections since no treatment or vaccine is available for avian influenza due to its strain variability (Huchzermeyer, 1999, 2002).
- Filariae: Very long and thin roundworms that have been found in the lungs and air sacs of ostriches. *Struthiofilaria megalocephala* have been isolated from the air sacs of a South African ostrich. Isolations have been rare and they appear to be harmless (Huchzermeyer, 1994, 1999).
- Chaetotoxy: Rhinitis, sinusitis and airsacculitis were found in an ostrich infested with quill mites that normally cause severe damage to the feathers. Treatment with ivermectin relieves the respiratory symptoms of chaetotoxy (Huchzermeyer, 1994, 1998b).
- Pneumonia: It is relatively rare in ostriches due to the construction of the lungs (Huchzermeyer, 1998b).
- Anthracosis: Anthracosis and pneumoconiosis have been found to cause encapsulated granulomata in the lungs (Huchzermeyer, 1998b).

2.9 Poultry Mycoplasma Vaccines

Several vaccines for the treatment of poultry mycoplasmas, as well as methods of administering a vaccine are available. However, none of these vaccines have specifically been developed for ostriches which emphasize the need for the development, trial and registration of a specific vaccine.

Pectora roborant cultus recti

Vaccines have four immunological requirements, and according to Ada (1994) they are:

(i) antigen processing and interleukin production must be initiated by the activation of antigen-presenting cells;

(ii) a high yield of memory cells by activation of T and B cells;

(iii) variation in immune response in the population due to major histocompatibility complex (MHC) polymorphism must be overcome by the generation of Th and Tc cells to several epitopes; and

(iv) antibodies must be continually present

Immunization must also be successful, and therefore the following criteria according to Ellison *et al.* (1992) must be met:

(i) the vaccine must contain protective immunogen(s);

(ii) if a live vaccine, it must be genetically and phenotypically stable;

(iii) a protective respiratory mucosal immune response must be elicited via the route and presentation; and

(iv) protection of the vaccine must not induce toxic reactions or adverse immune abnormalities

The above mentioned requirements are applicable to all vaccines for humans and animals. However, the question still stands on what the ideal poultry and ostrich mycoplasma vaccine should be like. Whithear (1996) suggested the following regarding poultry mycoplasmas, and this is therefore also relevant for an ostrich mycoplasma vaccine. The ideal mycoplasma vaccine should be safe to use and cost-effective. Safety is more important in the case of live vaccines than with bacterins. Live vaccines should not cause disease in the vaccinated animal, or spread to neighbouring flocks and cause disease. Regression to a virulent form should not occur in an attenuated strain. Lifelong immunity, preferably from a single dose, must be initiated by the vaccine. Manufacture of the vaccine must be cheap, and it must be derived from properly defined seed stock with a consistently high potency and purity. Administration of the vaccine to a large number of birds should be cheap and convenient. In the case of a flock, performance should improve to exceed the cost of purchase and administration. The ideal mycoplasma vaccine does not exist yet, since vaccines currently available still have disadvantages associated with their use (Whithear, 1996).

2.9.1 Vaccines

Two types of vaccines are available for poultry, namely killed whole cells (bacterins) or living cultures, both having their advantages and uses (Jordan, 1990b; Pattison and Cook, 1996). At the Onderstepoort Veterinary Institute, South Africa, vaccines for the following diseases have been used in ostriches: anthrax, botulism and clostridial enterotoxaemia, but none for mycoplasmas (Huchzermeyer, 1998b). Live vaccines have been developed for *M. gallisepticum* and *M. synoviae* strains, but antibiotics have also been used as treatment for poultry mycoplasmas.

2.9.1.1 Killed vaccines (bacterins)

Bacterins are made up of inactivated organisms suspended either in aluminium hydroxide adjuvants or an aqueous oil emulsion. They provide high and extended levels of immunity, and must be injected (Jordan, 1990b; Pattison and Cook, 1996; Whithear, 1996). An advantage of using bacterins above live vaccines is that they are non-infectious, and thus will not revert to virulence or cross-infect to other stock. However, they are expensive and birds need to be vaccinated individually. Bacterins of *M. gallisepticum* are used commercially in several countries, but bacterins of *M. synoviae* are not used widely in the poultry industry (Whithear, 1996; Levisohn and Kleven, 2000). Oil emulsion bacterins are not recommended for use in ostriches, since they cause large abscesses and granulomas underneath the skin. If they are to be used, the vaccine must be centrifuged in advance in order for the oil to be separated. The oil level is removed and the oil free vaccine can then be used for subcutaneous injection (Dr. A. Botes, 2005, personal communication).

2.9.1.2 Live vaccines

Live vaccines usually contain only one antigen which can either be a naturally occurring strain of moderate virulence, or an artificially attenuated strain of low virulence. These vaccines can be administered through various methods to an individual bird or a flock. The mycoplasma replicates rapidly in the target organ(s) and therefore only a small amount of antigen is required (Jordan, 1990b; Pattison and Cook, 1996; Whithear, 1996). A significant quality of a live mycoplasma vaccine strain is that it should provide long-term immunity without causing disease or spreading to other vulnerable birds. The ability of certain mycoplasma species to interact synergistically with other infectious agents complicates this delicate balance. Severe diseases can be produced from these

synergistic interactions if the birds are subjected to physiological and/or environmental stress (Whithear, 1996).

2.9.1.3 **M. gallisepticum *vaccines***

When using a *M. gallisepticum* vaccine, there are three definite objectives that protection should be provided for, namely (i) disease in the respiratory tract, (ii) fall in egg production, and (iii) transmission of *M. gallisepticum* through the egg (Whithear, 1996).

Currently, there are four strains of live *M. gallisepticum* vaccines that are used commercially worldwide. These are the F strain, ts-11 and 6/85.

The F strain occurs naturally, has moderate virulence in chickens and high virulence in turkeys. Transmissibility of this strain is also low. Administering of the F strain vaccine can be via several routes including intranasal, intraocular and drinking water, but coarse spray is used most often. Vaccination with this strain prevents egg production losses effectively, and it stimulates immunity against infection by challenge or wild-type infection (Whithear, 1996; Levisohn and Kleven, 2000).

Strain ts-11 is an artificially attenuated strain with low virulence and low tendency to spread between birds. Administering of this vaccine is via eye drops. Protection is induced after challenge with *M. gallisepticum* through the development of circulating antibodies. The ts-11 strain provides lifelong immunity by remaining in the upper respiratory tract for the rest of the life of the vaccinated flock (Whithear, 1996; Levisohn and Kleven, 2000).

Strain 6/85 is also artificially attenuated, has low virulence and does not spread easily from bird to bird. This vaccine induces resistance against virulent *M. gallisepticum*. No humoral antibody response is stimulated although the vaccine can be detected in the upper respiratory tract for four to eight weeks after administration by spray. The primary use of this vaccine is to prevent egg production losses (Whithear, 1996; Levisohn and Kleven, 2000).

The newly available Nobilis MG 6/85 vaccine, a live *M. gallisepticum* vaccine which is a commercially available from Intervet, appears to be an almost ideal vaccine. Research has shown that it is genetically stable, non-pathogenic, suitable for convenient storage as

Pectora roborant cultus recti

well as mass administration, and it also prevents drops in egg production related to *M. gallisepticum* (Nobilis MG 6/85, 2005).

The ts-11 and 6/85 strains are preferred to the F strain because of their low virulence as well as low potential to be transmitted to unvaccinated flocks.

Control of stock is the preferred method of keeping them free from *M. gallisepticum* infection, but in cases where this is not possible, vaccination is the alternative method (Levisohn and Kleven, 2000). Current vaccines do have a disadvantage, namely that there is no serological technique that can accurately distinguish between a naturally infected and vaccinated flock (Whithear, 1996).

2.9.1.4 **M. synoviae** ***vaccines***

Currently, the MS-H strain, an attenuated strain of *M. synoviae,* is used as a vaccine against *M. synoviae*. It is administered by eye drops, after which it colonises the respiratory tract of chickens, stimulates a measurable serum antibody response, and remains in the respiratory tract for at least 55 weeks after vaccination. No lesions were caused after inoculating it into the air sacs or by administration via aerosol to chickens. At the time of vaccination, the success of the MS-H strain depends on the bird being free from exposure to the wild-type *M. synoviae* (Whithear, 1996).

2.9.1.5 DNA vaccines

Wolff *et al.* (1990) originally described the concept of a DNA vaccine. Although details regarding the mechanisms of action of a DNA vaccine are still unclear, the principle is relatively simple. Genes encoding the immunogenic protein(s) are inserted into a suitable eukaryotic expression plasmid that can be replicated in bacteria. After large-scale production and purification steps, the DNA vaccine can be directly inoculated, usually by intramuscular injection, into the animal to be vaccinated. Subsequently the plasmid insert is expressed by the host cells and the protein produced initiates an immune response (Wolff *et al.*, 1990).

The use of a DNA vaccine is a very powerful tool and it has several advantages as well as disadvantages. Advantages of DNA immunization include the following: (i) it mimics live attenuated vaccination; (ii) correct MHC I presentation of antigen is provided; (iii)

concurrent administration is allowed; (iv) genetic stability of immunizing plasmid; and (v) modification of the immune response may be permitted (Webster, 1998; Oshop *et al.*, 2002).

Disadvantages of DNA immunization include (i) induction of tolerance; (ii) integration of the DNA into the host genome; and (iii) induction of auto-immunity and anti-DNA antibodies (Webster, 1998; Oshop *et al.*, 2002).

Although the concept of DNA vaccination is still in its early stages in the poultry industry, it has been found to be advantageous. Progeny have high levels of maternal antibodies due to vaccination of the hens, and interference of passive maternal antibodies is also minimal (Oshop *et al.*, 2002).

2.9.2 Administration of vaccine

As mentioned above, two types of vaccines are available for poultry, namely killed or live vaccines. Although there are several ways of administering a vaccine, a killed vaccine must be injected and a live vaccine can be sprayed over the facial area. Live vaccines can also be administered via the drinking water, through eye drops or injection. Killed vaccines are normally supplied in suspension or emulsion, whilst live vaccines are normally supplied in a freeze-dried form in vials (Jordan, 1990b; Pattison and Cook, 1996). Administration of vaccines and medication for an individual bird as well as a flock will be discussed briefly.

Pectora roborant cultus recti

2.9.2.1 Individual vaccination

The individual bird, or ostrich, can be vaccinated via one of the following ways:

- Dosing by mouth: Also known as drenching. Liquid is poured over the larynx into the oesophagus, and care must be taken not to pour it down the trachea (Huchzermeyer, 1998a).
- Injection: Killed or live vaccines are either given intramuscularly, into the breast or leg, or subcutaneously under the loose skin at the back of the neck. In the case of the ostrich, injections are given subcutaneously and the leg muscles must be avoided at all times since it is the most valuable meat, and injection marks downgrade the skin. A less diluted vaccine can cause kidney damage due to premature excretion of the vaccine via the renal portal system which

drains the posterior half of the ostrich's body (Jordan, 1990b; Pattison and Cook, 1996; Huchzermeyer, 1998a).

- Eye drop: The most effective method of administering a live vaccine is through eye drops or the intranasal route. Accuracy is important which makes immunisation a bit time consuming (Jordan, 1990b; Pattison and Cook, 1996).
- Wing web: This method, via the wing web, is the principal method of administration of the fowl pox vaccine. Seven to fourteen days post-vaccination a slightly raised and swollen area should appear at the application site. This indicates that the vaccine was absorbed (Jordan, 1990b; Pattison and Cook, 1996).
- *In ovo:* One of the vaccination methods that have not been used widely in ostriches but would be possible is vaccination via the egg. An example of this method is the administration of Marek's disease vaccines via inoculation of fertile chicken eggs at 18 days. This system will hopefully some day be suitable for administration of various live vaccines (Pattison and Cook, 1996).

2.9.2.2 Flock vaccination

In some cases, individual vaccination is not necessary and the whole flock can be vaccinated at the same time. Administration methods for the flock include the following:

- Drinking water: Live vaccines in particular, can be administered via drinking water. They should be reconstituted in clean cold water containing powdered milk. The powdered milk acts as a stabilizer and protects the live vaccine from harmful substances that might occur in the water. Vaccines are usually diluted according to the age of the birds, but the water consumption of the bird also has to be considered (Jordan, 1990b; Pattison and Cook, 1996). In the case of the ostrich, this route of administering live vaccines is not recommended. Their drinking behaviour is irregular, the water troughs are exposed to the ultraviolet rays of the sun and the life span of the vaccine virus is shortened in the water (Huchzermeyer, 1998a; Perelman, 1999).
- In feed: This method is the best in cases where medication has to be given over a prolonged period, and has been used to distribute live Newcastle disease vaccine to small backyard flocks. The results, however, have been quite variable (Pattison and Cook, 1996; Huchzermeyer, 1998a).

Pectora roborant cultus recti

- Spray or aerosol: The live vaccine, reconstituted in distilled water, can also be administered by spray or aerosol over the facial area. Droplet sizes of less than 5 µm in diameter can penetrate the respiratory system into the lungs. A coarse spray contacts only the upper respiratory tract with droplet sizes being larger than 10 µm. This method of vaccination is normally more efficient in a controlled environment than in an open sided house (Jordan, 1990b; Pattison and Cook, 1996).
- Inhalation: By fogging or fumigating an enclosed room with ostrich chicks, antifungals and antimicrobials can be administered to them (Huchzermeyer, 1998a).

2.9.3 Previous studies with poultry mycoplasma vaccines

One of the first persons to detect that chickens had immunity to *M. gallisepticum* infection, was Nelson in 1935 (as referred to in Adler and Lamas Da Silva, 1970). He noted that after recovery from chronic coryza caused by *M. gallisepticum* in the chickens, they were resistant to a second exposure (Adler and Lamas Da Silva, 1970). However, birds that have some degree of immunity after recovery from an infection still carry the organism and can transmit the disease to susceptible stock either by contact or through egg transmission to their progeny. It has also been observed that antibodies remained in chickens that recovered from an infection by *M. gallisepticum*, and they had a faster rate of *M. gallisepticum* elimination upon re-exposure (Yoder, 1984; Ley and Yoder, 1997).

Although Lin and Kleven (1984) stated that while the use of bacterins as vaccines does not provide effective immunity against challenge with *M. gallisepticum*, they do have the advantage of not reverting to virulence or cause vaccine reactions. Bacterins also elicit a more consistent and reliable immune response (Droual *et al.*, 1990). Panigrahy and co-workers did a study in 1981 in which they compared the immunogenic potency of an oil emulsion bacterin versus an aqueous preparation. They found that oil emulsified *M. gallisepticum* bacterins are highly antigenic and they also induce significantly higher antibody titers than the aqueous preparation (Panigrahy *et al.*, 1981).

One disadvantage of using a bacterin is the lesions that are sometimes caused when injecting chickens intramuscularly. These lesions, which are mostly cysts with thin fibrous capsules, are sometimes associated with lymphocytic aggregates but less often with a

granulomatous reaction (Droual *et al.*, 1990). From another study by Droual *et al.* (1993), it was suggested that the vaccine materials follow paths of least resistance, hence the negative effect they can have depending on the route of injection. Therefore it is better to inject oil-adjuvanted killed vaccines subcutaneously rather than intramuscularly in the leg which could lead to lameness (Droual *et al.*, 1993). In the case of ostriches, the forming of abscesses under the skin is also seen with the use of oil emulsion bacterins. Despite the disadvantages of oil-adjuvanted bacterins, they are associated with stronger immunogenic responses as found by Droual *et al.* (1993) which make these vaccines popular for use.

As discussed previously, the live *M. gallisepticum* F-strain vaccine occurs naturally and has high virulence to turkeys but only moderate virulence to chickens (Lin and Kleven, 1984; Whithear, 1996; Levisohn and Kleven, 2000; Ferraz and Danelli, 2003). In a study done by Lin and Kleven (1984), they noticed that eye-drop vaccination of the F-strain possibly does not provide adequate immunity against *M. gallisepticum*. Penetration of the vaccine might not be deep enough into the respiratory tract and multiply as rapidly and therefore the immune system has less exposure to the antigen. The use of aerosol is recommended as vaccination method rather than vaccination via eye-drop (Lin and Kleven, 1984).

All three live *M. gallisepticum* vaccines were compared to each other in a study by Abd-El-Motelib and Kleven (1993) in young chickens. They found that the F-strain was more virulent than the ts-11 and 6/85 strains which elicited little or no vaccination reaction. The F-strain provided better protection against air sacculitis and was also more effective in preventing colonization by challenge strains.

In a recent study by Biró *et al.* (2005) on the *M. gallisepticum* ts-11 vaccine, their results showed that the ts-11 vaccine is safe to use, and it does not cause any pathological lesions or clinical signs. Their results were based on a challenge with the virulent *M. gallisepticum* R strain. However, Ferraz and Danelli (2003) found that it is difficult to distinguish between a vaccinated and naturally infected flock with the use of the ts-11 strain since no molecular marker is available. Noormohammadi *et al.* (2002a) failed to detect antibodies after ts-11 vaccination, but they found that after administering higher doses of vaccine higher antibody levels were produced.

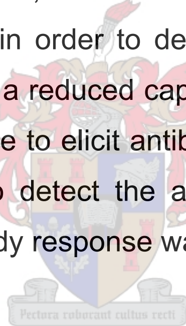
The *M. gallisepticum* 6/85 strain is safe to use due to its low virulence. Spreading of the 6/85 vaccine from bird to bird is also very poor. Its safety was evaluated by Zaki *et al.* (2004), and

Pectora roborant cultus recti

they found that its pathogenicity might be slightly more for turkeys than for chickens. No evidence of reversion to virulence was observed.

The avirulent *M. gallisepticum* strain R_{high}, was reconstituted to form the live *M. gallisepticum* vaccine GT5. GT5 expresses the major cytadhesin GapA on its surface, yet has low levels of *in vitro* cytoadherence. During a study to test its efficacy, Papazisi *et al.* (2002b) found that GT5 could stimulate a protective immune response. Two weeks after vaccination only modest amounts of IgG and little, if any, secretory IgA or IgM anti-*M. gallisepticum* were found in tracheal washings. After challenge with virulent *M. gallisepticum* strain R_{low}, ample amounts of specific IgA were found which suggests its role in clearing the infection rather than giving protection. It is thus hypothesized that tracheal IgG gives protection against R_{low} since it was elicited by GT5 vaccination. Immunization with GT5 thus provides short term protection against challenge with wild type *M. gallisepticum* R_{low}.

In the case of *M. synoviae* vaccines, the live attenuated MS-H strain vaccine was studied by Noormohammadi *et al.* (2002b) in order to determine whether low levels of antibodies in vaccinated chickens were due to a reduced capability of the antigen in detecting antibodies, or the limited ability of the vaccine to elicit antibodies. They found that the antigens used in serological tests were unable to detect the antibodies, hence the lower levels, and the highest detectable level of antibody response was only seen after 100 days of vaccination.


Pectora roborant cultus recti

2.9.4 Antibody response

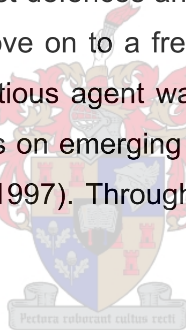
Infectious diseases usually have a classic antibody response which can be divided into three phases: (i) during weeks 1 to 3 of the disease, antibodies are produced rapidly; (ii) the antibody levels peak at 2 to 4 weeks after infection; and (iii) antibody levels show a gradual decrease months to years after recovery from the infection (Kenny, 1992).

In a study by Blignaut *et al.* (2000), the antibody response to Newcastle disease virus (NDV) in South African ostriches was tested. Two vaccine trials were launched in which birds for slaughtering (age 2.5 months up to 14 months) as well as young birds (age 5 weeks up to 2.5 months) were vaccinated at different time intervals. From the results that were obtained for both trials, a peak in antibody response could already be seen after 14 days, thus 2 weeks, but the response was better after 21 days, thus 3 weeks. The assumption could be made that the antibody response against mycoplasma infections in the ostriches would be more or less the same.

The occurrence of antibodies to *M. gallisepticum* and *M. synoviae* as well as other common avian pathogens was determined in a study by Ley *et al.*, (2000) in 163 commercially raised slaughter-age ostriches in Ohio and Indiana. They found that these ostriches had minimal exposure to any of the pathogens and therefore no antibody reaction. These results confirmed earlier findings by Shane and Tully in 1996, where no *M. gallisepticum* or *M. synoviae*-positive serum was reported in any common commercial ratite species. In contrast to this, 11% of 149 ostriches in Zimbabwe had antibodies that bound to *M. gallisepticum* and/or *M. synoviae* coating antigens in an ELISA test (Ley *et al.*, 2000).

2.10 Pathogenicity of Mycoplasmas

Mycoplasmas are known as the ideal parasite because they seldom kill their host and rather live in harmony. In order to be a successful pathogen, it must have a way of entering its host, reach the target tissue and possibly adhere to the target. It should invade the target tissue and multiply whilst evading the host defences and causing some damage to the host. Finally, it must be able to escape and move on to a fresh host (Bradbury, 2005). The first report in humans of mycoplasmas as infectious agent was in the 1930's and 1940's, and since then the impact of mycoplasma species on emerging diseases have increased in humans as well as animals (Baseman and Tully, 1997). Through adhesion, mycoplasmas are pathogenic to their hosts.



2.10.1 Adhesion to host cell

For a mycoplasma to colonize and infect a host, adhesion is essential. Its pathogenicity is dependent on adhesion to the host, and without adhesion the mycoplasma is avirulent (Razin and Jacobs, 1992; Rottem, 2003). The process of adhesion is multifactorial and accessory membrane proteins are also involved (Razin *et al.*, 1998). When a mycoplasma attaches to its host, it can interact with membrane receptors or adjust transport mechanisms. The cell membrane of the host is also sensitive to toxic materials, such as hydrogen peroxide and superoxide radicals, generated by adhering mycoplasmas. It is believed that they cause oxidative stress in the host cell which leads to damage to the cell membrane (Rottem and Naot, 1998; Rottem, 2003).

The cell components responsible for attachment are proteins and are termed adhesins, and are part of the cell membrane (Razin and Jacobs, 1992). Surface-exposed adhesins have been identified in *Mycoplasma pneumoniae*, namely P1 and P30, as well as accessory

proteins named HMW1, HMW2, HMW3, A, B and C. P1 is regarded as the main *M. pneumoniae* adhesin, but shares a number of characteristics with P30. The accessory proteins are necessary for proper functioning of the adhesins, but they could not be defined as adhesins as they are not directly involved in cell adherence. Without P1, *M. pneumoniae* is unable to attach properly to its host and is therefore avirulent (Razin and Jacobs, 1992; Krause, 1998; Razin *et al.*, 1998; Krause and Balish, 2001; Chaudhry *et al.*, 2005; www.mgc.ac.cn/, 2005). In *M. genitalium* the major adhesin is termed MgPa which is the counterpart or analogue of P1, and their roles in attachment are apparently similar (Carson *et al.*, 1992; Razin and Jacobs, 1992; Razin *et al.*, 1998; Razin, 1999). Other adhesins that have been identified include those of *M. gallisepticum*, namely GapA and CrmA (cytadherence-related molecule), and *M. pirum* which is named P1-like adhesin (Papazisi *et al.*, 2000). In the case of *M. fermentans* and *M. hominis* no tip structure is present (Razin *et al.*, 1998), but *M. hominis* can adhere to its host via two cytoadhesins, namely the membrane proteins P50 and P100 (Henrich *et al.*, 1993).

On the host cell membrane, receptors responsible for mycoplasma attachment have been identified as sialoglycoconjugates (Razin and Jacobs, 1992; Razin *et al.*, 1998; www.mgc.ac.cn/, 2005). These are receptors for *M. pneumoniae*, *M. genitalium*, *M. gallisepticum* as well as *M. synoviae* (Razin and Jacobs, 1992). For *M. pneumoniae* as well as the other mycoplasmas there is more than one type of receptor (Razin, 1999; Rottem, 2003; www.mgc.ac.cn/, 2005). It has been found that several mycoplasma species are able to survive in nonphagocytic cells (Rottem and Naot, 1998). It is believed that mycoplasmas stay on the epithelial cell's surface, but a few that are not naturally pathogenic have evolved mechanisms to penetrate host cells (Rottem, 2003). In the case of *M. penetrans*, invasion of the host cell begins by binding to the cell surface which is followed by internalization. Immediate and intimate contact between the mycoplasma membrane and cytoplasmic membrane of the host cell is due to the absence of a rigid cell wall, and this may lead to cell fusion. Mycoplasmas requiring unesterified cholesterol for growth have fusogenic activity (Rottem and Naot, 1998; Rottem, 2003). *M. pneumoniae*, *M. genitalium*, *M. fermentans* and the poultry mycoplasma *M. gallisepticum* are, however, all known to be surface parasites (Rottem, 2003).

For a mycoplasma to survive in its host, it has to elude the immune system. One way of escaping the host's immune system, is by varying its antigenic repertoire which prevents it from being recognized which is commonly used by a variety of other pathogens as well.

Pectora roborant cultus recti

Antigenic variation includes variation by homopolymeric repeats, variation by reiterated coding sequence domains or variation by chromosomal repeats. Molecular mimicry and phenotypic plasticity are also mechanisms which guarantee that mycoplasmas are not entirely or efficiently recognized by the host's immune system (Wise, 1993; Rottem and Naot, 1998; Rottem, 2003). Although some mycoplasmas can reside intracellularly, their ability to multiply within the host cell still needs credible evidence (Rottem, 2003).

2.10.2 Interaction with the host immune system

Mycoplasma-induced specific acquired immunity as well as non-specific innate immunity is involved when a mycoplasma interacts with a host's immune system. The host's immune system can either be activated or suppressed by certain mycoplasma species. These are the actions used to evade host immune responses (Razin *et al.*, 1998; Nicolson *et al.*, 1999).

Specific mechanisms of acquired immunity include stimulation of cell-mediated immunity, production of local as well as systemic anti-mycoplasmal antibodies, and phagocytosis and opsonization of organisms. Non-specific immune reactions have an effect on cells making up the immune system. Influences include inducing B-cell differentiation, inhibiting or stimulating development of normal lymphocyte subsets; inducing cytokines which include tumour necrosis factor-α (TNFα), interferons, interleukin-1 (IL-1), IL-2, IL-4, IL-6, and granulocyte macrophage-colony stimulating factor (GM-CSF) from B-cells as well as other cell types; increasing the cytotoxicity of T cells, macrophages and natural killer cells; enhanced expression of cell receptors; and activation of the complement cascade. Mycoplasmas can also secrete soluble factors that inhibit growth and differentiation of immune competent cells or stimulate maturation (Razin *et al.*, 1998; Nicolson *et al.*, 1999).

Immune-modulating substances, for example the mycoplasmal lipoprotein spiralin, can be secreted by mycoplasmas in human and murine species. Apoptosis can also be initiated or enhanced by mycoplasmas that suppress the host immune system directly, such as the AIDS-associated mycoplasma, *M. fermentans* (Nicolson *et al.*, 1999).

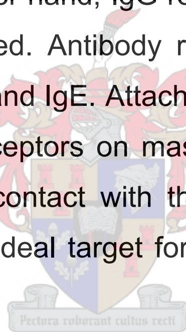
Knowledge on interactions between the avian mycoplasmas and the host immune system is very limited. It has been reported that *M. gallisepticum* can induce transient immunosuppression in turkeys infected with avian pneumovirus, and *M. meleagridis* as well as *M. iowae* can cause immunosuppression in turkeys. More recently it was shown that a

Pectora roborant cultus recti

virulent strain of *M. gallisepticum* can cause temporary T cell suppression in infected chickens (Bradbury, 2005).

The mycoplasma's ability to either suppress or stimulate the host's immune system contributes to its pathogenic properties. A chronic, persistent infection is the result of the mycoplasma being able to evade or suppress the host defence mechanism. Therefore, clinical symptoms in humans and animals are more indicative of damage due to the immune and inflammatory responses of the host itself, than to the direct toxic effects of mycoplasma cell components (Razin *et al*., 1998; Bradbury, 2005).

Responses from the major antibody classes, IgM, IgG, IgA and IgE are also elicited upon mycoplasma infection. IgM and IgG are found in the serum of infected animals and humans and could therefore be used for serodiagnosis of mycoplasma infection. In the case of IgM responses, they decline after the infection is cleared and can only be used as an indication of an active infection. On the other hand, IgG responses can remain high for a considerable time after an infection is resolved. Antibody responses due to mucosal infections in the airways are associated with IgA and IgE. Attachment of pathogens to the mucosal surface is blocked by IgA. IgE binds to receptors on mast cells which results in the local release of inflammatory substances upon contact with the mycoplasma. Therefore the tip structure which aids in attachment is the ideal target for a vaccine against mycoplasmas (Simecka, 2005).



2.10.3 Other possible virulence causal factors

Other characteristics that have been implied as virulence causal factors of mycoplasmas include (Simecka *et al*., 1992; Baseman and Tully, 1997):

(i) the cause of oxidative stress and host cell membrane damage by adhering mycoplasmas due to the generation of hydrogen peroxide and superoxide radicals;

(ii) disruption of host cell maintenance and function for competition and depletion of nutrients or biosynthetic precursors;

(iii) increased integrity of the mycoplasma surface and immunoregulatory activities due to the existence of capsule-like material and electron-dense surface layers or structures;

(iv) surface diversity and potential of escaping the host's immune defence through high-frequency phase and antigenic variation;

(v) localized tissue disruption, disorganization and chromosomal aberrations in the host cell milieu because of secretion or introduction of mycoplasmal enzymes; and

(vi) circumventing of mycoplasmicidal immune mechanisms and selective drug therapies through intracellular residence

Although mycoplasmas have multiple pathways of interactions, the tip structure, the primary adhesion organelle, is still the key to its infectivity. Without adhesion, no adaptation to host microenvironment accompanied by rapid changes in the cell surface adhesion receptor for better binding and entry as well as antigen mimicry can take place, and hence no pathogenicity (Nicolson *et al.*, 1999). For this reason, the objective of this study is to target the tip structure components as potential vaccine candidates, similar molecules to GapA in *M. gallisepticum* in the ostrich mycoplasmas are good vaccine candidates as they represent the first step in pathogenicity. The mycoplasma genome and genes involved in adhesion will therefore be discussed next.

2.11 The Mycoplasma Genome

As outlined before, mycoplasma genomes can be very small and they survive with a minimum amount of genes. In this section, characteristics that feature in all mycoplasma genomes will be discussed first as this is key to their survival. The genome of *M. gallisepticum* will be discussed thereafter in greater detail since it is a poultry mycoplasma and the research done in this project was largely based on *M. gallisepticum* and the results achieved compared to it. This will be followed by a comparison of genes that are involved in the structuring of attachment organelles, such as GapA of *M. gallisepticum* and P1 of *M. pneumoniae,* as well as membrane proteins of *M. hominis.* As mentioned before, these are very important as they enable the mycoplasma to attach to its host (Razin *et al.*, 1998).

2.11.1 General characteristics of the genome

The first large-scale attempts to sequence entire mycoplasma genomes commenced around 1990 (Razin *et al.*, 1998). The circular double-stranded mycoplasma genome is the smallest of all prokaryotes and is approximately a quarter of the size of *E. coli* (4 700 kb). Genome sizes have been found to vary from 580 to 1 350 kb. The smallest reported mycoplasma

genome is that of the human pathogen *M. genitalium* with a size of 580 kb (Herrmann, 1992; Dybvig and Voelker, 1996). The largest genome sequenced so far is that of *M. penetrans*, with a size of 1 358 kb. In *M. synoviae*, the genome size is 800 kb which is smaller than the genomes of obligate intracellular pathogens (Bencina, 2002; Papazisi *et al.*, 2003). The genome size can even vary between strains of the same species (Razin *et al.*, 1998). Eight genomes of the genus *Mycoplasma* have been sequenced successfully, and this includes those of *M. pneumoniae*, *M. genitalium*, *M. penetrans* and *M. gallisepticum* (Razin *et al.*, 1998; Binnewies *et al.*, 2005). Recently, the sequencing of the genome of *M. synoviae* has also been completed (Vasconcelos *et al.*, 2005).

It has been found that there is no correlation between the size of the genome and the average G+C content, which is in the range of 24 to 33 mol% with a few exceptions, such as *M. pneumoniae* with the highest value of 41% (Razin, 1992; Bové, 1993; Rottem and Barile, 1993). This is still low when compared to other bacteria, such as *E. coli* with a 48 to 52 mol% G+C. In the case of the poultry mycoplasmas, their mol% G+C is 31.8-35.7% for *M. gallisepticum*, 25.0% for *M. iowae*, 27.0-28.6% for *M. meleagridis* and 28% for *M. synoviae* (Herrmann, 1992; Vasconcelos *et al.*, 2005). The distribution of the G+C content in the genome is very uneven. Due to the low G+C content, the genome is exceptionally A+T rich (Rottem and Barile, 1993; Razin *et al.*, 1998).

Another characteristic of the mycoplasma genome is the fact that the structure and organization of important genes is highly conserved between different species. Thus according to Rottem and Barile (1993) groups of genes are conserved within the genome. This statement is in contradiction with Rocha and Blanchard (2002) who stated that the gene order is poorly conserved, and thus the relative position of a gene in the genome is not conserved.

The variation from the universal genetic code is also an important characteristic. UGA, which is the universal termination codon, is read as a tryptophan by mycoplasmas (Rottem and Barile, 1993). Only UAA and UAG are used as termination codons with preference to UAA (Bové, 1993; Razin *et al.*, 1998; Marin and Oliver, 2003). The start codon, AUG, is at the beginning of most of the mycoplasmal genes' coding regions, but GUG and UUG have been found as substitute start codons (Dybvig and Voelker, 1996). Codons with an A and U specifically in the wobble (3') position are favoured, but also in the first and second position (Razin *et al.*, 1998; Fadiel *et al.*, 2005). This results in fewer Gly, Pro, Ala and Arg residues in mycoplasmal proteins (Razin *et al.*, 1998).

Through genome analysis it has been found that many proteins with functions associated with catabolism and metabolite transport are encoded by mycoplasmal genes, whereas only a few anabolic proteins are encoded. This is in accordance with the fact that mycoplasmas acquire the necessary nutrients from their host and environment as a result of their limited anabolic capabilities. Pathways used in order to supply energy, their ATP synthesis as well as essential enzymes that are absent have been discussed under Morphology and Biochemistry in section 2.6. Through an approximate calculation using theoretical and experimental approaches, it was determined that the minimum number of important genes for a mycoplasma is between 265 and 350 (Papazisi *et al.*, 2003).

In the following section, more detail will be given on the genome of *M. gallisepticum* since it is a poultry mycoplasma.

2.11.2 The *M. gallisepticum* strain R_{low} genome

The complete genome of *M. gallisepticum* strain R_{low} has been sequenced and is available in GenBank under accession number AE015450. General features of the genome are illustrated in Figure 2.2 and discussed below.

The *M. gallisepticum* genome consists of 996 422 bp with a total G+C content of 31 mol%. It includes 742 reported coding DNA sequences (CDSs) which represents a 91% coding density. Only 469 of the CDSs have a function assigned to them, 150 are conserved hypothetical proteins and thus similar to genes in other bacterial species, and 123 are unique hypothetical proteins (Papazisi *et al.*, 2003; Browning and Markham, 2004). The average CDS G+C content is 32 mol% (17-45 mol%), and the average CDS length 1 206 nt (108-5 928 nt). The average of the third nucleotide position containing a G/C is 24%. Thirty-three tRNA genes were identified and they are complementary to all of the typically found twenty amino acids. As in the other mycoplasma species, only UAA and UAG are used as termination codons. Two copies of the rRNA genes are present in the genome: one set is arranged as an operon with 16S, 23S and 5S genes beside each other; and upstream of the 5S gene is a second copy of the 16S rRNA gene (Papazisi *et al.*, 2003).

The origin of replication (*oriC*) of mycoplasma genomes is believed to be supposed DnaA boxes in the area nearby the *dnaA* gene, which is the *oriC* for most bacteria. The gene order of the *oriC* region in the phylogenetic cluster, which contains *M. gallisepticum*, seems to be conserved (Papazisi *et al.*, 2003).

The *vlhA* gene family, previously termed pMGA, has the important function of generating antigenic diversity during chronic infections to make it possible for the mycoplasma to escape the host's immune system. The family contains 43 genes and makes up a total of 10.4% (103 kb) of the genome. These 43 genes are spread among five loci containing 8, 2, 9, 12 and 12 genes respectively. They are numbered in accordance with their locus and position (e.g. *vlhA*1.01). This gene family forms the largest paralogous gene family in the genome (Jan *et al.*, 2001; Papazisi *et al.*, 2003; Allen *et al.*, 2005). A change in the expression of this gene family and cytadhesin genes can affect *M. gallisepticum*'s adherence (Bencina, 2002).

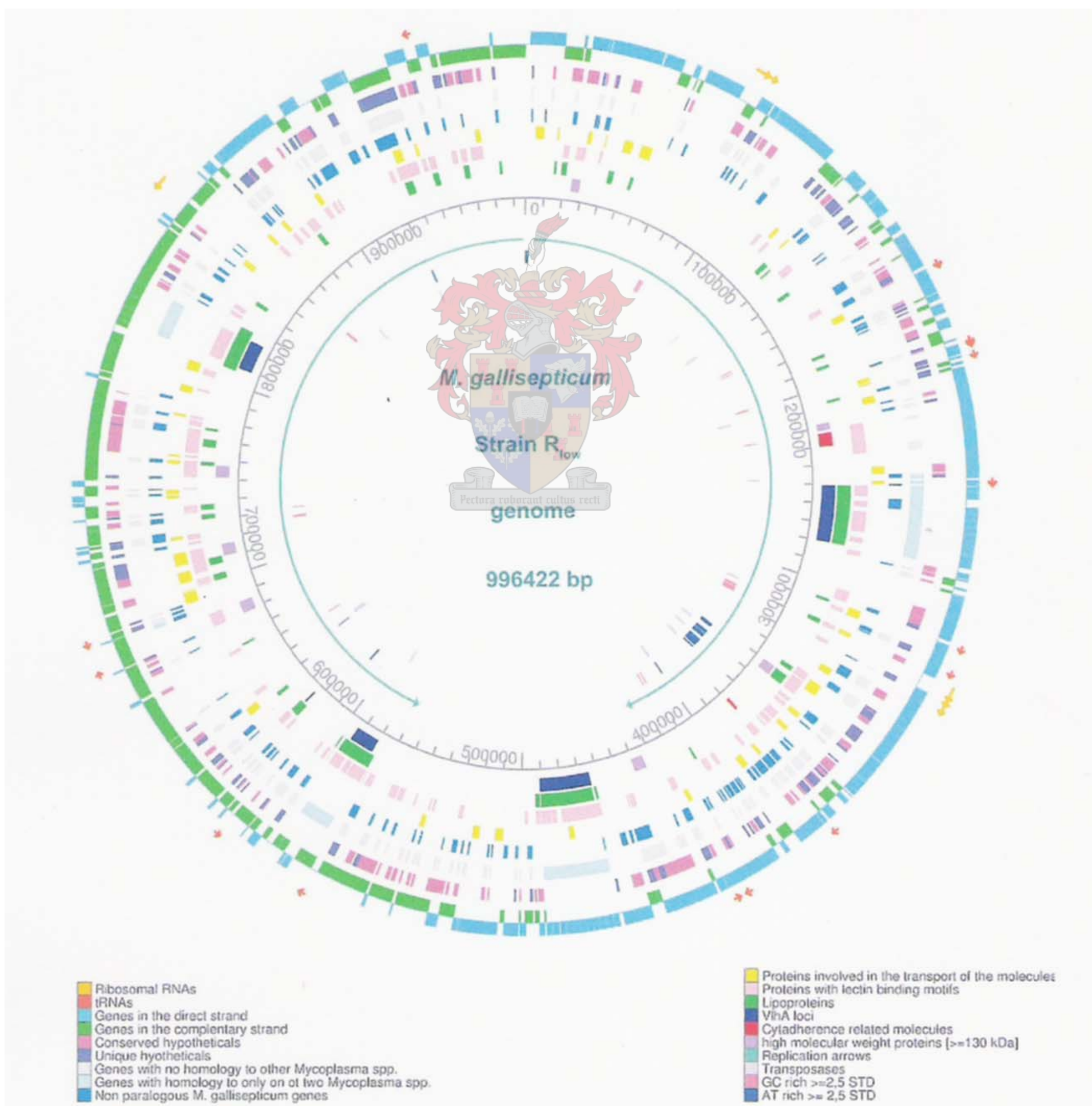


Figure 2.2 Complete genome of *M. gallisepticum* strain R_{low} (Papazisi *et al.*, 2003).

In *M. gallisepticum,* expression of both the *gapA* and *crmA* gene is necessary for cytadherence and pathogenesis (Papazisi *et al*., 2003). Through an experimental infection in chickens with different *M. gallisepticum* strains, it was found that a low (R_{low}) as well as high-passage population (R_{high}) of strain R colonizes the trachea, but only R_{low} causes air sac lesions. Their ability to invade non-phagocytic eukaryotic cells *in vitro* also differs (Winner *et al*., 2000; Much *et al*., 2002). It is also the expression of GapA that distinguishes R_{low} from R_{high} in which it is absent (Much *et al*., 2002). The *gapA* gene is the equivalent of *M. pneumoniae* cytadhesin P1, and *crmA* shows 41% amino acid homology with the ORF6 protein of *M. pneumoniae* which also plays an accessory role in cytadherence. Downstream of the *gapAcrmA* operon are two CDSs, *crmB* and *crmC*, that encode proteins possibly sharing homology to GapA and CrmA (Papazisi *et al*., 2003). Another alleged cytadhesin-related protein in *M. gallisepticum* is PvpA. This adhesin molecule is variable in size among strains and exists only as a single chromosomal copy (Boguslavsky *et al*., 2000; Liu *et al*., 2001).

A large percentage of the genome is dedicated to membrane-associated molecules. Ten percent of all CDSs are assumed to be lipoproteins normally revealed on the mycoplasma surface, and almost 20% contain multiple transmembrane domains (Papazisi *et al*., 2003; Browning and Markham, 2004). The ABC transporter molecules make up the second-largest paralogous family in *M. gallisepticum* with 24 CDSs (Papazisi *et al*., 2003).

Pectora roborant cultus recti

Although almost one-third of the genes are still undefined in terms of function, approximately 17% of the *M. gallisepticum* genes seem to be unique. Further studies into the genomics and metabolism of this pathogen will clarify the role of genes in its virulence mechanisms (Papazisi *et al*., 2003; Browning and Markham, 2004).

2.11.3 The genes and proteins involved in host cell adhesion

The poultry mycoplasmas *M. gallisepticum* and *M. synoviae*, as well as other mycoplasmas, possess the ability to adhere to their respective hosts, and this ability allows them to become pathogenic. It must be assumed that the ostrich mycoplasmas, Ms01, Ms02 and Ms03, possess adherence mechanisms to enable them to be pathogenic. As one of the objectives of this study is to identify a gene(s) in the ostrich mycoplasmas, Ms01, Ms02 and Ms03, with a role in cytadherence and possibly pathogenesis, an overview of the present knowledge of mycoplasma adherence will be given.

In the case of *M. gallisepticum*, GapA and CrmA have been identified as adhesion proteins. GapA has a definite role in adherence to host cells. GapA provides the pathogen with variable adhesive properties while it propagates, due to the phase variation it undergoes in expression. Attachment variation may encourage consecutive colonization of several hosts or of various niches in a single host (Winner *et al.*, 2003). It is a 105 kDa protein encoded by the *gapA* ORF of 2 895 bp, and is believed to be the primary cytadhesin molecule (Goh *et al.*, 1998; Mudahi-Orenstein *et al.*, 2003). It has an A+T content of 64 mol%, and a high proline content which is located primarily at the carboxyl terminus. The conformation of the polypeptide chain is possibly influenced by the proline-rich region in a way to aid the topological organization of the cytadhesin. At the amino-terminal region are two cysteine residues. The *gapA* gene exists as a single copy in all *M. gallisepticum* strains, but variation in its molecular mass has been observed (98, 105 and 110 kDa) (Goh *et al.*, 1998).

CrmA is a 116 kDa protein located downstream of the *gapA* gene and is part of the same operon. This single operon encodes two proteins that belong to the ADP1 family, which is a conserved mycoplasma adhesion family. CrmA has also been found to share 41% amino acid homology with ORF6 protein of *M. pneumoniae* which also plays an accessory role in cytadherence. On its own, neither CrmA nor GapA is adequate for cytadherence. Apparently, coexpression is essential for efficient cytadherence and virulence (Papazisi *et al.*, 2000, 2002a; Mudahi-Orenstein *et al.*, 2003). Downstream of the *gapAcrmA* operon are two CDSs, namely *crmB* and *crmC* (see Figure 2.3) which encode proteins sharing homology with GapA and CrmA (Papazisi *et al.*, 2003).

Pectora roborant cultus recti

Figure 2.3 The *gapA* operon of *M. gallisepticum*. The *gapA* gene is 3344 bp and encodes for GapA, 22 bp downstream of this is the *crmA* gene (3188 bp) encoding for CrmA. Another 162 bp downstream is the *crmB* gene (2765 bp) encoding for CrmB with the *crmC* gene (2567 bp) encoding for CrmC next to it (Papazisi *et al.*, 2003; *Mycoplasma gallisepticum* R, complete genome, NCBI accession number NC_004829).

The coexpression necessity of GapA and CrmA might be due to the lectin-like characteristics of the extracellular portions of mycoplasma cytadherence molecules. Sequence analysis has indicated that the GapA and CrmA cytoplasmic tails have features that may interact with one another at this intracellular location. The cytoplasmic tails share

critical sequence as well as structural homology with the protein family motifs and proteins involved in DNA binding and protein-protein interactions (Papazisi *et al.*, 2002a).

The human pathogen *M. pneumoniae* has a specialized tip-like attachment organelle which mediates cytadherence. The major surface adhesin P1 has a molecular mass of 170 kDa, and the adhesin-related P30 has a molecular mass of 30 kDa. P1 as well as P30 is directly involved in receptor binding, and although the accessory proteins HMW1 to HMW5 and proteins A, B, and C are not adhesins, they are required for proper functioning (Layh-Schmitt *et al.*, 2000; Chaundry *et al.*, 2005). The P1 operon, situated next to the P1 gene, consists of three open reading frames in the order ORF4-P1-ORF6 (Figure 2.4), and the gene has an A+T content of 46.5%. Two membrane proteins, 40 kDa and 90 kDa (also known as C and B respectively), are the products of the ORF6 gene. Together with HMW1-HMW3, the 40 kDa and 90 kDa proteins are required for tip structure formation as well as clustering of the P1 protein in the tip. It has been found that *M. pneumoniae* mutants lacking the membrane proteins of 40 kDa and 90 kDa form a structure which is round or ovoid making them unable to attach. As a result of this they are also avirulent (Razin and Jacobs, 1992; Ruland *et al.*, 1994; Layh-Schmitt and Harkenthal, 1999; Layh-Schmitt *et al.*, 2000).

Figure 2.4 Operon of *M. pneumoniae* surface adhesin P1. The ORF4 gene is 974 bp and is situated 13 bp upstream of the P1 gene (4883 bp) which encodes for the cytadhesin P1. The ORF6 gene (3656 bp) is situated 6 bp downstream of P1 and encodes for two membrane proteins (Razin and Jacobs, 1992; *Mycoplasma pneumoniae* M129, complete genome, NCBI accession number NC_000912).

In *M. genitalium*, another human pathogen, MgPa is the gene equivalent to the P1 adhesin. Adhesion to its host is also mediated by a specialized tip-like structure. The A+T content of the gene is 60.1% and it is organized in the genome as a three-gene operon consisting of ORF1-MgPa-ORF3 (Figure 2.5). The MgPa protein has a high molecular mass of 160 kDa, but it is smaller than P1, and ORF1 a 29 kDa protein and ORF3 a 114 kDa protein. As with P1 and GapA, the C-terminus is proline rich but cysteine is absent (Razin and Jacobs, 1992; Razin, 1999).

Figure 2.5 The MgPa operon of *M. genitalium*. In this three-gene operon, ORF1 encodes a 29 kDa protein, MgPa encodes for MgPa, and ORF3 encodes a 114 kDa protein (Razin and Jacobs, 1992).

The three above-mentioned mycoplasmas adhere with a protein-enriched tip structure as mediator, but in contrast to this, *M. hominis* has membrane proteins as adhesins (Henrich *et al.*, 1993, 1996). Two cytadhesins have been identified by Henrich and co-workers (1993), namely the membrane proteins P50 and P100. The p50 gene occurs as a single copy gene and exists in all *M. hominis* isolates. Repetitive domains A, B and C make up three-quarters of the P50 adhesin. Adherence of the organism to its host is not the only important role of P50 as this membrane protein also allows evasion of the host immune system through mutation and variation (Henrich *et al.*, 1998).

The *M. hominis* P100, which is species specific, is organized within an operon structure. It is a cysteine-anchored lipoprotein expressed as a precursor polypeptide. Four open reading frames putatively encoding the four core domains of an ABC transport system, OppBCDF, are localized downstream of P100. This suggests that the cytadherence-associated lipoprotein P100 functions as the substrate-binding domain OppA of an oligopeptide permease (Opp) of *M. hominis.* The first ORF, encoding a putative protein with homologies to OppB domains of other species, starts 15 bp downstream of P100 gene. One bp downstream of the *oppB* gene, the second ORF encodes for OppC. The third ORF encodes a protein with homologies to the ATP-binding domain OppD, and the *oppF* gene completes the cluster with an overlap of 4 bp at the 3' end of *oppD.* Figure 2.6 illustrates the physical map of the *opp* operon in *M. hominis* (Henrich *et al.*, 1999).

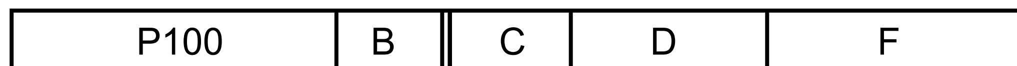


Figure 2.6 The *M. hominis opp* operon consisting of P100 and OppBCDF downstream of it. P100 is 961 amino acids; OppB is 381 amino acids; OppC is 424 amino acids; OppD is 388 amino acids and OppF is 842 amino acids respectively (Henrich *et al.*, 1999).

Although the Opp transport system of *M. hominis* shares little overall sequence similarity with the respective domains of other species, it still has the typical features namely the four core domains OppBCDF and P100 as the substrate-binding domain OppA. The homologies of OppB and OppC with other species range from 22-50%, and the two ATP-binding domains OppD and OppF show homologies of up to 41.9% with respective domains of other species. The oligopeptide-binding proteins as well as the entire oligopeptide transport system can be involved in bacterial adhesion, but this still needs further analysis (Henrich *et al.*, 1999).

In a comparison of CrmA with other mycoplasma cytadhesin-related molecules, an overall amino acid identity of 41% was revealed with *M. pneumoniae* ORF6 and *M. genitalium* MgpC. Through protein sequence analysis and hydrophobicity profiles, homology of the last 250 amino acids of the C termini of these three proteins were revealed, and they appear to be divided into two domains, namely domain A and domain B (Papazisi *et al.*, 2000).

Domain A, which represents a surface exposed region, is shared by *M. gallisepticum* CrmA, *M. pneumoniae* ORF6 and *M. genitalium* MgpC. An overall amino acid identity of 55% is shared among these cytadhesin-related molecules (Papazisi *et al.*, 2000).

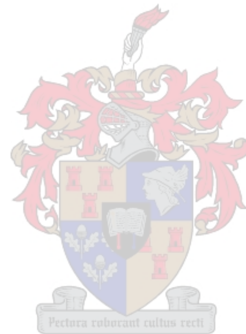
Domain B, which represents the transmembrane region and intracytoplasmic tail, shares an overall amino acid identity of 63% between *M. gallisepticum* CrmA, *M. pneumoniae* ORF6 and *M. genitalium* MgpC. This region is not only shared among the cytadhesin-related molecules, but also among other mycoplasma cytadhesins namely *M. gallisepticum* GapA, *M. pneumoniae* P1, *M. genitalium* MgPa and *M. pirum* P1-like adhesion. An overall amino acid identity of 49% is shared among all seven proteins in domain B. The high degree of sequence identity among cytadhesin-related *M. gallisepticum* CrmA, *M. pneumoniae* ORF6 and *M. genitalium* MgpC in domain A as well as in domain B, suggests a functional conservation among molecules associated with and essential for effective cytadherence in pathogenic mycoplasmas (Papazisi *et al.*, 2000).

Pectora roborant cultus recti

In the other poultry pathogen, *M. synoviae*, no adhesion related gene has been identified previously, only a 55 000 molecular weight (MW) antigen that cross-reacted with polyclonal rabbit antiserum specific for the P1 protein of *M. pneumoniae.* In an amino acid alignment with the P1 protein, a 90 amino acid portion of *M. synoviae* had 27.8% identity (Morsy *et al.*, 1993). However, recently four MgPa-like protein CDSs were identified by Vasconcelos and

co-workers (2005) in the genome of *M. synoviae*. CDSs that encode for most of the other tip organelle components were not identified (Vasconcelos *et al.*, 2005).

By using the comparisons made in the literature, it was possible to develop a strategy for the isolation of genes from the three ostrich mycoplasmas encoding for proteins involved in cytadherence. These approaches will be outlined in Chapter 3.



3. Genomic Investigations towards Vaccine Candidate Genes against Ostrich Mycoplasmas

3.1 Introduction

Three ostrich mycoplasmas have been identified in the South African ostrich, namely Ms01, Ms02 and Ms03 (Botes *et al.*, 2005a). It has been established that these mycoplasmas are pathogenic (Botes *et al.*, 2005b) and cause significant economical losses in the ostrich industry. For this reason, the development of suitable vaccines against ostrich mycoplasmas has become a primary research objective for the ostrich industry. As ostrich mycoplasmas are difficult to cultivate, and no attenuated strains are known, both live and killed vaccine approaches cannot be considered at present. Instead, the development of DNA vaccines based on membrane attachment protein genes, also referred to as cytadhesin genes, was investigated.

Possible vaccine candidate genes include the genes and proteins involved in host cell adhesion. These have been discussed in section 2.11.3 and will only be mentioned again. In the poultry mycoplasma *M. gallisepticum*, GapA has been identified as cytadhesin protein and CrmA, CrmB and CrmC as cytadhesin-related proteins (Papazisi *et al.*, 2003). In the case of *M. pneumoniae* P1 is a vaccine candidate gene (Razin and Jacobs, 1992), for *M. pirum* P1-like (Papazisi *et al.*, 2000), MgPa for *M. genitalium* (Razin and Jacobs, 1992; Razin 1999) and in the case of *M. hominis* the membrane proteins P100 oppBCDF and P50 (Henrich *et al.*, 1993) could serve as target.

In the isolation of such genes, the order of genes in the mycoplasma genome is important in an isolation strategy. If gene order was conserved, primers that bind to genes adjacent to membrane attachment protein genes could be designed, and used for their amplification and subsequent isolation. Contradictory opinions about the order of genes in the mycoplasma genome exist. Rottem and Barile (1993) stated that the structure and organization of important genes are highly conserved in the genomes of different mycoplasma species. In contradiction with this, Rocha and Blanchard (2002) stated that the gene order is poorly conserved.

Papazisi *et al.* (2000) in a study of the *M. gallisepticum* cytadhesin genes of the R_{low} and R_{high} strains, developed primers for the amplification of overlapping segments of the whole of the

gapA gene. Potentially these primers could therefore be used for the amplification of the *gapA* and related genes of other mycoplasmas.

The objective of this study was to isolate cytadhesin genes from ostrich mycoplasmas, with the eventual goal of using these genes in DNA vaccines. In the development of a strategy for the isolation of cytadhesin genes, it was important to determine whether or not the gene order of mycoplasma genomes is conserved. For this reason, gene plots were performed on the fully sequenced genomes of a number of mycoplasma species. Secondly, several primer approaches with primers for adhesins based on those designed by Papazisi *et al.* (2000) and Henrich *et al.* (1996), as well as primers that were developed from sequence alignments, were used in polymerase chain reactions (PCRs) with ostrich mycoplasma DNA. These PCR products were used for sequencing. Thirdly, some of the PCR products were cloned, and subsequently sequenced. Finally, all the generated sequences were compared to genes, and more specifically adhesin genes, of other mycoplasma species by alignment and by using the Basic Local Alignment Search Tool (BLAST) with a view to identifying the cytadhesin genes of ostrich mycoplasmas.

3.2 Materials and Methods

3.2.1 Gene order comparisons of mycoplasma genomes

Pectora roborant cultus recti

In the development of a strategy to identify cytadhesin genes or cytadhesin-related genes in the ostrich mycoplasmas Ms01, Ms02 and Ms03, it would be essential to know whether or not the order of genes is conserved within the mycoplasma genome. If the gene order is conserved, neighbouring genes can be targeted for primer binding regardless of their relatedness to adhesion genes. If no conservation is observed, the search for a gene should be limited to the adhesin operon.

In order to test this, a comparison of the gene order in different mycoplasmas was undertaken using the Gene plot tool on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/). This tool compares the order of genes of different genomes with each other, and can also be used to compare the order of genes in the genomes of different species with each other.

The genomes of *M. gallisepticum* R (poultry), *M. hyopneumoniae* 232 (pig) and *M. pulmonis* UAB CTP (human) were compared with the Gene plot tool. Only fully annotated genomes

such as the above three can be compared with the Gene plot tool. For this reason, none of the other poultry mycoplasma genomes that have been sequenced could be compared to *M. gallisepticum*. The genome of *M. gallisepticum* was compared to *M. gallisepticum* itself, and to *M. hyopneumoniae* and *M. pulmonis. M. hyopneumoniae* and *M. pulmonis* were also compared to each other since they are closely related (they fall in the same phylogenetic clade, see Figure 2.1) and therefore their gene order could be expected to be very similar.

3.2.2 Primer development

Four primer approaches were followed in this study. The mycoplasma genome is very A+T rich and therefore primers were developed with the least amount of A's and T's next to each other to minimize random annealing. All of these approaches were aimed at the amplification of genes or gene segments in adhesin gene operons. Several primer combinations were used in the PCR reactions.

Primers for the first approach were developed by Papazisi *et al.* (2000) for the amplification of *M. gallisepticum* GapA and CrmA. DAPSA was used for the alignment of mycoplasma sequences with *M. gallisepticum* domain B in order to develop primers for the second primer approach within the *gapA* domain B region as well as *crmA*. The primer developed in the third primer approach was based on the alignment of *M. synoviae* against *M. gallisepticum* GapA domain B. Two more primers, one in *M. gallisepticum* GapA and the other *M. gallisepticum* GapA domain B, were developed in the fourth primer approach.

The melting temperature (T_m) of each primer was calculated with Primer Designer (V1.01). All four primer approaches were used for the amplification of fragments from the genomes of Ms01, Ms02 and Ms03.

3.2.2.1 Primer approach 1

Papazisi *et al.* (2000) used a set of primers for the amplification of the *M. gallisepticum* GapA and CrmA genes. These primers were named A – E respectively and used in different combinations with each other. The position of primers A to E relative to *gapA* and *crmA* as well as their expected product sizes are illustrated in Figure 3.1. A summary of these primers and their relative positions are given in Table 3.1.

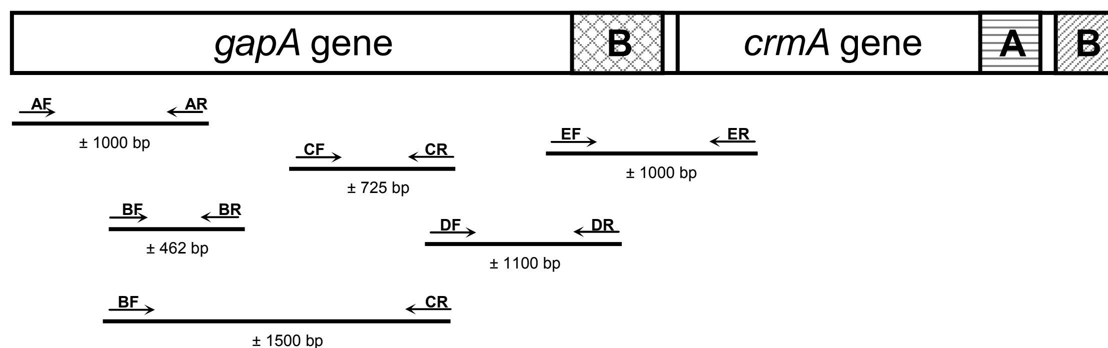


Figure 3.1 Primer approach 1: Primer pairs used for amplification of *M. gallisepticum* GapA and CrmA (Papazisi *et al.,* 2000). Primers A – E (F = forward, R = reverse) as well as combinations are illustrated relative to the genes. The expected product sizes are indicated beneath the line. The two conserved areas referred to as domain A and B, are indicated by "A" and "B" respectively.

Table 3.1 Primers A – E used in primer approach 1. Base pair positions given are relative to the *M. gallisepticum gapA* and *crmA* genes.

Primer	Sequence	bp-position	Tm (ºC)*
AF	5' AGA CCA AAC TTC CCT AAC '3	1[a]	58
AR	5' TAG TGC TGC TGG AGG AGG '3	990[a]	67
BF	5' GCC GGA TTG ATT TGT ATG '3	644[a]	64
BR	5' TC CTA CTG CTT CTA CTT CTG '3	1086[a]	60
CF	5' TGA TAA TCC TAA TGC TGT '3	1407[a]	55
CR	5' GG AAA CAC AAA ACA AGT '3	2155[a]	54
DF	5' ATT AGT AAG CCA GCT GGT '3	2137[a]	60
DR	5' CA ATG TCT CAA AAC CGT AAG '3	3452[b]	64
EF	5' TAA CGT AAT CGG TCA AGG TGC '3	3042[a]	71
ER	5' CT AAG TGA TGA TTT TGC TGG '3	4072[c]	64

*Tm calculated with Primer Designer (V1.01)

F = Forward primer, R = Reverse primer

[a]Based on gene sequence of *M. gallisepticum gapA*

[b]Based on gene sequence of *M. gallisepticum gapA* (Domain B)

[c]Based on gene sequence of *M. gallisepticum crmA*

In this primer approach these primers were used for the amplification of such fragments from the genomes of Ms01, Ms02 and Ms03.

3.2.2.2 Primer approach 2

In the second primer approach, the computer program for DNA and Protein Sequence Alignment (DAPSA) was used to align the sequences of *M. gallisepticum* GapA and CrmA, *M. pneumoniae* P1 and ORF6, *M. genitalium* MgpB and MgpC, as well as *M. pirum* P1-like (DNA sequences were retrieved from GenBank). The sequences were aligned manually with the *M. gallisepticum* GapA (domain B) on nucleotide as well as amino acid level. DAPSA was used to convert the DNA sequences to amino acid sequences. From the nucleotide and amino acid alignments, conserved areas were revealed in the cytadhesin and cytadhesin-related molecules. The conserved areas are highlighted in the amino acid alignment which is illustrated in Figure 3.2. The nucleotide alignment is added as Appendix A, and the conserved areas are also highlighted.

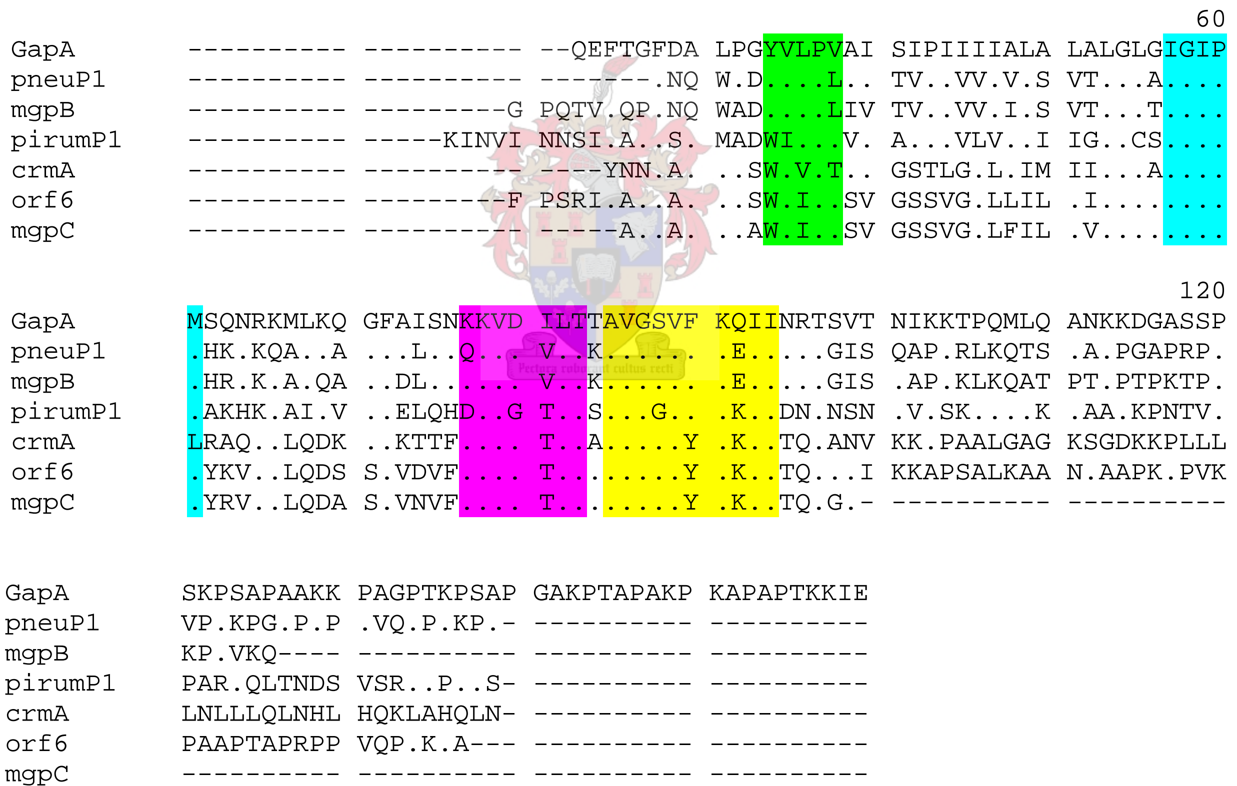


Figure 3.2 Amino acid alignment of the domain B region of mycoplasma cytadhesin as well as cytadhesin-related molecules. The computer program DAPSA was used for manual multiple sequence alignment of the protein sequences of *M. gallisepticum* GapA and CrmA (GapA and crmA), *M. pneumoniae* P1 and ORF6 (pneuP1 and orf6), *M. genitalium* MgpB and MgpC (mgpB and mgpC) and *M. pirum* P1-like (pirumP1). Primers were developed within the conserved areas which are highlighted in the alignment.

Since domain B is present in the cytadhesin as well as cytadhesin-related molecules, it could serve as a possible target in finding a gene related to adhesion in the ostrich mycoplasmas. The assumption was made that *M. gallisepticum gapA* and *crmA* are situated next to each other, and therefore primers in the *gapA* domain B region as well as *crmA* were developed for the amplification of fragments from the genomes of Ms01, Ms02 and Ms03. Two forward primers, DB1F and DB2F, were designed to potentially bind in the *M. gallisepticum* GapA domain B, and three reverse primers, DA1R, DA2R and DB3R in the *M. gallisepticum* CrmA domain A and domain B respectively.

The two forward primers were also combined with primer ER from the first approach. The position of the primers relative to *gapA* and *crmA* as well as their expected product sizes are illustrated in Figure 3.3. A summary of these primers and their relative positions are revealed in Table 3.2.

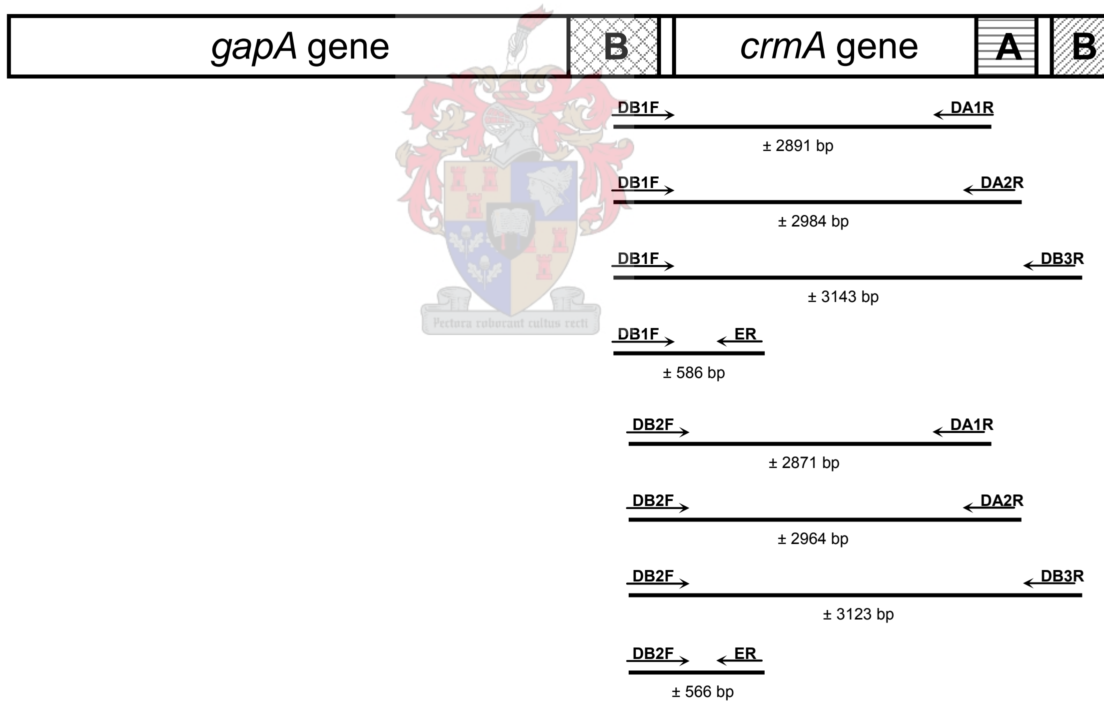


Figure 3.3 Primer approach 2: Primers developed from the nucleotide as well as amino acid alignment of mycoplasma cytadhesin and cytadhesin-related sequences. *M. gallisepticum* GapA domain B is combined with CrmA based on the assumption that they are situated next to each other. The direction of the primer pairs as well as expected product sizes are illustrated (F = forward, R = reverse). The two conserved areas referred to as domain A and B, are indicated by “A” and “B” respectively.

Table 3.2 Sequence of the primers used in primer approach 2, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes.

Primer	Sequence	bp-position	Tm (ºC)*
DB1F	5' AA(A/G) GTT GAT (A/G)(T/C)(T/C/G/A) (C/T)TG AC(A/C/T) '3	3506[b]	51
DB2F	5' GC(C/G/A/T) GTT GGT AGT GT(G/C/T) '3	3536[b]	56
DA1R	5' ATT AGC (A/T)GG (A/G)GT GAA '3	6382[d]	47
DA2R	5' CAT CTA AGT A(T/C)T (C/G)GA TC '3	6472[d]	39
DB3R	5' TA(A/T) (A/T)GG (A/G)AT (A/T/C)CC (G/A)AT '3	6634[e]	48

*Tm calculated with Primer Designer (V1.01)

F = Forward primer, R = Reverse primer

[b]Based on gene sequence of *M. gallisepticum gapA* (Domain B)

[d]Based on gene sequence of *M. gallisepticum crmA* (Domain A)

[e]Based on gene sequence of *M. gallisepticum crmA* (Domain B)

In this primer approach these primers were used for the amplification of such fragments from the genomes of Ms01, Ms02 and Ms03.

3.2.2.3 Primer approach 3

This approach was an extension of primer approach 1. A reverse primer in the area between EF and DR, but still in domain B (see Figure 3.1), was required in order to potentially obtain a smaller and single product. Therefore, for the third primer approach, *M. synoviae* sequences were used since it is related to Ms02 (from the phylogenetic relationship, Figure 2.1) and the 55 000 MW antigen cross-reacted with antiserum for *M. pneumoniae* P1 (see page 50). It was aligned manually against *M. gallisepticum* GapA and *M. gallisepticum* GapA domain B using DAPSA. The nucleotide alignment of the domain B region is illustrated in Figure 3.4.

```
                                                                    60
GapA       ATGGCGAATA CGTTGCTGTT CCACAAGCTA ATAGTGTGTT TGTGTCTGAC ----------
GapADB     .......... .......... .......... .......... .......... ----------
Synoviae   .......... .......... .......... .......... .......... ----------

                                                                   120
GapA       ---------- --CAAGAATT TACTGGTTTT GATGCGCTTC CAGGTTATGT ATTACCAGTA
GapADB     ---------- --........ .......... .......... .......... ..........
Synoviae   ---------- --........ .......... .......... .......... ..........

                                                                   180
GapA       GCGATCTCGA TTCCGATCAT CATAATTGCC TTGGCATTAG CTTTAGGTCT AGGTATTGGT
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   .......... ...-...... .......... .......... .......... .......---

                                                                   240
GapA       ATTCCAATGT CTCAAAACCG TAAGATGTTG AAACAAGGAT TTGCGATTTC AAACAAAAAA
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   ---------- ---------- ---------- ---------- ---------- ----------

                                                                   300
GapA       GTTGATATTC TGACAACAGC CGTTGGTAGT GTGTTCAAAC AAATTATTAA TCGAACATCT
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   ---------- ---------- ---------- ---------- ---------- ----------

                                                                   360
GapA       GTGACAAATA TTAAGAAGAC YCCACAAATG CTTCAAGCCA ACAAGAAAGA TGGAGCATCT
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   ---------- ---------- ---------- ---------- ---------- ----------

                                                                   420
GapA       TCACCAAGCA AGCCATCAGC TCCAGCTGCT AAGAAACCAG CAGGACCAAC TAAACCATCT
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   ---------- ---------- ---------- ---------- ---------- ----------

                                                                   480
GapA       GCTCCAGGGG CAAAACCAAC AGCACCAGCT AAACCAAAAG CTCCAGCACC AACTAAGAAA
GapADB     .......... .......... .......... .......... .......... ..........
Synoviae   ---------- ---------- ---------- ---------- ---------- ----------

GapA       ATTGAATAA
GapADB     ......---
Synoviae   ---------
```

Figure 3.4 Nucleotide alignment of *M. synoviae* (Synoviae) against *M. gallisepticum* GapA and domain B (GapA and GapADB respectively). The computer program DAPSA was used, a . indicates a match and _ no match. Only domain B is illustrated, and the region showing limited homology is highlighted in yellow.

The nucleotide alignment showed that there is limited homology between *M. gallisepticum* GapA domain B and *M. synoviae*, as well as in the region before domain B. Based on the sequence of *M. synoviae*, primer E2R was developed. This primer is more or less halfway in the area between the beginning of domain B and primer DR. Its position relative to

gapA and *crmA* as well as expected product size in combination with EF is illustrated in Figure 3.5. The relative positions of these primers are shown in Table 3.3.

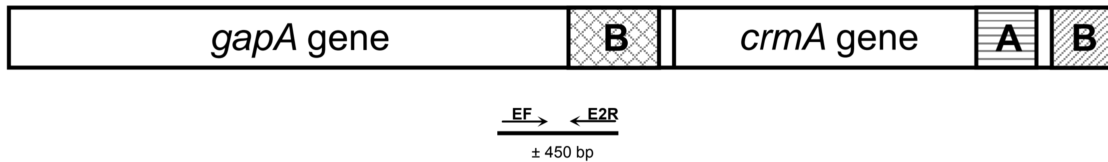


Figure 3.5 Primer approach 3: Primer E2R was developed for the area between EF and DR, but still in *M. gallisepticum* GapA domain B. This primer is based on the sequence of *M. synoviae* which shares homology with Ms02 as well as *M. gallisepticum* domain B. The expected product size in combination with EF is illustrated (F = forward, R = reverse). The two conserved areas referred to as domain A and B, are indicated by "A" and "B" respectively.

Table 3.3 Sequence of primer E2R developed for primer approach 3 and primer EF, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes.

Primer	Sequence	bp-position	Tm (ºC)*
EF	5' TAA CGT AAT CGG TCA AGG TGC '3	3042[a]	71
E2R	5' CGG AAT CGA GAT CGC TAC TG 3'	3383[b]	71

*Tm calculated with Primer Designer (V1.01)

F = Forward primer, R = Reverse primer

[a]Based on gene sequence of *M. gallisepticum gapA*

[b]Based on gene sequence of *M. gallisepticum gapA* (Domain B)

In this primer approach these primers were used for the amplification of such fragments from the genomes of Ms01, Ms02 and Ms03.

3.2.2.4 Primer approach 4

Based on the sequence alignment of *M. synoviae* with *M. gallisepticum* GapA that was done for primer approach three, two new primers were developed, namely E2F and E3R. The forward primer, E2F, is situated in the *M. gallisepticum* GapA region before EF. The reverse primer, E3R, is situated before E2R but still in domain B since it is a conserved area. These primers were used in combination with EF and E2R, and their positions relative to *gapA* and *crmA* as well as expected product size is illustrated in Figure 3.6. A

summary of the two primers developed for this approach, primers E2F and E3R, and their relative positions is given in Table 3.4.

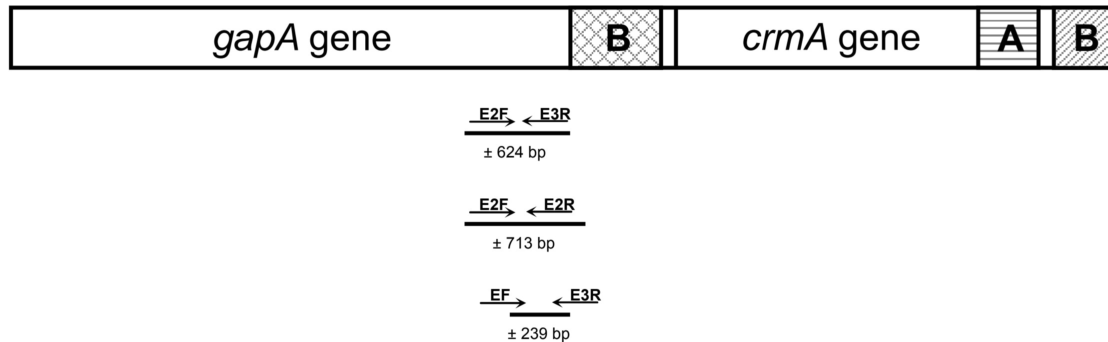


Figure 3.6 Primer approach 4: Primers E2F and E3R were developed from the alignment of *M. synoviae* with *M. gallisepticum* GapA. Domain B is still included in the primer area since it is a conserved area. These primers were also combined with primers EF and E2R (F = forward, R = reverse). The expected product sizes are also illustrated. The two conserved areas referred to as domain A and B, are indicated by "A" and "B" respectively.

Table 3.4 Sequence of primers developed for primer approach 4, as well as their base pair positions relative to the *M. gallisepticum gapA* and *crmA* genes.

Primer	Sequence	bp-position	Tm (ºC)*
E2F	5' GCG CTT ACT TAT CAT CAA CTG G '3	2660[a]	70
E3R	5' GTG GAA CAG CAA CGT ATT CG '3	3294[a]	69

*Tm calculated with Primer Designer (V1.01)

F = Forward primer, R = Reverse primer

[a]Based on gene sequence of *M. gallisepticum gapA*

In this primer approach these primers were used for the amplification of such fragments from the genomes of Ms01, Ms02 and Ms03.

3.2.3 Isolation of genomic DNA

For the isolation of genomic DNA from ostrich mycoplasma-containing solid agar, the *N*-cetyl-*N,N,N*-trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987), originally developed for the extraction of genomic DNA from fresh plant tissue, was used. To the mycoplasma-containing agar, 500 µl of 2 x CTAB buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% v/v, CTAB; 0.2%, v/v, 2-mercaptoethanol) was added

and incubated at 60 ºC for 1 h. After incubation, 500 µl chloroform-isoamylalcohol (24:1, v/v) was added and mixed gently for 10 min followed by centrifugation at 7 000 x g for 5 min. The upper aqueous phase was removed and a 2/3 volume of cold isopropanol was added to this and mixed gently. To allow the precipitation of nucleic acids, the sample was incubated overnight at -20 ºC. The sample was subsequently centrifuged at 3 000 x g for 2 min. After the supernatant was decanted, the pellet was resuspended in 1.5 ml wash buffer (40 mM ammonium acetate:absolute ethanol, 1:3) and incubated at room temperature for 20 min. The incubation was followed by centrifugation at 3 000 x g for 1 min, after which the supernatant was once again decanted and the pellet air-dried to remove any ethanol. The DNA pellet was finally redissolved overnight at 4 ºC in 250 µl TE-buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).

Using this procedure, genomic DNA of Ms01, Ms02 and Ms03 was isolated. The identity of these mycoplasmas was confirmed by 16S rRNA sequencing (Botes *et al.*, 2005a). This also ensured that contamination with any other mycoplasma DNA could be excluded. As this DNA was subsequently used for PCR amplification of adhesin genes using primers based on *M. gallisepticum* sequences, it would have been advantageous to have used *M. gallisepticum* DNA as a positive control. However, *M. gallisepticum* does not infect ostriches, for which reason it could not be obtained from our collaborators at the Klein Karoo Group. The Western Cape Regional Veterinary Laboratory in Stellenbosch (Department of Agriculture, Western Cape) was approached to obtain a *M. gallisepticum* culture from them. Unfortunately, they could not supply this material, as there is very strong control over *M. gallisepticum* infection in poultry as a result of which it is rarely isolated. Although this may be a serious disadvantage in the primer approaches, the lack of a positive control was not viewed to be essential for the initial investigations.

3.2.4 PCR amplification

For each primer combination, amplification reactions were carried out in 20 µl volumes. Table 3.5 summarises the master mix for each primer pair as well as annealing temperature and PCR program that was used. For each primer combination reaction, 2 µl 10 x Reaction Buffer (RB, JMR-Holdings, USA) was used and the reaction volume increased to 20 µl with deionized water. Each PCR amplification reaction contained 2 µl DNA sample from Ms01, Ms02 or Ms03. In the case of primer pair E2F+E3R, the DNA sample was diluted 10 x with sterilised MilliQ water. All the primers were synthesized by the DNA Synthesis Laboratory, Department of Molecular and Cellular Biology, University of Cape Town. The

deoxynucleotides (dATP, dGTP, dCTP and dTTP) were supplied by Advanced Biotechnologies Ltd., UK, and the $MgCl_2$ as well as Super-therm Taq polymerase by JMR-Holdings, USA.

Table 3.5 Summary of master mix for individual primer combinations. PCR amplification reactions were carried out in 20 µl volumes. In addition to products in the table, each reaction contained 2 µl 10 x RB Buffer, 2 µl DNA sample and the reaction volume increased to 20 µl with deionized water.

Primer combination	Annealing temp (ºC)	PCR program	dNTP		Primer		$MgCl_2$ (mM)	Taq (U)
			µM	µl/20µl	pmol/µl	µl/20µl		
Primer approach 1								
AF+AR	46+GRA 13	GapA1	250	4.0	20	0.4	2.5	1.5
BF+BR	45+GRA 14	GapA1	250	0.5	20	0.4	2.0	1.5
CF+CR	40+GRA 6	GapA1	250	4.0	20	0.4	2.5	1.5
DF+DR	45+GRA 15	GapA1	250	4.0	20	0.4	2.5	1.5
EF+ER	50+GRA 4	GapA1	250	4.0	20	0.4	2.5	1.5
BF+CR	40+GRA 8	GapA1	250	4.0	20	0.4	2.5	1.5
EF+DR	59+GRA 7	Domain	200	0.8	20	0.4	2.0	1.0
Primer approach 2								
DB1F+DA1R	37.9	Domain	200	0.8	20	0.4	2.0	1.0
DB1F+DA2R	41.9	Domain	200	0.8	20	0.4	2.0	1.0
DB1F+DB3R	35.0	Domain	200	0.8	20	0.4	2.0	1.0
DB2F+DA1R	37.7	Domain	200	0.8	20	0.4	2.0	1.0
DB2F+DA2R	41.9	Domain	200	0.8	20	0.4	2.0	1.0
DB2F+DB3R	35.0	Domain	200	0.8	20	0.4	2.0	1.0
DB1F+ER	42.0	Domain	200	0.8	20	0.4	2.0	1.0
DB2F+ER	44.7	Domain	200	0.8	20	0.4	2.0	1.0
Primer approach 3								
EF+E2R	30+GRA 10	GapA1	250	1.0	30	0.4	4.0	1.6
Primer approach 4								
E2F+E3R	37.0	GapA2	200	0.8	20	0.4	4.0	1.0
E2F+E2R	34.0	GapA2	200	0.8	20	0.4	4.0	1.0
EF+E3R	36.0	GapA2	200	0.8	20	0.4	4.0	1.0

Pectora roborant cultus recti

All the amplification reactions were performed in a P x 2 Thermal Cycler (Hybaid). In cases where GRA is indicated in the annealing temperature column, a gradient was set. This enabled the optimization of the amplification of DNA from Ms01, Ms02 and Ms03 in the same cycle since their annealing temperatures differed only slightly. The three PCR programs that were used, namely GapA1, Domain and GapA2, are summarised in Table 3.6.

Table 3.6 PCR programs used in DNA amplification reactions for Ms01, Ms02 and Ms03. The annealing temperature (ºC) for each primer combination is given in Table 3.6.

PCR program	Stage	Temperature (ºC)	Time	Cycles
GapA1	1	94.0 (see table 3.6) 72.0	30 sec 30 sec 1 min	35
	2	72.0 15.0	6 min *Hold*	1
Domain	1	94.0 (see table 3.6) 72.0	45 sec 45 sec 1.5 min	35
	2	72.0 15.0	6 min *Hold*	1
GapA2	1	95	5 min	1
	2	94.0 (see table 3.6) 72.0	30 sec 30 sec 1 min	35
	3	72.0 15.0	6 min *Hold*	1

3.2.5 Detection of PCR products

Agarose gel electrophoresis was used to analyse the amplified DNA. Of each PCR product, 10 µl was mixed with a 0.1 volume of gel loading buffer (50% glycerol; 0.1% v/v bromophenol blue; 50 mM EDTA; 100 mM Tris-base, pH 8.0) and separated on a 2% agarose gel (Molecular Grade Agarose D1-LE, Whitehead Scientific) in 1 x TAE buffer (Tris-base; glacial acetic acid; 0.5 M EDTA, pH 8.0). Ethidium bromide (0.175 µg/ml) was included in the gel for ultraviolet (UV) visualization of the DNA.

3.2.6 Cloning of PCR products

With the various primer approaches with the ostrich mycoplasmas as outlined before, more than one PCR product was amplified in many instances. As a result of this, it was difficult to determine the sequence of a cytadhesin or cytadhesin-related gene in these mycoplasmas directly using direct sequencing of PCR products. In order to overcome this problem, the PCR products from each of the primer approaches were used for cloning. A convenient vector for the cloning of PCR products is the pGEM-T Easy vector (Figure 3.7; Promega).

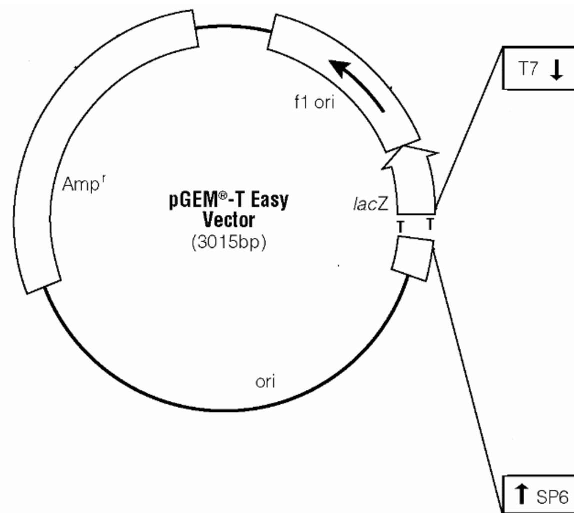


Figure 3.7 The pGEM-T Easy Vector circle map used for cloning of PCR products. The transcription initiation site of T7 is at bp position 1 and that of SP6 at bp position 141. The T7 promoter (-17 to +3) is from bp position 2999-3 and the SP6 promoter (-17 to +3) from bp position 139-158 (www.promega.com/vectors/).

The high copy number pGEM-T Easy vector contains two RNA polymerase promoters, T7 and SP6, besides a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Successful insertion of a PCR product inactivates the α-peptide coding sequence and colonies containing the insert can be identified directly by colour screening on indicator plates. Once the plasmid DNA has been isolated, the T7 and SP6 promoter primers (Table 3.7) can be used for the sequencing of the cloned insert.

Pectora roborant cultus recti

Table 3.7 T7 and SP6 promoter primers used for sequencing of cloned inserts. The bp-position is that of the pGEM-T Easy vector.

Primer	Sequence	bp-position
T7	5' TAA TAC GAC TCA CTA TAG GG '3	2999-3
SP6	5' ATT TAG GTG ACA CTA TAG AA '3	139-158

Since there is only one insert per vector, the problem of multiple products as well as poor sequencing was eliminated.

3.2.6.1 Ligation of PCR product into pGEM-T Easy Vector

For the ligation reaction, a specific insert:vector molar ratio was not used, but rather two definite volumes of PCR product since the concentration of many of the PCR products were very low after amplification. One µl (which is the same as a 1:1 ratio) and 3 µl of PCR product were used in two separate ligation reactions. The ligation reaction for the standard reactions, positive control, as well as background control, is shown in Table 3.8.

Table 3.8 Protocol for the ligation reaction of standard reactions for cloning PCR products into pGEM-T Easy Vector (Promega), as well as positive control and background control. Ligation reactions were incubated overnight at 4 ºC.

Reaction Components	Standard reaction		Positive control	Background control
	1 µl DNA	3 µl DNA		
2x Rapid Ligation Buffer	5 µl	5 µl	5 µl	5 µl
pGEM T-Easy Vector (50 ng/µl)	1 µl	1 µl	1 µl	1 µl
PCR product	1 µl	3 µl	-	-
Control Insert DNA (4 ng/µl)	-	-	2 µl	-
T4 DNA Ligase	1 µl	1 µl	1 µl	1 µl
Deionized water	2 µl	-	1 µl	3 µl
Final volume	10 µl	10 µl	10 µl	10 µl

All the ligation reactions were incubated overnight at 4 ºC to ensure maximal ligation.

Pectora roborant cultus recti

3.2.6.2 Transformation of E. coli *with ligation products*

For each ligation reaction, two Luria-Bertani (LB) plates (10 g Bacto-tryptone; 5 g Bacto-yeast extract; 5 g NaCl; in 1 l deionized water; pH 7) with agar (15 g agar/1 l LB medium) were prepared. These plates also contained ampicillin (100 µg/ml; Ampicillin (D [-]-α-Aminobenzylpenicillin) sodium salt, SIGMA), isopropyl ß-D-thiogalactopyranoside (IPTG, 0.1 M; used at 160 µl per 100 ml LB medium; Promega) and X-gal (50 mg/ml; 100 mg 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Promega) dissolved in 2 ml N,N'-dimethyl-formamide; used at 80 µl per 100 ml LB medium). The prepared LB/ampicillin/IPTG/X-Gal plates were kept at room temperature while the transformation was performed.

JM 109 (*E. coli*) high efficiency competent cells (Promega) were used for transformation of the ligation reactions. The JM 109 cells were removed from -80 ºC storage and thawed on ice before transferring 50 µl to a sterile polypropylene tube (e.g. 17 x 100 mm Falcon tube) on ice for each ligation reaction. After centrifuging the tubes containing the ligation

reactions, 2 µl of each ligation reaction was added to a tube with JM 109 cells and mixed by gently flicking the tube. Incubation on ice for 20 min was followed by heat-shocking the cells for 45-50 sec in a water bath at 42 ºC, and immediately returning the tubes to ice for another 2 min. LB medium (950 µl, room temperature) was added to the tubes containing cells transformed with ligation reactions and incubated at 37 ºC for 1.5 h while shaking (200 rpm). A volume of 50 µl and 150 µl of each transformation mixture with a standard reaction was plated onto the LB/ampicillin/IPTG/X-gal plates respectively. In the case of the positive control and background control transformations, 100 µl was plated out in duplicate onto the plates. The plates were incubated overnight (16-24 hours) at 37 ºC, followed by further incubation at 4 ºC to facilitate blue colour development. A successful transformation was indicated by a white colony. Plates were stored at 4 ºC afterwards.

3.2.6.3 Diagnostic PCR

A relatively quick method of testing for a successful insertion the pGEM plasmid in a white colony is a diagnostic PCR. This was done by using a toothpick scrape of the colony in a PCR amplification reaction. A blue colony was used as a negative control. Amplification of the possible insert using primer pair T7 and SP6 was carried out in 10 µl volumes. Each reaction mixture consisted of 1 µl 10 x RB, 0.4 µl of 5 mM of each deoxynucleotide (dATP, dTTP, dCTP and dGTP), 0.5 µl of each primer (20 pmol/µl), 0.6 µl $MgCl_2$, 0.1 µl of Super-therm Taq polymerase (0.02 units), 6.9 µl deionized water and a toothpick scrape of the selected colony.

The amplification was performed in a P x 2 Thermal Cycler programmed to preheat for 5 min at 94 ºC. This was followed by 25 cycles of 94 ºC (30 sec), 55 ºC (30 sec) and 72 ºC (30 sec), followed by a final extension reaction for 7 min at 72 ºC. Detection of the PCR product and hence cloned insert was analyzed by loading 10 µl of the PCR reaction onto a 2% agarose gel. Gel electrophoresis was performed as described in section 3.2.5.

3.2.6.4 Overnight culture of recombinant colonies

After visualization of the diagnostic PCR gel under UV light and confirmation of the cloning of the PCR product, colonies were selected to be cultured overnight. The recombinant colonies with an insert, as well as a blue colony as negative control, were inoculated into a 17 x 100 mm Falcon tube containing 5 ml LB medium and 5 µl ampicillin (100 µg/ml). This was incubated overnight (± 16 h) at 37 ºC while shaking at 200 rpm.

3.2.6.5 Isolation of recombinant plasmid DNA

For isolation of the recombinant plasmid DNA from the overnight culture, the Plasmix minipreps Protocol B (Talent), which is a plasmid DNA purification system from 1-3 ml of bacterial culture, was used according to the manufacturer's instructions. The isolated plasmid DNA samples were each concentrated to 15-20 µl by centrifugal evaporation on a Savant Speedvac. Only 1 µl of the plasmid DNA was loaded onto a 2% agarose gel and analyzed by gel electrophoresis (as described in section 3.2.5) in order to ensure that plasmid DNA was isolated successfully.

3.2.6.6 Insert check PCR

In order to verify the size of the insert DNA, a PCR amplification reaction was done with the isolated plasmid DNA. This was necessary since the PCR product that was used for cloning, sometimes had multiple bands as a product. A 10 µl volume PCR amplification reaction was carried out using primer pair T7 and SP6. Each reaction mixture consisted of 1 µl 10 x RB, 0.4 µl of 5 mM of each deoxynucleotide (dATP, dTTP, dCTP and dGTP), 0.5 µl of each primer (20 pmol/µl), 0.6 µl $MgCl_2$, 0.1 µl of Super-therm Taq polymerase (0.02 units), 5.9 µl deionized water and 1 µl of a 1000 x diluted isolated plasmid DNA.

Amplification was performed in a P x 2 Thermal Cycler programmed to preheat for 5 min at 94 ºC. This was followed by 25 cycles of 94 ºC (30 sec), 55 ºC (30 sec) and 72 ºC (30 sec), followed by a final extension reaction for 7 min at 72 ºC. Detection of the isolated plasmid DNA was analyzed by loading 10 µl of the PCR reaction onto a 2% agarose gel. Gel electrophoresis was performed as described in section 3.2.5, but the gel was stained afterwards with ethidium bromide for 20 min in order to visualize them under the UV light since the bands were sometimes very faint.

3.2.7 Sequencing

3.2.7.1 Sequencing of PCR products

For sequencing of PCR products, the PCR products were electrophoresed on a 2% agarose gel for 2 h at 150 V in 1 x TAE buffer containing ethidium bromide as described previously. DNA containing bands in the expected product size area were excised under a UV light. The Wizard SV Gel and PCR Clean-Up System (Promega) was used according to the manufacturer's instructions to purify the DNA from the excised band.

Sequencing reactions were carried out in 10 µl reaction volumes using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequencing reactions were done for each sample, one with the forward primer and the other with the reverse primer. Each sequencing reaction mixture contained 5 µl 5 x Sequencing buffer, 2 µl Terminator mix, 1 µl primer (0.8 pmol/µl), 0.5 µl, 1 µl or 1.5 µl DNA depending on the intensity of the band on the gel, and filled up to 10 µl with deionized water.

Amplifications were performed in a P x 2 Thermal Cycler programmed to perform 35 cycles of 96 ºC (10 sec), 52 ºC (30 sec) and 60 ºC (4 min), followed by a final extension reaction for 10 min at 60 ºC. Analysis of the sequencing PCR reaction products were performed on an ABI PRISM® 373 DNA Sequencer at the DNA sequencing facility of the University of Stellenbosch.

3.2.7.2 Sequencing of isolated plasmid DNA

Isolated plasmid DNA, which was the final product of the cloning procedure, was also used for sequencing. A 10 µl sequencing reaction contained 4 µl Terminator mix, 3 µl of primer T7 (3.3 pmol/µl) and 3 µl isolated plasmid DNA.

Amplifications were performed in a P x 2 Thermal Cycler programmed to perform 35 cycles of 96 ºC (10 sec), 52 ºC (30 sec) and 60 ºC (4 min), followed by a final extension reaction for 10 min at 60 ºC. Analysis of the isolated plasmid DNA sequencing products were performed on an ABI PRISM® 373 DNA Sequencer at the DNA sequencing facility of the University of Stellenbosch.

3.2.8 Analysis of sequences

Sequences of the PCR products as well as isolated plasmid DNA, were compared to each other using the DNA and Protein Sequence Alignment (DAPSA) program (Harley, 1998). In the case of sequences of the isolated plasmid DNA, the vector sequences were trimmed off the ends. The automatic alignment function was used, but manual alignment of sequences was used to refine the alignments. Sequences were also aligned manually with the following sequences: *M. gallisepticum* GapA, domain A and domain B of *M. gallisepticum* CrmA, domain B of *M. gallisepticum* GapA, *M. pneumoniae* P1 as well as *M. synoviae*.

3.2.9 Comparison of mycoplasma sequences using BLAST

The BLAST search engine, which is available on-line on the NCBI website (http://www.ncbi.nlm.nih.gov/blast), was used for sequence similarity searches. Several BLAST search programs, each with a different search strategy, are available. For this study, BLASTN, which compares a nucleotide query sequence against a nucleotide sequence database, as well as TBLASTX, which compares a translated nucleotide query sequence against a translated nucleotide sequence database, was used. These searches were done in order to see whether any of the generated sequences of Ms01, Ms02 or Ms03 showed similarity to other mycoplasma species, especially with cytadhesin or cytadhesin-related genes of avian mycoplasmas.

One of the most important parameters in a BLAST search is the Expect (E)-value. This indicates the statistical significance of an alignment between the query sequence and a sequence in a database. The default E-threshold setting is 10, which means that for a particular query, all possible alignments for which 10 or less hits of similar bit score are expected to occur by chance in a database of similar size will be returned in the search. A bit score reflects the length of the alignment between a query sequence and a sequence in a database.

The E-value of a particular match is dependent on the bit score and the size of the database. The lower the E-value, the more likely it is that the alignment did not occur randomly, but reflects true sequence similarity. In most cases, results with E-values higher than 0.1 as well as bit scores lower than 50, are not regarded to reflect statistically significant sequence similarity.

The BLASTN 2.2.12 program was used with the nr database (all non-redundant GenBank+EMBl+DDBJ+PDB nucleotide sequences, excluding EST, STS, GSS or HTGS sequences), comparisons were made with all organisms and the default Expect (E) value threshold was 10 for all searches. Default search settings were used throughout.

A TBLASTX 2.2.12 search was also done with all the sequences of Ms01, Ms02 and Ms03. The nr database and genetic code 4, which include the Mycoplasma/Spiroplasma code, was used to translate the query. The Expect (E) value was changed to 1 and Blosum62 was selected as matrix option since it is the best for detecting weak protein similarities.

For the BLASTN as well as TBLASTX results, comparisons with an E value higher than 0.1 and a bit score lower than 50 were not regarded as statistically significant.

3.3 Results

The results that were obtained during the study in order to find a vaccine candidate gene(s) related to cytadhesion in the three ostrich mycoplasmas will be discussed next.

3.3.1 Gene order comparisons of mycoplasma genomes

From the results from Gene plot it is clear that a straight line will be produced if the gene order is homologous in the genomes compared, as illustrated in Figure 3.8 A where *M. gallisepticum* was compared and plotted against itself. The operon which includes the cytadhesin genes GapA, CrmA, CrmB and CrmC is situated in the area where the two grey lines cross. In the case where the genome of *M. gallisepticum* was compared to the genomes of *M. hyopneumoniae* and *M. pulmonis* (Figure 3.8 B and C respectively), the dots were placed largely at random. This indicates that there was no homology in the arrangement of genes between these genomes. However, the order of the GapA, CrmA, CrmB, CrmC operon remained the same, but the order of adjacent genes differed. The genes that are positioned in the area where the two grey lines cross represent the GapA operon, but the rest of the genome does not have the same gene order.

Pectora roborant cultus recti

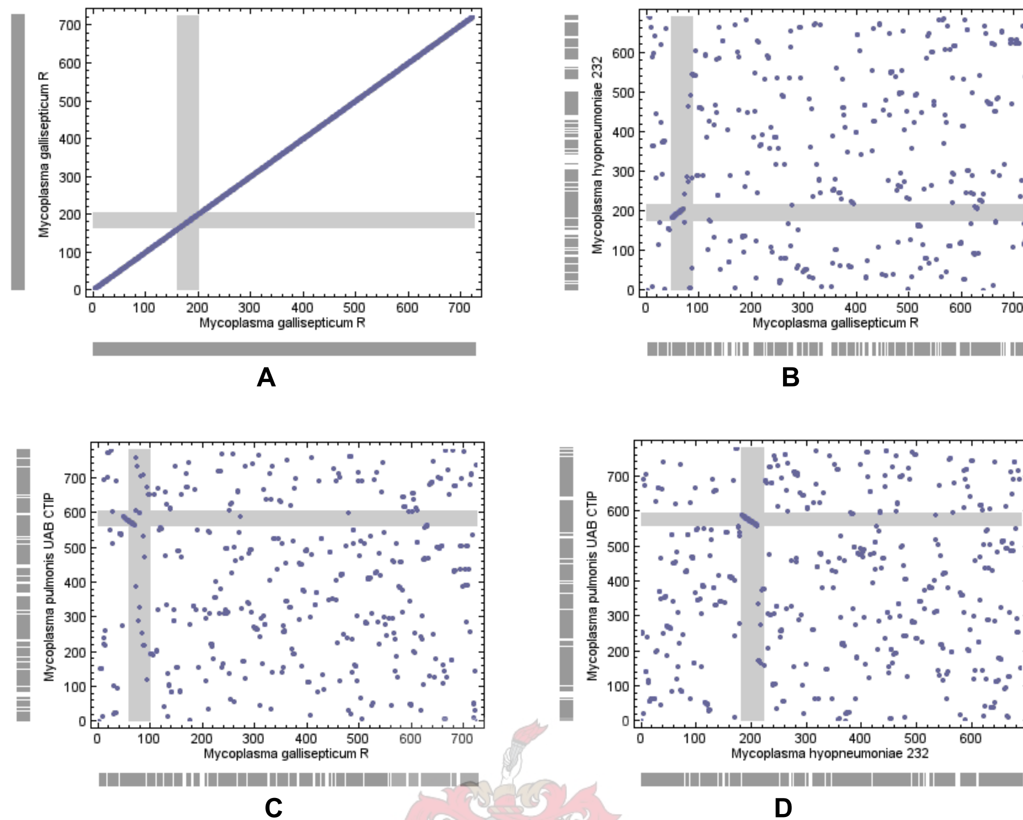


Figure 3.8 Comparison of mycoplasma genomes using the Gene plot tool on the NCBI website. A: *M. gallisepticum* R versus *M. gallisepticum* R; B: *M. gallisepticum* R versus *M. hyopneumoniae* 232; C: *M. gallisepticum* R versus *M. pulmonis* UAB CTP; D: *M. hyopneumoniae* 232 versus *M. pulmonis* UAB CTP.

In the comparison of the genomes of *M. hyopneumoniae* and *M. pulmonis* (Figure 3.8 D) only random dots were largely observed. This indicates that although they are placed in the same clade, their genome order was not conserved. This analysis therefore supports the conclusion of Rocha and Blanchard (2002) that the gene order is poorly conserved in mycoplasma genomes. Thus, even though the operon encoding for proteins related to cytadhesin was conserved, its position in the mycoplasma genome was not conserved. Based on these results, it was assumed that the three ostrich mycoplasmas would also not show a conserved gene order. Therefore adjacent genes should not be used as targets, but rather genes “within” the operon, for example a cytadhesin gene such as GapA and a cytadhesin-related gene such as CrmA of *M. gallisepticum*.

3.3.2 PCR amplification

The gene approaches that were followed included several primer combinations. For each primer approach that was followed, the sequence of the primer, annealing temperature

used, as well as base pair position relative to the *M. gallisepticum gapA* or *crmA* gene will be given. Subsequently, the PCR amplification results obtained with genomic DNA for Ms01, Ms02 as well as Ms03 will be given.

3.3.2.1 Primer approach 1

Primers A – E as well as combinations of these primers were used for the first primer approach. For each primer combination, a certain product size was expected. However, this size was not always obtained with Ms01, Ms02 or Ms03. The amplification products that were obtained with PCR reactions for Ms01, Ms02 and Ms03 are summarized in Table 3.9. A gel electrophoresis example of some of the products that were amplified using these primers is shown in Figure 3.9.

Table 3.9 Expected amplification products as well as actual amplification products obtained with primers A – E for primer approach 1. Primers were also used in combination with each other. A 100 bp DNA ladder was loaded onto the gel to determine the size of the amplification product.

Primer combination	PCR Product size (bp)			
	Expected	Ms01	Ms02	Ms03
AF+AR	± 1100	-	-	-
BF+BR	± 462	-	-	-
CF+CR	± 725	> 1500 ± 1500 ± 700 300-400	± 1500 1000-1500 ± 700 ± 500 ± 350	± 1500 ± 350
DF+DR	± 1100	± 1500 ± 900	-	± 1500 1000-1500 ± 900 300-400
EF+ER	± 1000	-	-	-
BF+CR	± 1500	> 1500 1000-1500 ± 900 500-600	± 900	± 1500 ± 900 ± 600 ± 500
EF+DR	± 430	> 1500 ± 1500 ± 900	-	> 1500

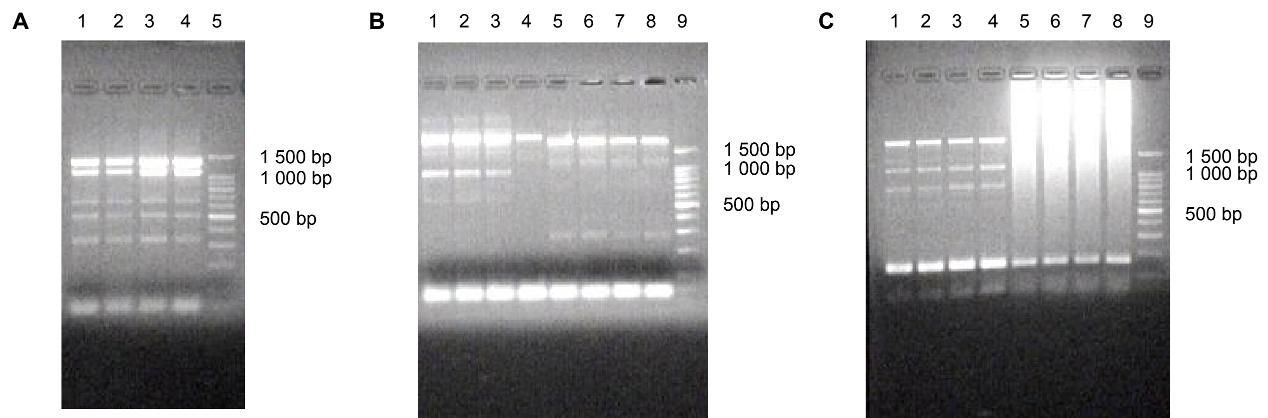


Figure 3.9 Gel electrophoresis of amplification products during optimisation of PCR reactions for primer approach 1. A: Primer combination CF+CR; lanes 1-2: Ms02 at 42.5 ºC, lanes 3-4: Ms02 at 43.3 ºC. B: Primer combination DF+DR; lanes 1-4: Ms01 at 49.4 ºC – 51.4 ºC; lanes 5-8: Ms03 at 45.1 ºC – 46.3 ºC. C: Primer combination BF+CR; lanes 1-4: Ms01 at 44.4 ºC – 45.5 ºC; lanes 5-8: smears of Ms02 generated as product of non-optimised PCR reaction. A 100 bp DNA ladder was loaded onto the last well of each gel.

From Table 3.9 and Figure 3.9 it is clear that the expected product size was not always obtained with Ms01, Ms02 or Ms03. For primer combinations AF+AR, BF+BR and EF+ER no amplification products were produced. Primer combinations DF+DR and EF+DR only produced products with Ms01 and Ms03, but none with Ms02. Only primer combinations CF+CR and BF+CR produced products for all three ostrich mycoplasmas. In most instances multiple products were produced, which did not always include the expected product size.

Pectora roborant cultus recti

From the first primer approach, the amplification products of Ms01 used for cloning, were CF+CR, ± 700 bp and BF+CR, ± 900 bp. In the case of Ms02, only products of primer combination CF+CR were used for cloning, namely ± 1500 bp and ± 700 bp. The amplification product of Ms03 of primer combination BF+CR, ± 1500 bp was used for cloning. Although there were more PCR products with the different primer combinations all of them could not be used for cloning, as some PCR products had a too low concentration. Other PCR products were not of the expected size and therefore not appropriate for cloning. The ± 900 bp of BF+CR of Ms02 was cloned in spite of the fragment not being ± 1 500 bp in size, because it was the only amplification product obtained.

3.3.2.2 Primer approach 2

For the second primer approach, primers DB1F, DB2F, DA1R, DA2R and DB3R were used. Primer ER from the first primer approach was also used in combination with the two forward primers.

As with the first primer approach, a certain product size was expected for each primer combination. However, these sizes were not always obtained using Ms01, Ms02 or Ms03 DNA as a template. The amplification products that were obtained with PCR reactions from Ms01, Ms02 and Ms03 are summarized in Table 3.10. Figure 3.10 illustrates the gel electrophoresis of some of the obtained amplification products.

Table 3.10 Expected amplification products as well as actual amplification products obtained with primers used in primer approach 2. A 100 bp DNA ladder was loaded onto the gel to determine the size of the amplification product.

Primer combination	PCR Product size (bp)			
	Expected	Ms01	Ms02	Ms03
DB1F+DA1R	± 2891	± 1500 ± 1000 ± 600 300-400	± 1500 ± 1000 ± 600	800-900
DB1F+DA2R	± 2984	-	-	-
DB1F+DB3R	± 3143	± 1000	-	-
DB2F+DA1R	± 2871	± 1500 900-1000 ± 500	± 1500 ± 1000 ± 900 ± 700 ± 500	1000-1500 ± 500
DB2F+DA2R	± 2964	-	-	-
DB2F+DB3R	± 3123	-	-	-
DB1F+ER	± 586	> 1500 ± 1500 ± 800 ± 500	1000-1500 ± 900 700-800 500-600	> 1500 1000-1500 ± 700
DB2F+ER	± 566	± 1500 700-800 500-600	± 1500 ± 900	± 1500

Pectura roborant cultus recti

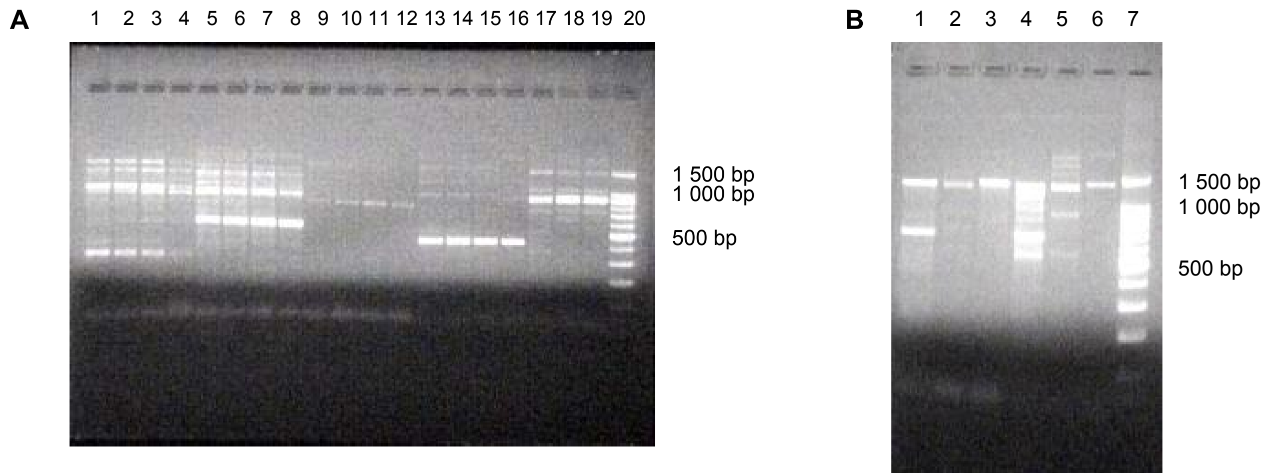


Figure 3.10 Gel electrophoresis of amplification products during optimisation of PCR reactions for primer approach 2. A: a temperature gradient of 37.3 ºC – 44.2 ºC was used in both primer combinations; lanes 1-12: primer combination DB1F+DA1R; lanes 1-4: Ms01; lanes 5-8: Ms02; lanes 9-12: Ms03; lanes 13-19: primer combination DB2F+DA1R; lanes 13-16: Ms01; lanes 17-19: Ms02. B: lanes 1-3: primer combination DB1F+ER at 40.9 ºC; lane 1: Ms01; lane 2: Ms02; lane 3: Ms03; lanes 4-6: primer combination DB2F+ER at 44.7 ºC; lane 4: Ms01; lane 5: Ms02; lane 6: Ms03. A 100 bp DNA ladder was loaded onto both gels.

The expected product sizes from the primer combinations used for primer approach 2 were quite large in four cases (Table 3.10). From Table 3.10 and Figure 3.10 it can be seen that the results obtained with Ms01, Ms02 and Ms03 were unsatisfactory when compared to the expected results. A possible explanation for this is that primer combinations which work in one mycoplasma genome would not necessarily work as well in another mycoplasma genome. Another possible reason is that the degeneracy of the primers lead to mispriming especially since mycoplasmas are very A+T rich. The third reason may be that the product sizes were underestimated because a ladder with a maximum product size of 1 500 bp was used. For primer approach 2, primer combinations DB1F+DA1R and DB2F+DA1R amplified products with all three ostrich mycoplasmas, but none were of the expected size. Primer combination DB1F+DB3R only amplified a product with Ms01, and primer combinations DB2F+DA2R and DB2F+DB3R amplified no products with the ostrich mycoplasmas.

When the two forward primers were used separately in combination with primer ER from the first primer approach, the expected product sizes were much smaller and PCR products were amplified from all three ostrich mycoplasmas. Amplification products of Ms01, Ms02 as well as Ms03 were used for cloning and these include the following for Ms01: for primer combination DB1F+ER products of ± 1 500 bp, ± 800 bp and ± 500 bp were used, and for primer combination DB2F+ER a 500-600 bp product was used. In

spite of the fragments for DB1F+ER not being the expected ± 586 bp in size, these were the only amplification products obtained. Cloning of products of Ms02 included the following: for primer combination DB2F+DA1R ± 500 bp and for primer combination DB1F+ER products of 1 000-1 500 bp and ± 900 bp were used. In the case of Ms03, products 1 000-1 500 bp and ± 500 bp from primer combination DB2F+DA1R, as well as products of 1 000-1 500 bp from primer combination DB1F+ER were used for cloning.

3.3.2.3 Primer approach 3

In the third primer approach, primer E2R was developed and used in combination with primer EF in order to potentially obtain a smaller and single product. A single PCR product of ± 450 bp was expected for this primer combination. The products that were amplified with Ms01, Ms02 and Ms03 are summarized in Table 3.11. Gel electrophoresis of the amplified DNA of Ms01 and Ms03 is illustrated in Figure 3.11.

Table 3.11 Amplification products expected as well as products obtained from primer combination for primer approach 3. A 100 bp DNA ladder was loaded onto the gel to determine the size of the amplification product.

Primer combination	PCR Product size (bp)			
	Expected	Ms01	Ms02	Ms03
EF+E2R	± 450	> 1500 1000-1500 ± 800 650-700 550-600 400-500	-	1000-1500 ± 900 800-900 700-800 600-700 ± 500

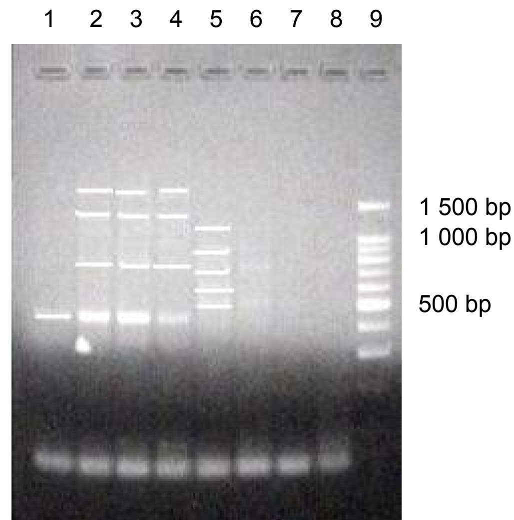


Figure 3.11 Gel electrophoresis of amplification products for primer approach 3 with DNA from Ms01 and Ms03 using primer combination EF+E2R. The annealing temperature ranged from 30.0 ºC – 40.1 ºC to optimize PCR conditions. Lanes 1-4: Ms01; lanes 5-8: Ms03; lane 9: 100 bp DNA ladder. Some of the bands were intensified in order to make them more visible in the photo.

In Table 3.11 it can be seen that products were only amplified with Ms01 and Ms03, but none with Ms02. These amplification products can be seen in Figure 3.11. Although a single, smaller PCR product was not amplified for Ms01 or Ms03, a product in the range of 450 bp was amplified with both. However, the ± 500 bp product obtained with Ms03 was very faint and thus had a low concentration, and was therefore not suitable for cloning. Re-amplification of the ± 500 bp product with Ms03 was also unsuccessful since the concentration was still too low for cloning. In the case of Ms01, products of 400-500 bp, 650-700 bp as well as ± 800 bp were used for cloning.

3.3.2.4 Primer approach 4

With the final primer approach, the aim was to amplify regions within domain B since it is a conserved area. The forward primer, E2F, was used in combination with primer E2R from the third primer approach, and the reverse primer, E3R in combination with forward primer EF from primer approach 1. The amplification products of Ms01, Ms02 and Ms03 with the primer combinations are summarized in Table 3.12. Gel electrophoresis of the amplification products at 36 ºC are illustrated in Figure 3.12.

Table 3.12 Expected amplification products as well as products amplified with primer combinations used in primer approach 4. A 100 bp DNA ladder was loaded onto the gel in order to determine the size of the amplification product.

Primer combination	PCR Product size (bp)			
	Expected	Ms01	Ms02	Ms03
E2F+E3R	± 624	> 1500 ± 1500 ± 1000 ± 800 ± 600 300-400	1000-1500 ± 1000	1000-1500 1000-1500 900-1000 ± 600 400-500
E2F+E2R	± 713	± 700 ± 500	-	> 1500 700-800
EF+E3R	± 239	> 1500 1000-1500 ± 1000 ± 900 800-900 200-300	± 1500 ± 900 700-800 ± 500	± 1000 ± 700 500-600

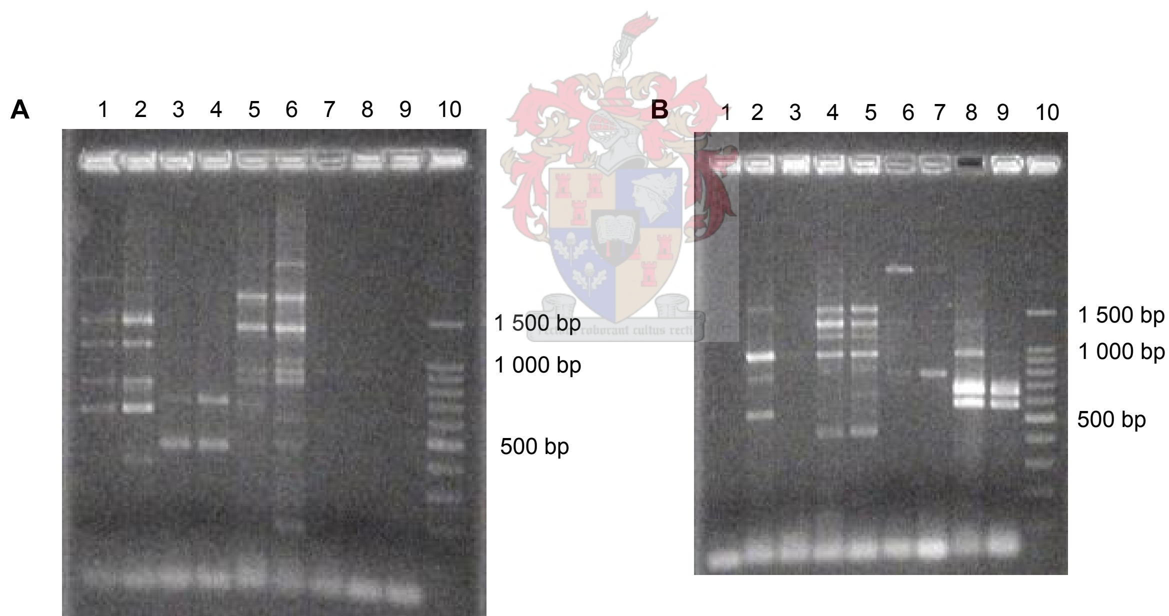


Figure 3.12 Gel electrophoresis of amplification products during optimisation of PCR reactions at 36 ºC for primer approach 4. A: lanes 1-2: E2F+E3R, Ms01; lanes 3-4: E2F+E2R, Ms01; lanes 5-6: EF+E3R, Ms01; lanes 7-8: E2F+E3R, Ms02; lane 9: E2F+E2R, Ms02, lane 10: 100 bp DNA ladder. B: lane 1: E2F+E2R, Ms02; lanes 2-3: EF+E3R, Ms02; lanes 4-5: E2F+E3R, Ms03; lanes 6-7: E2F+E2R, Ms03; lanes 8-9: EF+E3R, Ms03; lane 10: 100 bp DNA ladder.

Amplification products were obtained with Ms01, Ms02 as well as Ms03 for primer combinations E2F+E3R and EF+E3R (Figure 3.12). In the case of primer combination EF+E3R the obtained products were much larger than the expected product size. Primer

combination E2F+E2R only amplified products with Ms01 and Ms03 (see Table 3.12). With primer combination E2F+E3R, a product of ± 600 bp was amplified with Ms01 which is close to the expected product size of ± 624 bp. This product was then used for cloning. Since products conforming to the expected sizes of amplification products were amplified repeatedly with Ms01, the focus was shifted to Ms01 and therefore none of the other products of Ms02 and Ms03 were used for cloning at this time.

3.3.3 Cloning of PCR products

Since more than one PCR product was amplified in many instances, the PCR products of the four primer approaches that were used for cloning are summarised in Table 3.13.

Table 3.13 Summary of the PCR products of the four primer approaches that were used for cloning with the pGEM-T Easy Vector System. In most cases, the final product of the cloning procedure, namely isolated plasmid DNA, was used for sequencing.

	Mycoplasma	**Primer pair**	**Product size (bp)**
Primer approach 1	Ms01	BF + CR	± 900
		CF + CR	± 700
	Ms02	CF + CR	± 1500
		CF + CR	± 700
	Ms03	BF + CR	± 1500
Primer approach 2	Ms01	DB1F + ER	± 1500
		DB1F + ER	± 800
		DB1F + ER	± 500
		DB2F + ER	500-600
	Ms02	DB1F + ER	1000-1500
		DB1F + ER	± 900
		DB2F + DA1R	± 700
		DB2F + DA1R	± 500
	Ms03	DB1F + ER	1000-1500
		DB2F + DA1R	1000-1500
		DB2F + DA1R	± 500
Primer approach 3	Ms01	EF + E2R	± 800
		EF + E2R	650-700
		EF + E2R	550-600
		EF + E2R	400-500
	Ms02	-	-
	Ms03	-	-
Primer approach 4	Ms01	E2F + E3R	± 600
	Ms02	-	-
	Ms03	-	-

In most of the cases, cloning of the PCR products of all four primer approaches was successful. From plates containing cloned inserts of Ms01, Ms02 and Ms03, white colonies

were randomly selected to perform diagnostic PCRs. In most instances, a single bright PCR band was observed, and compared to the diagnostic PCR of a blue colony – which has no insert – an increase in size indicated that an insert was present in the clone. The white colonies with an insert from Ms01, Ms02 and Ms03, were then cultured overnight, after which the plasmid DNA was isolated. The isolated plasmid DNA was then used in an insert check PCR in order to determine if the size of the insert was approximately the size of the original PCR product. Figure 3.13 is an example of the gel electrophoresis after an insert check PCR was performed using primers T7 and SP6. Successful (lanes 1-3, 5, 7 and 8) as well as unsuccessful (lanes 4, 6 and 9) cloning with DNA from a PCR product is illustrated.

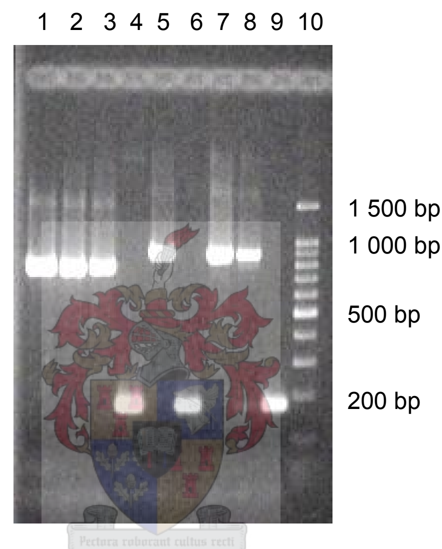


Figure 3.13 Gel electrophoresis of insert check PCR using primers T7 and SP6. DNA from Ms01, primer combination EF+E2R was used in this cloning reaction. A 400-500 bp PCR product (lanes 1-3) and a 650-700 bp PCR product (lanes 4-9) was used in the ligation reaction. Products of successful cloning, lanes 1-3, 5, 7 and 8, were subsequently used in sequencing reactions. Lanes 4, 6 and 9 indicate unsuccsessful cloning and only the vector area between T7 and SP6 (170 bp) was amplified. A 100 bp DNA ladder was used to estimate the product sizes.

It was expected that the isolated plasmid DNA of Ms02 or Ms03 would give better PCR results than that of Ms01 since they are more closely related to the poultry mycoplasmas. However, the insert check PCR results with Ms01's cloned DNA were better since the band intensity was brighter and the results could be repeated. In the case of Ms02 and Ms03, the band intensity of the insert check PCR product of the plasmid DNA was either very low, or the PCR product was absent. This was probably due to the low concentration of the DNA that was used for cloning. For the purpose of sequencing, most of the isolated plasmid DNA products of Ms01 were used, but only those of Ms02 and Ms03 of which the insert check PCR product, with primers SP6 and T7, could still be seen clearly.

3.3.4 Alignment of sequences

For all the mycoplasma DNA that was submitted for sequencing, either from a PCR product or a cloning product, a printout of the sequence was also requested. This printout was used as a quick method to determine whether the sequence was “good” or “bad”. A “good” sequence was identified by the correct short cloning vector sequence at the start and the end of amplification product, by the identification of the primer pair at the both ends of the amplification product that was used and by the length of the sequence which had to correspond to the expected insert size and by mostly single peaks i.e. unambiguous base calling. On the other hand, a sequence in which the correct short cloning vector sequence at the start and the end of amplification product could not be identified, or in which both primers could not be identified at the ends of the amplification product, or of which the amplification product was not of the correct size, or in which there were significant numbers of ambiguities were regarded as a “bad” sequence and these sequences were not analysed further. In a number of instances inserts were detected in which only one primer could be identified, or in which the forward and reverse primer had simply joined to each other by apparent blunt end fusion, or in which vector sequences were largely present, and these were rejected.

Alignment of the “good” sequences was done with the computer program DAPSA. All alignments were done manually, since the automatic alignment of the unknown sequences with known sequences was unsatisfactory which was in all likelihood the result of a lack of even short stretches of identical sequence. For alignment purposes the following sequences of other mycoplasma species were available from GenBank: *M. gallisepticum* GapA (whole sequence), *M. gallisepticum* CrmA, *M. gallisepticum* GapA domain B, *M. pneumoniae* P1, *M. pneumoniae* ORF 6, *M. synoviae*, *M. genitalium* MgpB, *M. genitalium* MgpC and *M. pirum* P1-like.

After editing of the ostrich mycoplasma sequences of Ms01, Ms02 and Ms03 to remove cloning vector sequences, alignment with the above sequences with other mycoplasma species was undertaken. Sequences were mostly aligned with *M. gallisepticum* GapA, CrmA as well as domain B of GapA since it is one of the most important poultry mycoplasmas in which the adhesion genes have been identified. With the manual alignment of the sequences many spaces had to be inserted into the unknown sequence in order to align with *M. gallisepticum*, especially GapA and CrmA. As a result of this, the ostrich mycoplasma sequences were cut up into short sequences with many deletions in between, but without these deletions the percentage sequence similarity was very low (under 40%).

This was a problem with the sequences of Ms01, Ms02 as well as Ms03. For this reason, additional alignments were done in order to compare the ostrich mycoplasma sequences not only with *M. gallisepticum*, but also with other mycoplasma species. For this purpose a BLAST search was done and will be discussed in the next section. (Refer to Appendix A for sequence alignment example)

3.3.5 Sequence analysis of cloned DNA fragments using BLAST

BLAST searches, which included a BLASTN as well as TBLASTX, were performed using the sequences obtained directly from PCR fragments and from the cloned DNA fragments. Sequences generated directly from PCR products of Ms01, Ms02 and Ms03 only gave hits with the primers used in the amplifications. When the primer sequences were trimmed from the sequences, no significant hits with other mycoplasma species were found. Sequences from the cloned DNA fragments in which the functional part could easily be isolated were subsequently used in the BLAST searches. Firstly, only sequences generated with primer pairs CF+CR, EF+E2R and E2F+E3R from Ms01 were used in the searches. A total of 71 sequences from Ms01 were used in the searches. Sequences generated with primers from Ms02 and Ms03 were all identified as "bad" sequences (see page 82 for definition) and were therefore not used in the BLAST searches.

Pectora roborant cultus recti

The settings that were used for the BLASTN and TBLASTX searches are summarised in section 3.2.9. In both searches, several mycoplasma species had sequences which showed similarity to the different sequences generated from Ms01. Although Ms01 is not related to poultry mycoplasmas, it was hoped that the sequences would align with any of the adhesin or adhesin-related genes of the mycoplasma species, or one of the poultry mycoplasmas. In the BLASTN searches, only *M. synoviae* was hit with sequences of PCR products generated with primers CF+CR, with sequences of PCR products generated with primers EF+E2R only *M. gallisepticum* was hit, and with sequences of PCR products generated with primers E2F+E3R, the poultry mycoplasmas *M. synoviae* and *M. gallisepticum* were hit. In both cases where *M. gallisepticum* sequences were hit, the alignment was not regarded as significant. In the case of the TBLASTX search with sequences of PCR products generated with primers CF+CR as well as primers EF+E2R *M. synoviae* sequences were hit, and with sequences of PCR products generated with primers E2F+E3R *M. synoviae* as well as *M. gallisepticum* sequences were hit. Once again the hits with *M. gallisepticum* were not significant. None of the hits were with *M. gallisepticum* GapA or CrmA although most of the primers were developed from their gene sequences.

A summary of the mycoplasma species that produced a significant alignment with the BLASTN search with sequences of Ms01 is given in Table 3.14. The best mycoplasma alignments with the TBLASTX search are summarised in Table 3.15. Among the non-poultry mycoplasma species that were hit was *M. hominis* (human pathogen), *M. mobile* (fish mycoplasma), *M. pulmonis* (rats and mice as host) and *M. hyopneumoniae* (swine mycoplasma).

Table 3.14 Summary of significant hits of Ms01 with Mycoplasma species with BLASTN search.

Primer	Sequence	Query (letters)	*Mycoplasma* sequence producing significant alignment	Score (bits)	E-value	Identities (%)
CF&CR	1C00001F	659	M. hominis P100, oppB, oppC, oppD, oppF genes	145.0	2.00E-31	139/161 (86%)
	1C00001F	659	M. synoviae 53 complete genome	50.1	0.009	49/57 (85%)
EF&E2R	1E00025F	582	M. gallisepticum strain R section 1 of 4 of the complete genome	46.1	0.12	23/23 (100%)
	1E00025F	582	M. gallisepticum cytadhesin (gapA) pseudogene, complete genome	46.1	0.12	23/23 (100%)
E2F&E3R	1T7	822	M. mobile 163K complete genome	85.7	2.00E-13	106/127 (83%)
	1T7	822	M. pulmonis (strain UAB CTIP) complete genome, segment 1/3	77.8	5.00E-11	54/59 (91%)
	3T7	365	M. mycoides subsp. mycoides SC genomic DNA, complete sequence; segment 1/4	77.8	2.00E-11	51/55 (92%)
	5T7	365	M. synoviae 53, complete genome	83.8	3.00E-13	69/78 (88%)
	7T7	847	M. hyopneumoniae 232, complete genome	79.8	1.00E-11	90/104 (86%)
	7T7	847	M. hyopneumoniae J, complete genome	71.9	3.00E-09	89/104 (85%)

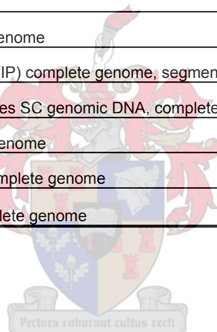


Table 3.15 Summary of most significant hits of Ms01 sequences with Mycoplasma species with the TBLASTX search.

Primer	Sequence	Query	*Mycoplasma* sequence producing significant alignment		Score	E-value	Identities
		(letters)	Organism	Features in sequence	(bits)		(%)
CF&CR	1C00004F	654	M. hominis P100, oppB, oppC, oppD, oppF genes		380	1.00E-102	159/201 (79%)
	1C00004F	654	M. synoviae 53 complete genome	Oligopeptide ABC transporter, ATP-binding protein	279	2.00E-72	121/187 (64%)
	1C00004F	654	M. pulmonis (strain UAB CTIP) complete genome; segment 2/3	Oligopeptide ABC transporter, ATP-binding protein	167	2.00E-65	70/124 (56%)
	1C00004F	654	M. mobile 163K complete genome	Bidomainal protein	175	9.00E-65	74/137 (54%)
	1C00004F	654	M. hyopneumoniae 7448, complete genome	Oligopeptide ABC transporter, ATP-binding protein	164	1.00E-62	67/127 (52%)
EF&E2R	1E00005F	458	M. mobile 163K complete genome	Predicted kinase	53.3	5.00E-13	20/41 (48%)
	1E00005F	458	M. penetrans HF-2 DNA, complete genome	Fatty acid-phospholipid synthesis protein	56.1	1.00E-05	28/82 (34%)
	1E00012F	459	M. mycoides subsp. Mycoides SC genomic DNA, complete sequence; segment 2/4	Fatty acid/ phospholipid synthesis PlsX	60.6	5.00E-07	27/51 (52%)
E2F&E3R	1T7	822	M. hyopneumoniae 7448, complete genome	Glucose inhibited division protein A	318	1.00E-93	137/198 (69%)
	1T7	822	M. hyopneumoniae J, complete genome	Glucose inhibited division protein A	317	3.00E-93	136/198 (68%)
	6T7	810	M. synoviae 53, complete genome	Glucose inhibited division protein A	321	7.00E-93	131/202 (64%)
	6T7	810	M. hyopneumoniae 232, complete genome	Glucose inhibited division protein A	319	5.00E-92	137/198 (69%)
	6T7	810	M. pulmonis (strain UAB CTIP) complete genome; segment 1/3	Glucose inhibited division protein A	318	8.00E-91	131/198 (66%)
	7T7	847	M. mobile 163K complete genome	Glucose inhibited division protein A	291	5.00E-76	123/201 (61%)
	19T7	302	M. gallisepticum strain R section 3 of 4 of the complete genome	GidA	69.4	6.00E-10	38/77 (49%)
	2T7	818	M. synoviae 53, complete genome	Tyrosyl tRNA synthetase	98.2	9.00E-26	41/83 (49%)
	2T7	818	M. mycoides subsp. Mycoides SC genomic DNA, complete sequence; segment 3/4	Tyrosine-tRNA ligase	102	2.00E-19	52/151 (34%)
	2T7	818	Mycoplasma pulmonis (strain UAB CTIP) complete genome; segment 2/3	Tyrosyl tRNA synthetase1 (TYRRS 1)	81.3	5.00E-18	37/91 (40%)
	2T7	818	M. mobile 163K complete genome	Tyrosyl tRNA synthetase	71.6	3.00E-16	33/82 (40%)
	2T7	818	M. hyopneumoniae 7448, complete genome	Tyrosyl tRNA synthetase	84.0	1.00E-13	36/84 (42%)

Table 3.15 (continued)

Primer	Sequence	Query (letters)	*Mycoplasma* sequence producing significant alignment: Organism	Features in sequence	Score (bits)	E-value	Identities (%)
E2F&E3R	5T7	365	M. pulmonis (strain UAB CTIP) complete genome; segment 2/3	Elongation factor G (EF-G)	165	4.00E-38	66/112 (58%)
	5T7	365	M. penetrans HF-2 DNA, complete genome	Elongation factor G (EF-G)	159	2.00E-36	66/112 (58%)
	5T7	365	M. gallisepticum strain R section 3 of 4 of the complete genome	FusA	141	5.00E-31	60/114 (52%)
	20T7	366	M. hyopneumoniae 7448, complete genome	Elongation factor G (EF-G)	163	5.00E-38	69/112 (61%)
	20T7	366	M. hyopneumoniae J, complete genome	Elongation factor G (EF-G)	163	5.00E-38	69/112 (61%)
	20T7	366	M. hyopneumoniae 232, complete genome	GTP-binding protein chain elongation factor ef-g	163	5.00E-38	69/112 (61%)
	20T7	366	M. mycoides subsp. Mycoides SC genomic DNA, complete sequence; segment 1/4	Translation elongation factor G	152	9.00E-35	64/112 (57%)
	9T7	605	M. synoviae 53, complete genome	Conserved hypothetical protein	51.5	5.00E-04	23/57 (40%)
	16T7	602	M. gallisepticum strain R section 3 of 4 of the complete genome	Conserved hypothetical	54.2	7.00E-05	24/57 (42%)
	8T7	819	M. synoviae 53, complete genome	Endonuclease IV	130	5.00E-52	50/69 (72%)
	8T7	819	M. hyopneumoniae 7448, complete genome	Endonuclease IV	114	6.00E-42	45/70 (64%)
	8T7	819	M. hyopneumoniae J, complete genome	Endonuclease IV	112	2.00E-41	43/70 (61%)
	8T7	819	M. hyopneumoniae 232, complete genome	Endonuclease IV	112	2.00E-41	43/70 (61%)
	8T7	819	M. mobile 163K complete genome	Endonuclease IV	111	1.00E-38	46/71 (64%)
	8T7	819	M. gallisepticum strain R section 2 of 4 of the complete genome	Nfo	75.3	1.00E-23	32/59 (54%)

In Table 3.14 and 3.15 the query indicates the length of the sequence that was entered for the search. The identities indicate the length, and percentage, of the Ms01 sequence that aligned with the sequence of the mycoplasma species. In general, the score bits as well as E-values of the TBLASTX search were higher and more significant than that of the BLASTN search. Although the percentage identity of the BLASTN search was higher, the alignments with the TBLASTX search were even better. With the TBLASTX, amino acids that these gene regions encode for were aligned with each other, and the three base pairs that represent the amino acid might not be the same between the mycoplasma species, and therefore the percentage identity is lower.

The most significant hit of a Ms01 sequence with the BLASTN as well as TBLASTX was with the *M. hominis* P100, oppB, oppC, oppD, oppF genes. *M. hominis* P100 is a membrane protein, and the ABC transport system oppBCDF is located downstream of it in the same operon. With BLASTN the hit was with sequence 1C00001F (primers CF+CR), and with TBLASTX the hit was with sequence 1C00004F (also primers CF+CR). An alignment of the two sequences in DAPSA showed that sequence 1C00004F is the same as 1C00001F. It was surprising that *M. hominis* genes were hit since the primer pairs were originally developed for identification of *M. gallisepticum* GapA and CrmA. On the other hand, Ms01 falls in the *M. hominis* clade (see Figure 2.1) which could support this result. The BLAST statistics of these hits showed that the BLASTN score was 145, and out of the 659 bp that were submitted, 139 bp aligned with 161 bp of *M. hominis*. Using the TBLASTX the score was 380, and out of the 654 bp that was submitted, 159 bp aligned with 201 bp of *M. hominis*. In order to find out exactly which part of *M. hominis* P100, oppB, oppC, oppD, oppF genes aligned with the sequence of Ms01, the bp position (also given as part of the search result) of *M. hominis* was compared with the complete sequence available on GenBank (access number X99740). This revealed that when using BLASTN as well as TBLASTX the *M. hominis* oppD gene, which is 1 166 bp and has an oligopeptide transport ATP-binding protein homolog as product, aligned 79% with the sequence of Ms01. The shorter sequence of the BLASTN search was in the same region as the sequence of the TBLASTX search.

The second best hit of Ms01 was also with primer pair CF+CR (sequence 1C00001F and 1C00004F), and the hit was with *M. synoviae*. The TBLASTX search identified it as an ABC transporter, ATP-binding protein of an as yet unknown function. The search results did not specify the position of this hit, and therefore it is possible that this ABC transporter gene can be part of an adhesion gene, or it could be adjacent to an adhesin gene(s). For this reason,

Pectora roborant cultus recti

the position of an adhesin gene relative to the ABC transporter, ATP-binding protein was investigated in other mycoplasma species. Only mycoplasma species of which the complete genomes are available could be examined, and those that were used included *M. gallisepticum* (adhesin gene *gapA*), *M. pneumoniae* (adhesin gene *P1*) and *M. genitalium* (adhesin gene *mgpA*). Twenty genes and their products upstream as well as downstream of the adhesin gene were examined. In the case of *M. gallisepticum* and *M. pneumoniae*, no ABC transporter gene was found in this region. In the genome of *M. genitalium*, three ABC transporter genes were found in the area close to the adhesin gene *mgpA*. Two of these ABC transporters were permease proteins which were respectively 1 132 bp and 2 060 bp upstream of the adhesin gene. The ATP-binding protein was situated 3 052 bp upstream from the adhesin gene. Thus it appears that in some mycoplasma species the adhesin gene may contain an ABC transporter ATP-binding motif, but in other species the location of the ATP-binding protein is not necesserily adjacent or close to the adhesin gene.

With sequences 1E00005F, 1E00012F and 1E00025F (primer pair EF+E2R), no significant hits were found with *M. synoviae* (results not shown in table). Ms01 sequences 2T7, 6T7 and 8T7 (primer pair E2F+E3R) had significant hits with *M. synoviae* and these were the following: glucose inhibited division protein A, tyrosyl tRNA synthetase and endonuclease IV. Significant hits of sequences 5T7, 8T7 and 19T7 (primer pair E2F+E3R) with *M. gallisepticum* were the following: GidA (glucose inhibited division protein A), FusA (translation elongation and release factors) and Nfo. As with the ABC transporter genes in *M. gallisepticum*, none of these products are situated in the region 20 genes upstream or downstream of the adhesin gene *gapA*.

Pectora roborant cultus recti

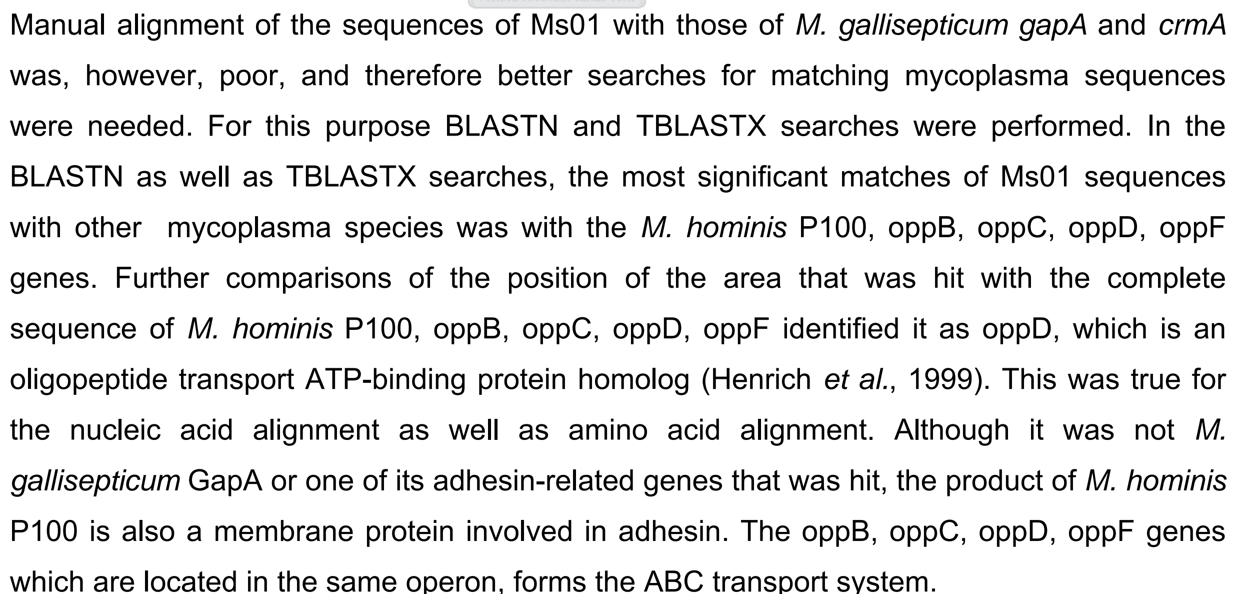
3.4 Discussion

One of the objectives of this study was to identify an adhesin, or adhesin-related gene in the three ostrich mycoplasmas Ms01, Ms02 and Ms03. In order to do this, the first step was to determine whether or not the gene order of mycoplasma genomes is conserved. This would reveal if adjacent genes could be used to target an adhesin gene. However, a comparison with Gene plot of those mycoplasma genomes which have been sequenced fully showed that the gene order is not conserved within mycoplasma genomes. The sequence of genes in operons in mycoplasma genomes was, however, found to be conserved. As a result, a search for an adhesin gene with primers must be restricted to the genes within an operon.

PCR's were performed in four primer approaches and this included several primer combinations. Primers for the first approach were based on the sequences of the *M. gallisepticum gapA* and *crmA* genes (Papazisi *et al*., 2000), and primers for the three other approaches were developed from the alignment of several adhesin and adhesin-related genes of mycoplasma species. Within the adhesin and adhesin-related genes, conserved areas, referred to as domain A and B, were recognised which are possibly also conserved in the adhesin genes of the ostrich mycoplasmas. Genomic DNA of Ms01, Ms02 as well as Ms03 was used in all the primer approaches and produced a range of PCR products. In most instances multiple PCR products were produced with DNA from all three ostrich mycoplasmas, and not only a single product of the expected size. Sequences that were generated directly from the PCR products were not satisfactory, and could not be used in BLAST searches.

In an attempt to generate readable sequences, PCR products of Ms01, Ms02 and Ms03 that were of the expected product size for a specific primer pair, were cloned into the pGEM-T Easy Vector. The cloning procedure was successful with PCR products from all three ostrich mycoplasmas. Sequences were generated from the final cloning product, namely the isolated plasmid DNA. Sequences from Ms01 were very good in that the functional part could be easily recognised. In the case of Ms02 and Ms03, the sequences were poor and could not be used successfully in BLAST searches.

Manual alignment of the sequences of Ms01 with those of *M. gallisepticum gapA* and *crmA* was, however, poor, and therefore better searches for matching mycoplasma sequences were needed. For this purpose BLASTN and TBLASTX searches were performed. In the BLASTN as well as TBLASTX searches, the most significant matches of Ms01 sequences with other mycoplasma species was with the *M. hominis* P100, oppB, oppC, oppD, oppF genes. Further comparisons of the position of the area that was hit with the complete sequence of *M. hominis* P100, oppB, oppC, oppD, oppF identified it as oppD, which is an oligopeptide transport ATP-binding protein homolog (Henrich *et al*., 1999). This was true for the nucleic acid alignment as well as amino acid alignment. Although it was not *M. gallisepticum* GapA or one of its adhesin-related genes that was hit, the product of *M. hominis* P100 is also a membrane protein involved in adhesin. The oppB, oppC, oppD, oppF genes which are located in the same operon, forms the ABC transport system.



ABC transport systems have been shown to be involved in the ATP-dependent transport of nutrients into microbial cells (Rottem, 2002). The *M. hominis* P100, oppBCDF operon

therefore appears to code for proteins involved in attachment and active transport as functional unit (Henrich *et al.*, 1993). From this finding it can be deduced that searches for ABC transporters may therefore also reveal attachment genes and was further examined in the subsequent gene searches in this study.

One of the poultry mycoplasmas that had a significant match with the sequences of Ms01 was *M. synoviae*, and it was also overall the second best hit with BLASTN and TBLASTX. The product of the *M. synoviae* gene that was hit with the TBLASTX is also an ABC transporter, ATP-binding protein. Thus it appears that the hits of *M. hominis* oppD and *M. synoviae* are similar in function. Further investigations revealed that in two other species, *M. gallisepticum* and *M. pneumoniae*, ABC transporter ATP-binding proteins are not adjacent to their adhesin genes (*gapA* and P1 respectively), adhesin-related genes, or in the nearby area. In the case of *M. genitalium*, the closest ABC transporter, ATP-binding protein is 3 052 bp upstream of its adhesin gene *mgpA*. This illustrates once again that the gene order of the mycoplasma genome is not conserved.

In the BLASTN and TBLASTX searches with other mycoplasma species, the hits were often with different lengths of the submitted Ms01 sequences. This could be because the genomic rearrangement between species is quite large, but this appears not to be a problem since the functional part of the sequence is short. With the primer approaches, the adhesin genes of other mycoplasma species were also amplified, which indicates that they possibly share conserved motives in the functional part of the membrane insertion and attachment genes, such as the domain A and B areas in *M. gallisepticum gapA* and *crmA*. This may explain why the *M. hominis* oppD gene was such a significant hit with the primers based on *M. gallisepticum gapA*.

The importance that these adhesin and adhesin-related proteins play in pathogenicity of mycoplasmas has recently been highlighted by the research done by Papazisi and co-workers (2000, 2002a, 2002b, 2003). They found that the expression of the adhesin and adhesin-related genes was essential for cytadherence and pathogenesis. If the *gapA* gene was expressed and the *crmA* gene was not, the mycoplasma lost its pathogenicity, but IgA antibodies that specifically bound to GapA were elicited after vaccination, and these in turn gave protection to infection with virulent strain (which expressed both GapA and CrmA) (Papazisi *et al.*, 2002b). This shows that the approach followed in this study, which has as its final goal to develop a DNA-vaccine against a specific adhesin shows considerable promise, as the vaccine would elicit immunity, but not cause pathogenesis.

In conclusion, it was found that the genes between mycoplasma species are not homologous, which is probably due to their different hosts. The primer approaches that were performed were not specific enough in that an adhesin or adhesin-related gene(s) was not found in the ostrich mycoplasmas, Ms01, Ms02 or Ms03. However, it forms a good basis for future studies since *M. hominis* oppD, which has 79% sequence identity with the sequence of Ms01, was identified as a possible probe for adhesin genes. Genomic mycoplasma DNA can be cut with a suitable restriction enzyme and the fragments cloned into a suitable plasmid vector, such as pSK Bluescript to generate a DNA library. Clones containing the adhesin genes could then be screened with the oppD probe. A suitably labelled oppD probe could then be used to select these clones through Southern Blotting. The *M. hominis* oppD gene is in the same operon as P100, which is the membrane protein involved in adhesion, and should therefore be an appropriate probe for the identification of a P100-like gene. Chromosome walking could then be used to reach the true adhesin genes of each of the ostrich mycoplasmas Ms01, Ms02 and Ms03, based on the assumption that their adhesin-related genes are in the same operon as their adhesin gene.

Once the adhesin operons of Ms01, Ms02 and Ms03 are isolated, they may be inserted into suitable DNA vaccine vectors and vaccines can be developed. Since Ms02 and Ms03 are more closely related to the poultry mycoplasmas, poultry mycoplasma vaccines can be used against them in the meantime. For this reason, a poultry vaccine trial was launched at Oudtshoorn in order to test whether or not mycoplasma vaccines elicit an efficient immune response. This vaccine trial will be discussed in Chapter 4.

Pectora roborant cultus recti

4. Trials with Poultry Mycoplasma Vaccines in Ostriches

4.1 Introduction

Currently, no registered vaccine is available against ostrich mycoplasmas. Due to the close relationship between the ostrich mycoplasma Ms02 and the poultry mycoplasma *M. synoviae* (see Figure 2.1), the possibility exists that anti-*M. synoviae* antibodies may cross-react with Ms02. In the initial phases of trying to identify which mycoplasmas infected ostriches, immunofluorescence antibody tests using antibodies against *M. synoviae* were found to recognise and bind to certain ostrich mycoplasmas. The initial deduction from this result was that ostriches were in actual fact infected with *M. synoviae*. The subsequent identification of the specific ostrich mycoplasmas and of the relationship of Ms02 and Ms03 to *M. synoviae* (Botes *et al.* 2004, 2005a) can however also explain this anomaly. Therefore a *M. synoviae* vaccine has the potential to elicit an effective immune response which may give protection against Ms02 and Ms03.

Several mycoplasma vaccines, bacterins and live vaccines, as well as vaccination methods are available. These subjects and previous studies with poultry mycoplasma vaccines have been discussed in Chapter 2, section 2.9. As *M. gallisepticum* is an important poultry pathogen, there has been an ongoing improvement in *M. gallisepticum* vaccines which give protection against this pathogen in poultry. Although *M. gallisepticum* has not been found in ostriches and is not closely related to any of the ostrich mycoplasmas, the advanced *M. gallisepticum* vaccines that were available warranted a trial in which their ability to elicit immune responses in ostriches was established.

Since the development of the enzyme-linked immunosorbent assay (ELISA) by Engvall and Perlmann (1971) this method has been widely used for the detection of antibodies to antigens immobilized on solid phases such as microtiter plates. In order to test the immune responses of vaccinated poultry, ELISA tests are therefore routinely used. Advantages of using ELISA as a testing method include its simplicity, specificity, rapidity, sensitivity and low cost. ELISA kits are also commercially available and adaptable (Crowther, 2000).

The objective of this study was to test whether poultry mycoplasma vaccines could be used effectively until an ostrich mycoplasma vaccine is available. In order to test whether poultry mycoplasma vaccines may elicit immune responses in ostriches, a vaccine trial using a *M. synoviae* vaccine and a *M. gallisepticum* vaccine, was launched at Oudtshoorn. In order to

test the level of antibody response in ostriches, a commercially available ELISA kit for the detection of *M. synoviae* antibodies in chicken and turkey serum was used and adapted in order to detect ostrich antibodies against *M. synoviae*.

4.2 Material and Methods

The following section describes the poultry mycoplasma vaccines used in the trial, the setup for the vaccine trial as well as adaptation of the commercial available ELISA kit that was used.

4.2.1 Poultry mycoplasma vaccines used in study

Two poultry mycoplasma vaccines were used in the trial, namely *M. synoviae* and *M. gallisepticum*. Both vaccines were obtained from Fort Dodge Animal Health, USA. Both vaccines were developed from field strains, the *M. gallisepticum* vaccine was isolated by West Virginia University and is referred to as Mg-bac, and the *M. synoviae* vaccine strain is unspecified and is referred to as Ms-bac.

Both vaccines were inactivated, oil emulsified vaccines and thus unable to multiply or spread to other birds. In the choice of vaccines, inactivated vaccines were purposely chosen so that live poultry mycoplasmas were not inadvertently introduced into ostriches. In the case of oil emulsified vaccines, the oil must be removed prior to use in ostriches in order to prevent granulomas and abscesses underneath the skin. The oil is removed by centrifuging the vaccine, but in the process the antigens are concentrated which could lead to a difference in the amount of antigen.

For immunization, 500 ml of both the *M. synoviae* as well as the *M. gallisepticum* bacterin, the vaccine was divided into 5 x 100 ml bottles, centrifuged and the oil removed.

4.2.2 Serum from ostriches included in the vaccine trial

Ostriches from three farms in the Oudtshoorn district were selected to be included in the vaccine trial. None of the ostriches that were used for the vaccine trial had mycoplasma symptoms (e-mail Dr A. Olivier, August 2005). On each farm, the ostriches were divided into the three groups, namely A, B and Control. The group of ostriches designated as Group A was vaccinated with *M. synoviae* vaccine, a Group B was vaccinated with *M. gallisepticum*

vaccine and a Control group was not vaccinated. Every ostrich on each farm, group, and age group was injected with a dosage of 1 ml oil free vaccine whilst the control group was not vaccinated. A summary of the ostriches used in the vaccine trial is presented in Table 4.1.

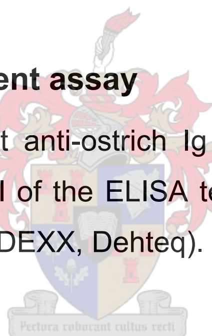
Table 4.1 Summary of the ostriches used in the poultry mycoplasma vaccine trial.

	Age of ostrich chicks	Group A	Group B	Control
Farm 1	3 months	10	10	10
Farm 2	4 - 5 months	10	10	10
Farm 3	6 - 7 months	20	20	20

Serum samples were taken on day 0, 7, 14 and 21 from each ostrich in each group. The serum was stored at 4 ºC, and for long term storage at -20 ºC. All the serum samples were used in the ELISA test in order to test the antibody response.

4.2.3 Enzyme-linked immunosorbent assay

Firstly for the ELISA test, rabbit anti-ostrich Ig was isolated and biotinylated. This was followed by the modified protocol of the ELISA test with a *Mycoplasma synoviae* Antibody Test Kit, namely FlockChek Ms (IDEXX, Dehteq).


Pectora roborant cultus recti

4.2.3.1 Isolation and biotinylation of rabbit anti-ostrich Ig

To precipitate the Ig fraction, 500 µl of day 74 rabbit anti-ostrich Ig serum was added to 1 ml PBS (0.15 M, pH 7.2) and 1.5 ml saturated ammonium sulphate. The sample was incubated at 4 ºC for 20 min followed by centrifugation at 27 200 x g (Model J-21B Centrifuge, Beckman) for 20 min. The supernatant was decanted, the pellet redissolved in 1 ml PBS, and 1 ml saturated ammonium sulphate was added. This mixture was incubated for 20 min at 4 ºC followed by centrifugation at 27 200 x g for 20 min. Supernatant was decanted once again and the remaining pellet redissolved in 500 µl PBS. The Ig fraction was dialyzed at 4 ºC overnight, ± 19 h, against carbonate buffer (0.1 M, pH 8.3). Changing of the carbonate buffer was done 4 h after starting dialysis (Hudson and Hay, 1980).

The Ig concentration was determined by absorption at 280 nm. Carbonate buffer was used as a blank, and the Ig sample was diluted 10 x in carbonate buffer. To obtain a 5

mg/ml concentration of rabbit anti-ostrich Ig, carbonate buffer was added to the Ig fraction. For biotinylation, a 2 mg/ml biotin reagent was prepared by adding biotinamidocaproate N-hydroxysuccinimide ester (Biotin, Sigma) to N,N dimethylformamid (DMF). For each ml Ig, 250 µl of the biotin reagent was added slowly to the Ig fraction while stirring at low speed for 2 h at room temperature. The prepared conjugate was dialyzed overnight, ± 19 h, against PBS (0.15 M, pH 7.2) at 4 ºC, and the buffer was changed to clean buffer 4 h after dialysis started. Finally, glycerol was added in a 1:1 ratio to the biotinylated rabbit anti-ostrich Ig preparation, mixed thoroughly and stored at -20 ºC.

The newly prepared biotinylated rabbit anti-ostrich Ig was compared to previously prepared biotinylated rabbit anti-ostrich Ig in an ELISA test, and found to give comparable results in an ostrich Newcastle Disease Virus antibody ELISA (results not shown).

4.2.3.2 ELISA for detection of humoral Ig antibodies to M. synoviae

The ELISA for the detection of humoral Ig antibodies to *M. synoviae* in ostriches is schematically presented in Figure 4.1.

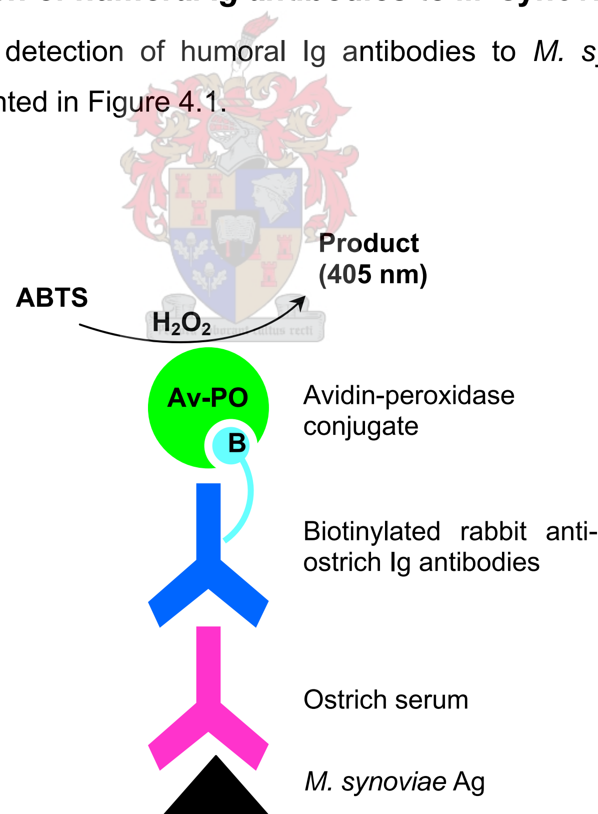


Figure 4.1 ELISA for detection of humoral Ig antibodies to *M. synoviae*.

From the *M. synoviae* Antibody Test Kit, only the coated plates (96 wells) and diluent buffer preserved with sodium azide (Reagent 5) was used for reasons previously given.

Ostrich serum from day 0, 7, 14 and 21 for each ostrich chick in each group from each farm, was prediluted 1:500 in the diluent buffer. Of the diluted serum, 100 µl was pipetted in duplicate into the wells, and the plate incubated for 3 h at 37 ºC. The serum was decanted from the plate, and the wells washed three times with PBS-Tween (PBS buffer with 0.1 % Tween-20).

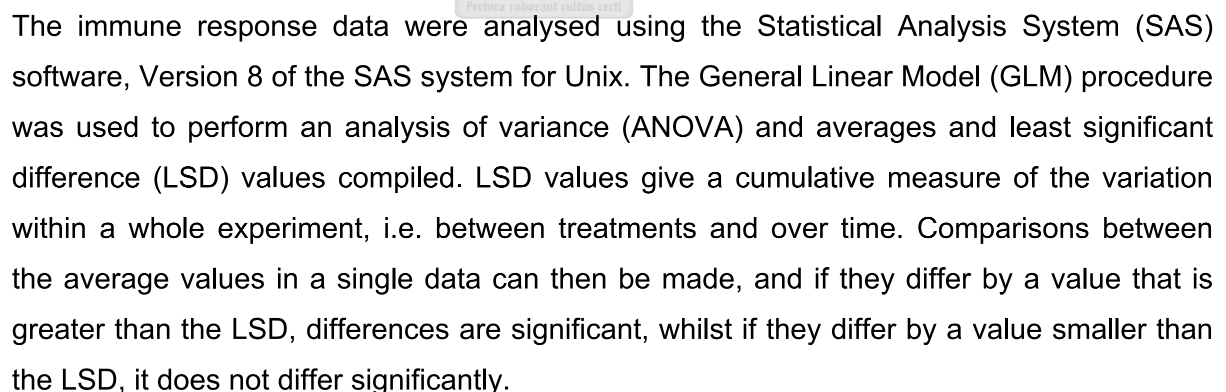
Biotinylated rabbit anti-ostrich Ig were diluted 100 x in casein-Tween (0.5 % casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02 % thiomersal, pH 7.6 with 0.1 % Tween), added to the plate, 100 µl/well, and incubated for 1 h at 37 ºC. The content of the plate was decanted before washing it three times with PBS-Tween.

After washing the plate, 100 µl of streptavidin peroxidase (AVPO), diluted 100 x in casein-Tween, was added to each well. The plate was incubated for 1 h at 37 ºC, after which the contents were decanted and the plate washed three times with PBS-Tween.

Finally, 100 µl of the substrate solution (0.05 % ABTS, 0.015 % H_2O_2 in 0.1 M citrate buffer, pH 5) was added to each well. After incubation for 30 min at 37 ºC, the absorbance was measured at 405 nm on a Titertek Multiscan spectrophotometer.

Pectora roborant cultus recti

4.2.4 Statistical analysis



The immune response data were analysed using the Statistical Analysis System (SAS) software, Version 8 of the SAS system for Unix. The General Linear Model (GLM) procedure was used to perform an analysis of variance (ANOVA) and averages and least significant difference (LSD) values compiled. LSD values give a cumulative measure of the variation within a whole experiment, i.e. between treatments and over time. Comparisons between the average values in a single data can then be made, and if they differ by a value that is greater than the LSD, differences are significant, whilst if they differ by a value smaller than the LSD, it does not differ significantly.

4.3 Results

4.3.1 Adaptation of ELISA

Plates were coated with *M. synoviae* antigen by the manufacturer and were ready for use. Antibodies against mycoplasma antigens present in the ostrich serum will bind to the *M.*

synoviae antigen on these plates. Along with the kit, (goat) anti-chicken/(goat) anti-turkey: horseradish peroxidase conjugate (HRPO) was supplied as secondary antibodies, but from previous experience it is known that antibodies against chicken antibodies do not react with ostrich antibodies (Blignaut *et al.*, 2000). Therefore, detection of the antibodies was by specific secondary antibodies, namely biotinylated rabbit anti-ostrich Ig antibodies, to which a strepavidin-enzyme conjugate was bound. The advantage of using the biotin-avidin system is its high sensitivity in amplifying the eventual signal and the low background levels it produces. A colourless substrate, 2,2'-Azino-di(3-ethylbenzthiazoline sulphonic acid-6) (ABTS) in combination with H_2O_2, that is converted to the radical cation of ABTS, and which in turn forms an azodication product with an absorbance maximum at 414 nm, was used for detecting the presence of the enzymes. The green coloured product could easily be detected at 405 nm (Figure 4.1).

As no ostrich sera containing antibodies to *M. synoviae* were available (no ostriches have been immunized with *M. synoviae* vaccines before) a number of preliminary experiments using the above ELISA were performed in an effort to validate it. Previously an ELISA procedure for the detection of antibodies against Newcastle Disease Virus (NDV) in poultry was adapted for use in ostriches in this laboratory (Blignaut *et al.*, 2000) and a similar approach was followed in the adaption of this ELISA procedure in that the same rabbit anti-ostrich antibodies as used in the anti-NDV ELISA were used here. In previous research it was found that high antibody levels against NDV could be detected 21 days after immunisation in 3 month old ostriches. Consequently, in this research, five randomly selected serum samples from birds taken before immunization and 21 days after immunization from Farm 1, i.e. birds that were three months old, and were used in the ELISA procedure for the detection of *M. synoviae* antibodies. In a comparison of the absorbance values obtained in the ELISA before and after immunization, it was also found that large differences in absorbance values could be measured in a majority of cases. The absorbance values obtained in this ELISA were similar to those obtained in the ELISA for NDV-specific antibodies in ostriches. As the ELISA plates were coated with *M. synoviae* antigens, it was concluded that the differences in absorbance values measured were an indication of specific antibody levels to the *M. synoviae* antigens.

4.3.2 Statistical analysis of ELISA results

The ELISA results that were used for statistical analysis with SAS are attached as Appendix B. The statistical analysis of the ELISA data with the SAS system (Appendix C) revealed

that although the treatments on Farm 1, 2 and 3 did not differ, the coefficient of variance was very large. With such a large difference it is difficult to compare the three farms directly to each other and treat it as one experiment, therefore each individual farm was analysed separately.

From the statistical analysis of the individual farms, the computed LSD value can be used as an indication of a statistically significant difference between the three groups. On Farm 1 (LSD value = 0.1937), there was no statistically significant difference between Group A, which received the *M. synoviae* vaccine, and Group B which received the *M. gallisepticum* vaccine. The difference between Group C, which received no vaccine, and Group A and Group B was statistically significant. On Farm 2 (LSD value = 0.1438), the difference between Group A and Group B is not statistically significant, but their difference with Group C was statistically significant. On Farm 3 (LSD value = 0.0568), the difference between Group A and Group B was also not statistically significant, but their difference with Group C was statistically significant. Thus no statistically significant differences were observed between the responses elicited by the two poultry mycoplasma vaccines on any of the three farms, but all the vaccinated ostriches elicited an immune response in comparison to the unimmunised controls.

4.3.3 Immune response of ostrich chicks

In the following section, the results of the ELISA tests for each farm are presented as graphs. For each graph, the average immune response of the group (A, B or Control) was plotted against time. The results of each bird at each time point were also analysed. Birds with a serum titer above 0.2 were regarded to have given a significant antibody response, and a titer value below 0.2 as a negative antibody response. The fraction of ostriches in each group on each farm that reacted to vaccination is summarised in tables.

4.3.3.1 Farm 1: 3 month old ostrich chicks

Figure 4.2 illustrates the average antibody response of the three groups to *M. synoviae* on the first farm in ostrich chicks which were 3 months old.

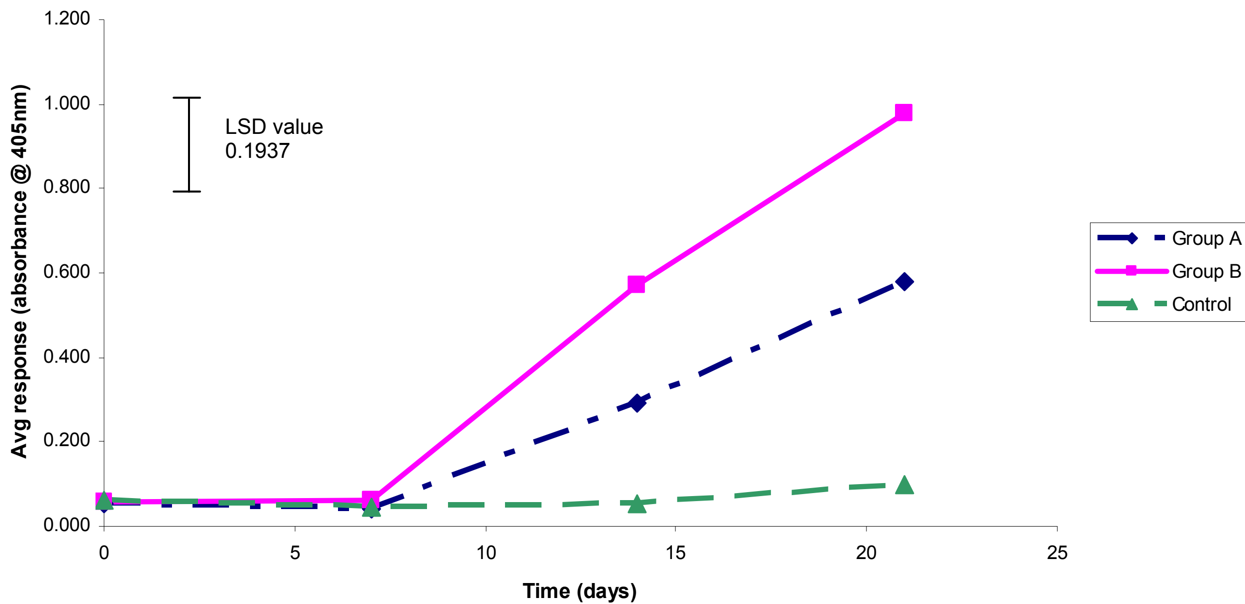


Figure 4.2 Average antibody response to *M. synoviae* of 3 month old ostrich chicks on Farm 1. Group A received *M. synoviae* vaccine (1 ml), Group B received *M. gallisepticum* vaccine (1 ml) and the Control group received no vaccine. The LSD value for Farm 1 is 0.1937.

From Figure 4.2 it can be seen that both vaccines elicited an immune response. Group B, which received *M. gallisepticum* vaccine had a tendency to a higher antibody response than Group A which received *M. synoviae* vaccine but the difference was not statistically significant. The antibody response rose significantly between days 7 and 14, and increased up to 21 days. Table 4.2 summarises the number of ostriches in each group that had an ELISA titer greater than 0.2.

Table 4.2 Fraction and percentage of the ostriches on Farm 1 that reacted to vaccination. For each group, only the ostriches with a positive antibody response, thus a titer greater than 0.2, on the respective days are indicated.

	Day 0		Day 7		Day 14		Day 21	
	Fraction	**%**	**Fraction**	**%**	**Fraction**	**%**	**Fraction**	**%**
Group A	0/10	0.00	0/10	0.00	3/10	30.00	5/10	50.00
Group B	0/10	0.00	0/9	0.00	7/9	77.78	9/9	100.00
Control	0/10	0.00	0/10	0.00	0/10	0.00	1/10	10.00

Although only 3 ostriches in Group A had a titer greater than 0.2 on day 14, the average of all 10 ostriches were still higher than 0.2 (see Figure 4.2). In Group B, 7 out of 9 ostriches had a positive antibody response with the *M. gallisepticum* vaccine after 14 days. After 21

days all the ostriches of Group B had a positive antibody response, but only 50% of Group A. In the Control group, one ostrich had a positive response after 21 days, and the other ostriches responded negatively.

4.3.3.2 Farm 2: 4-5 month old ostrich chicks

The average antibody response to *M. synoviae* of the 4-5 month old ostrich chicks on the second farm is illustrated in Figure 4.3. These ostriches have a larger body mass than those of Farm 1, and since all the ostriches received the same dosage of vaccine, namely 1 ml, the dosage per body mass is lower.

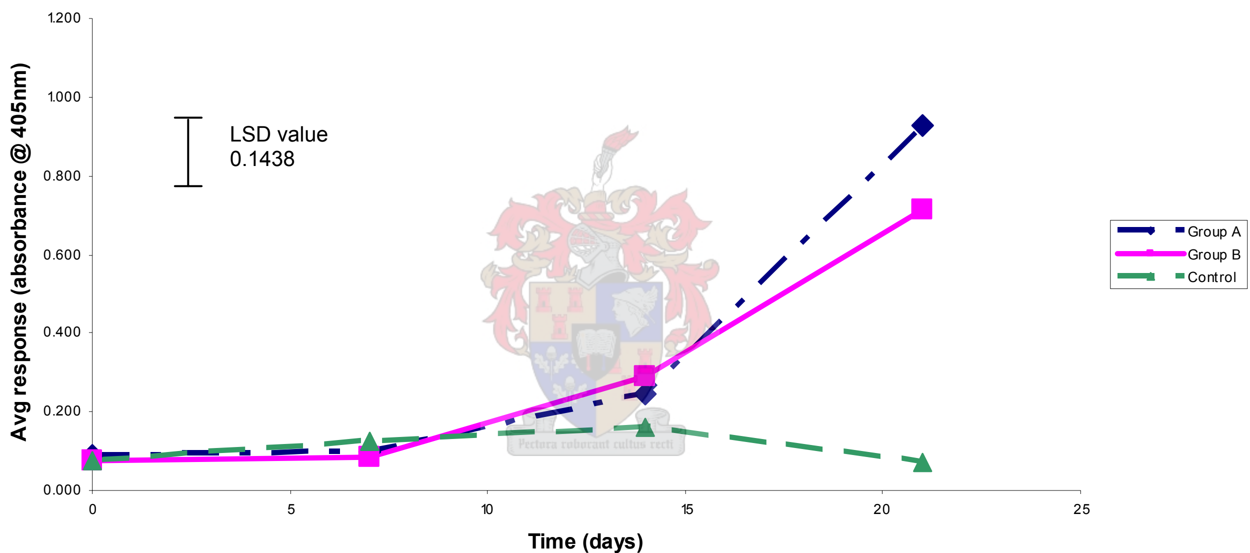


Figure 4.3 Average antibody response to *M. synoviae* of 4-5 month old ostrich chicks on Farm 2. Group A received *M. synoviae* vaccine (1 ml), Group B received *M. gallisepticum* vaccine (1 ml), and the Control group received no vaccine. The LSD value for Farm 2 is 0.1438.

The average antibody response of Group A, which received the *M. synoviae* vaccine was better than the average antibody response of Group B which received the *M. gallisepticum* vaccine, but the difference was not significantly different. A slight rise in average antibody response could be seen after 14 days. After 21 days the average antibody response showed a drastic increase in Group A and Group B. The fraction as well as percentage of the ostriches in each group that responded to vaccination is summarised in Table 4.3.

Table 4.3 Fraction and percentage of the ostriches on Farm 2 that reacted to vaccination. For each group, only the ostriches with a positive antibody response, thus a titer greater than 0.2, on the respective days are indicated.

	Day 0		Day 7		Day 14		Day 21	
	Fraction	%	Fraction	%	Fraction	%	Fraction	%
Group A	0/10	0.00	0/10	0.00	4/10	40.00	8/10	80.00
Group B	0/10	0.00	0/10	0.00	5/10	50.00	10/10	100.00
Control	0/10	0.00	1/10	10.00	2/10	20.00	0/9	0.00

A positive antibody response was only seen after 2 weeks of vaccination, and in Group A, this percentage doubled from day 14 to day 21 (from 40% to 80%). This was also the case for the ostriches in Group B where all of them had a positive response after 21 days. All the ostriches in the Control group had a negative antibody response, except for one ostrich on day 7 and two ostriches on day 14.

4.3.3.3 Farm 3: 6-7 month old ostrich chicks

The average antibody response of the three groups on Farm 3 is illustrated in Figure 4.4. These ostrich chicks of 6-7 months had the largest body mass of the ostriches used in this study, and therefore the lowest dosage per body mass.

Pectora roborant cultus recti

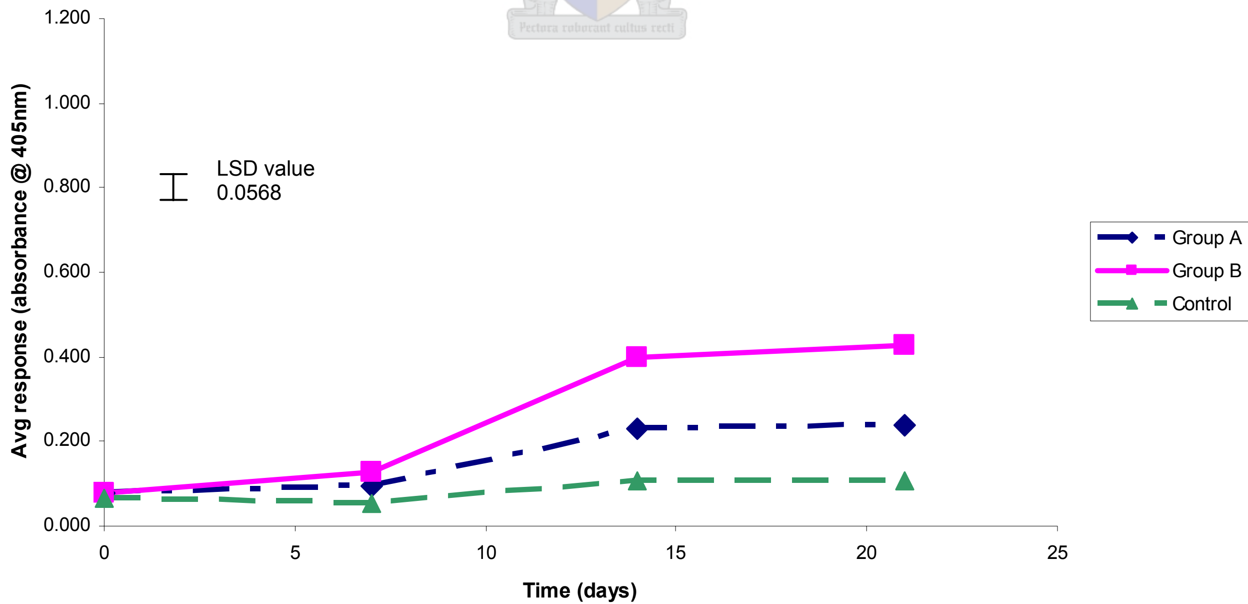


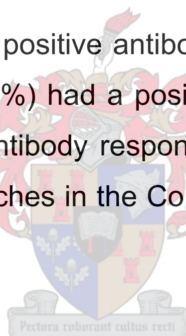
Figure 4.4 Average antibody response to *M. synoviae* of 6-7 month old ostrich chicks on Farm 3. Group A received *M. synoviae* vaccine (1 ml), Group B received *M. gallisepticum* vaccine (1 ml), and the Control group received no vaccine. The LSD value for Farm 3 was 0.0568.

The ostriches of Group B, which received the *M. gallisepticum* vaccine, had a better antibody response than Group A, but the difference was not large nor statistically different. An increase in antibody response could be seen from day 7 to 14, but after 21 days no further significant increase could be observed. The average of Group A was slightly above 0.2, and thus a positive antibody response. Table 4.4 summarises the number of ostriches in each group that had a positive antibody response.

Table 4.4 Fraction and percentage of the ostriches on Farm 3 that reacted to vaccination. For each group, only the ostriches with a positive antibody response, thus a titer greater than 0.2, on the respective days are indicated.

	Day 0		**Day 7**		**Day 14**		**Day 21**	
	Fraction	**%**	**Fraction**	**%**	**Fraction**	**%**	**Fraction**	**%**
Group A	0/20	0.00	1/20	5.00	7/20	35.00	9/20	45.00
Group B	0/20	0.00	2/20	10.00	13/20	65.00	14/20	70.00
Control	0/20	0.00	0/20	0.00	0/19	0.00	3/19	15.79

In Group A, one ostrich had a positive antibody response after 7 days, but after 21 days less than half of the group (45%) had a positive antibody response. In Group B, 65% of the ostriches had a positive antibody response after 14 days, but this only increased to 70% after 21 days. Three ostriches in the Control group had a positive antibody response after 21 days.



4.4 Discussion

The objective of this study was to test whether two poultry mycoplasma vaccines can elicit an immune response in ostriches since no mycoplasma vaccines have been tested in ostriches to date, nor are any mycoplasma vaccines registered for use in ostriches. The ELISA test results showed that the *M. synoviae* vaccine as well as the *M. gallisepticum* vaccine can be used to successfully elicit immune responses in ostriches. On two of the three farms, the *M. gallisepticum* vaccine had a tendency to elicit a higher immune response than the *M. synoviae* vaccine whilst on the third farm the opposite tendency was found. It must however be mentioned that these differences were never statistically significant.

A minimum antibody level in order to give effective protection against mycoplasmas could not be determined as the immunized birds could not be challenged. However, a cut-off value of 0.2 proved to be an indicator of protection against NDV in the vaccination trials in ostriches done by Blignaut *et al.* (2000). For this reason, this cut-off value was also used in this vaccine

trial. If *M. synoviae* vaccination is compared with *M. gallisepticum* vaccination, *M. gallisepticum* vaccination results in a higher percentage of birds that give significant immune responses.

As antibody responses to vaccination with *M. synoviae* and *M. gallisepticum* vaccines were measured using *M. synoviae* antigen coated plates, it could be expected that the measured responses to *M. synoviae* vaccination should be higher than *M. gallisepticum* vaccinations. Thus the antibody levels elicited by *M. gallisepticum* vaccination may be much higher than have been measured in these trials. However, this trial does show that the *M. gallisepticum* vaccines used does elicit immune responses in ostriches. The question of protection by these vaccines against the closely related ostrich mycoplasmas Ms02 and Ms03, will have to be determined by challenging ostriches vaccinated with the Mg-bac vaccine with live Ms02 and Ms03. However, as Ms02 and Ms03 have been found to be difficult to cultivate, they were not available for the challenging the ostriches vaccinated in this study.

In a comparison of three live *M. gallisepticum* vaccines, namely the F-, ts-11 and 6/85 strain, Abd-El-Motelib and Kleven (1993) found that the F-strain vaccine elicited strong serological responses and gave good protection to vaccinated birds, whilst the ts-11 and 6/85 strains were less effective. Biró *et al.* (2005) found that the ts-11 vaccine did elicit protective immunity in poultry and no pathological lesions were caused as a result of using this live *M. gallisepticum* vaccine. The *M. gallisepticum* 6/85 strain is also safe to use in poultry since it has a low virulence and spreads poorly from bird to bird whilst eliciting protective immunity (Zaki *et al.*, 2004). Another live *M. gallisepticum* vaccine GT5, which was reconstituted from the avirulent *M. gallisepticum* strain R_{high}, could also stimulate a protective immune response (Papazisi *et al.*, 2002b). In the case of *M. synoviae* vaccines, a study of the live attenuated MS-H strain by Noormohammadi *et al.* (2002b) revealed that the highest detectable level of antibody response was only seen after 100 days of vaccination since the antigens that were used in serological tests were unable to detect the antibodies. As live vaccine strains persist in birds after vaccination, the ostrich industry felt that it was a risk to use these vaccines in South African ostriches, as these live mycoplasma vaccine strains could perhaps establish themselves permanently leading to additional problems.

Although live vaccines could stimulate protective immune responses, killed bacterin vaccines are usually associated with more consistent and stronger immunogenic responses without the associated problems of strain persistence (Droual *et al.*, 1990, 1993). The *M. gallisepticum* bacterin, Mg-bac, has been used effectively in the vaccination of one-week-old chickens

(Karaca and Lam, 1987). For this reason, it was felt that this vaccine may also be effective in eliciting immune responses in ostriches. However, by removing the oil as was done in this vaccine trial, the effectiveness of the vaccine could have been influenced (Panigrahy *et al.*, 1981). This study shows that, in spite of the removal of the oil, the vaccine is capable of eliciting an immune response in ostriches.

From the results it is clear that the age and the mass of the ostrich chicks play a role in the immune responses after vaccination. All the ostriches received the same dosage of vaccine, and as their age increased, their body mass increased. The lower antibody response of the older ostrich chicks could have been influenced by their larger body mass and therefore a lower vaccine volume relative to the body mass. This dosage effect was also seen when ostriches were vaccinated with NDV vaccines (Blignaut *et al.*, 2000).

Over and above differences in age and therefore mass of the vaccinated ostriches, other factors that may have played a role in the differences in immune responses on the three farms, are genetic and environmental factors. As ostriches in the Oudtshoorn district are largely genetically uniform and environmental factors are the same on the three farms, it is unlikely that the differences in the immune responses between the farms can be ascribed to these factors.

In this vaccine trial only a single vaccination was given and the primary antibody response, and thus humoral response (B-cells), that followed was analysed. In order to test the secondary response, in which the antibody response is usually elicited faster, the ostriches should be vaccinated for a second time. After the two vaccinations, they should be challenged with live mycoplasmas. Currently there have been difficulties in culturing live mycoplasmas, and therefore a challenge could not have been performed in the trials conducted as part of this study. The amount of live mycoplasmas to be used and the route of administration in order to make the challenge effective also need to be determined. When challenging flocks of ostriches it is important to administer the live mycoplasmas via a natural route, and therefore a spray could be used. This route of infection would also be better for eliciting IgA responses.

In conclusion, both poultry mycoplasma vaccines can be used to vaccinate ostriches and will elicit significant immune responses if immunized in sufficient amounts in relation to age and body mass. In the future, a second vaccine trial with these poultry mycoplasma vaccines,

which include booster vaccinations, should be performed, followed by challenging the ostriches with live mycoplasmas to test the efficacy of vaccination.

5. Conclusion and Future Perspectives

Three ostrich specific mycoplasmas, Ms01, Ms02 and Ms03, have been identified by Botes *et al.* (2004, 2005a) as causative organisms of respiratory diseases in ostriches. For this reason, a need for effective vaccine(s) against these three ostrich mycoplasmas has arisen. Two potential approaches can be used to address this need, i.e. to develop a vaccine(s) and/or to use existing poultry vaccines to elicit protective immunity against these mycoplasmas. DNA vaccines have shown promise in poultry and for this reason a decision was taken to investigate this possibility. In DNA vaccine development, a suitable candidate gene encoding a protein involved in virulence has to be identified, isolated and inserted into the DNA vaccine vector. This preliminary investigation was therefore launched to (a) identify and isolate such candidate genes from the three ostrich mycoplasmas, and (b) to investigate whether poultry mycoplasma vaccines elicit immune responses in ostriches.

From the literature it was found that a specialized tip structure is involved in mycoplasma adhesion, and several adhesin as well as adhesin-related genes have been identified. These include *M. gallisepticum gapA* and *crmA*, of which coexpression is necessary for cytadherence and pathogenesis (Papazisi *et al.*, 2003). In the human pathogen *M. pneumoniae*, P1 mediates attachment and accessory proteins which are necessary for cytadherence and pathogenesis (Razin and Jacobs, 1992). In *M. genitalium*, *mgpA* is involved in adhesion (Razin and Jacobs, 1992). Membrane proteins can also be involved in adhesion, such as *M. hominis* P100 (Henrich *et al.*, 1993, 1996). Through a comparison of the adhesin as well as adhesin-related genes of *M. gallisepticum*, *M. pneumoniae* and *M. genitalium*, it was found that two conserved areas, known as domain A and B within these genes, are shared between these species (Papazisi *et al.*, 2000).

The strategy that was followed to address the first objective of the study started off with an investigation to determine if the gene order of the mycoplasma genome is conserved between species or not. By using the Gene plot tool available on the NCBI website, it was found that the genome order is not conserved, but operons were. Therefore, in order to identify an adhesin or adhesin-related gene(s) in the ostrich mycoplasmas, genes adjacent to these gene(s) should not be used as target, but rather a gene that is part of the operon, such as *M. gallisepticum gapA* or *crmA*. For this purpose, four primer approaches were developed that included several primer combinations in PCR reactions. The first primer approach consisted of primers developed by Papazisi *et al.* (2000) for the amplification of *M. gallisepticum gapA* and *crmA*. In the second primer approach, the domain B region of a number of mycoplasma adhesin and adhesin-related genes were aligned. Primers were developed in the *gapA* domain B and *crmA* domain B region, based on

the assumption that these two *M. gallisepticum* genes are situated next to each other. It was also assumed that the adhesin and adhesin-related genes of the three ostrich mycoplasmas are situated next to each other in the same operon. In the third primer approach, alignment of two poultry mycoplasmas *M. gallisepticum* and *M. synoviae*, which shares sequence similarity with Ms02, revealed homology in the domain B region. Another primer, to be used in combination with primers from the previous approaches, was developed in this area. In the fourth and final primer approach, two more primers were developed based on the alignment done in primer approach three. All the primer combinations of the four primer approaches were used for the amplification of fragments from the genomes of Ms01, Ms02 and Ms03.

Direct sequencing of the PCR products generated using the above primer approaches were not successful due to the heterogenicity thereof. For this reason, PCR products of Ms01, Ms02 and Ms03 were cloned into the pGEM-T Easy Vector System. Subsequently, sequences were generated from the cloned DNA of Ms01, Ms02 and Ms03. Manual alignment of these sequences in DAPSA with their parent sequences was poor, most probably as a result of an accumulation of mutations between these mycoplasmas over time. Consequently, these sequences were used in the web-based search engine BLAST to perform BLASTN and TBLASTX searches. Using these searches it was found that the primer approaches that were followed in this study were not specific enough to identify an adhesin or adhesin-related gene(s) in the three ostrich mycoplasmas, Ms01, Ms02 and Ms03. This illustrated that what works in one mycoplasma genome would not necessarily work in another mycoplasma genome since the genes are not sufficiently homologous between species. Sequences that were generated had a high diversity, but the *M. hominis* oppD gene sequence that was found to be the most significant hit (79% sequence identity) may be used as an appropriate probe in the future. The fact that oppD is in the same operon as P100, makes it even more advantageous. In future studies, DNA libraries constructed from Ms01, Ms02 as well as Ms03 could be screened using this fragment as probe, and although it is a long process it is currently the best next step in the search for vaccine candidate genes.

The second objective of this study was to isolate the adhesin or adhesin-related gene(s) after it has been identified in the three ostrich mycoplasmas. Since the first objective could not be achieved, this objective can only be accomplished once the DNA libraries for Ms01, Ms02 and Ms03 are compiled. The whole operon involved in adhesion could then be isolated for ostrich mycoplasmas Ms01, Ms02 as well as Ms03.

The third objective of this study was to test whether poultry mycoplasma vaccines can elicit an immune response in ostriches. In a vaccine trial, two inactivated oil emulsified vaccines of *M.*

synoviae and *M. gallisepticum*, were used. It was found that both vaccines elicited an immune response, and a high percentage of the ostriches responded to it. It was found that younger ostrich chicks gave higher antibody responses than older ostrich chicks when immunized with the same vaccine dose. The most likely reason for this was that they received a lower dosage of vaccine per body mass. Further investigations should include optimisation of the vaccine dosage as well as a second vaccine trial in which booster vaccinations are given, after which the ostriches are challenged with live mycoplasmas to test their efficacy.

This study has therefore contributed to the knowledge of vaccine candidate genes in ostrich mycoplasmas. It has also laid the groundwork for future studies into the development of an effective vaccine against ostrich mycoplasmas. This study also documents that poultry mycoplasma vaccines have the potential of protecting ostriches against ostrich mycoplasma infections. Both of these aspects of this study may therefore be of direct benefit to the South African ostrich industry.

Literature Cited

Abd-El-Motelib, T.Y. and Kleven, S.H. (1993). A comparative study of *Mycoplasma gallisepticum* vaccines in young chickens. *Avian Dis.*, **37**: 981-987.

Ada, G.L. (1994). Vaccines and immune response. In: Webster, R.G. and Granoff, A. (eds), *Encyclopedia of Virology (Vol. 3),* pp. 1503-1507. Academic Press, London.

Adler, H.E. and Lamas Da Silva, J.M. (1970). Immunization against *Mycoplasma gallisepticum. Avian Dis.,* **14**: 763-769.

Allen, J.L., Noormohammadi, A.H. and Browning, G.F. (2005). The vlhA loci of *Mycoplasma synoviae* are confined to a restricted region of the genome. *Microbiology,* **151**: 935-940.

Baseman, J.B. and Tully, J.G. (1997). Mycoplasmas: Sophisticated, reemerging, and burdened by their notoriety. *Emerg. Infect. Dis.,* **3**(1): 21-32.

Baum, S.G. (2000). Introduction to *Mycoplasma* diseases. In: Mandell, G.L, Bennett, J.E. and Dolin, R. (eds), *Principles and practice of Infectious Diseases,* pp. 2015-2018. Churchill Livingstone, Philadelphia, USA.

Bencina, D. (2002). Haemagglutinins of pathogenic avian mycoplasmas. *Avian Pathol.,* **31**: 535-547.

Bezuidenhout, A.J. (1999). Anatomy. In: Deeming, D.C. (ed), *The Ostrich: Biology, production and health,* pp. 13-50. CAB International, Cambridge.

Binnewies, T.T., Hallin, P.F., Staerfeldt, H-H. and Ussery, D.W. (2005). Genome update: proteome comparisons. *Microbiology,* **151**: 1-4.

Biró, J., Povazsan, J., Korösi, L, Glavits, R., Hufnagel, L. and Stipkovits, L. (2005). Safety and efficacy of *Mycoplasma gallisepticum* TS-11 vaccine for the protection of layer pullets against challenge with virulent *Mycoplasma gallisepticum* R-strain. *Avian Pathol.*, **34**: 341-347.

BLAST searches: www.ncbi.nlm.nih.gov/blast (Accessed several times).

Blignaut, A., Burger, W.P., Morley, A.J. and Bellstedt, D.U. (2000). Antibody response to La Sota strain vaccines of Newcastle Disease virus in ostriches (*Struthio camelus*) as detected by enzyme-linked immunosorbent assay. *Avian Dis.*, **44**: 390-398.

Boguslavsky, S., Menaker, D., Lysnyansky, I., Liu, T., Levisohn, S., Rosengarten, R., Garcia, M. and Yogev, D. (2000). Molecular characterization of the *Mycoplasma gallisepticum* pvpA gene which encodes a putative variable cytadhesin protein. *Infect. Immun.,* **68**(7): 3956-3964.

Pectora roborant cultus recti

Botes, A. (2004). Immunological and epidemiological investigations in South African ostriches and penguins. PhD, University of Stellenbosch, Stellenbosch: 63-108.

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

Botes, A., Peyrot, B.M., Olivier, A.J., Burger, W.P. and Bellstedt, D.U. (2005b). Investigations into mycoplasma infections in South African ostriches. Proceedings of the 3rd International Ratite Science Symposium, 14-16 October, Madrid, Spain.

Bové, J.M. (1993). Molecular features of Mollicutes. *Clin. Infect. Dis.,* **17**(Suppl1): S10-31.

Bradbury, J.M. (2005). Poultry mycoplasmas: sophisticated pathogens in simple guise. *Br. Poult. Sci.,* **46**(2): 125-136.

Browning, G.F. and Markham, P.F. (2004). Determination of the genomic sequence of *Mycoplasma gallisepticum. Rural Industries Research and Development Corporation,* **4**: 1-8.

Butcher, G.D. (2002). Factors to consider in serologic testing for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). Available: http://edis.ifas.ufl.edu (Accessed on 30 September 2005).

Carson, J.L., Hu, P-C and Collier, A.M. (1992). Cell structural and functional elements. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 63-72. American Society for Microbiology, Washington, D.C..

Chaudhry, R., Nisar, N., Hora, B., Chirasani, S.R. and Malhotra, P. (2005). Expression and immunological characterization of the carboxy-terminal region of the P1 adhesin protein of *Mycoplasma pneumoniae*. *J. Clin. Microbiol.,* **43**(1): 321-325.

Cline, J.L., Turner, K.S., O'Connor, R.J., Gomez, L.B. Jr., Kleven, S.H.and Rowland, G.N. (1997). Determination of colonization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in ostriches. *Am. Ostrich,* Special Annual Research Issue, April: 12-13.

Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (1994). Mycoplasmas. In: Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (eds), *Infectious Diseases of livestock with special reference to South Africa (Vol. 2),* pp. 1483-1484. Oxford University Press, Cape Town, South Africa.

Crowther, J.R. (2000). Preface. In: Walker, J.M. (series ed), *The ELISA guidebook, Methods in molecular biology, Vol 149,* pp. vii. Humana Press, Totowa, New Jersey.

Deeming, D.C. (1999). Introduction. In: Deeming, D.C. (ed), *The Ostrich: Biology, production and health,* pp. 1-11. CAB International, Cambridge.

Doyle, J. and Doyle, J. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.*, **19**: 11-15.

Droual, R., Bickford, A.A., Charlton, B.R. and Kuney, D.R. (1990). Investigation of problems associated with intramuscular breast injection of oil-adjuvanted killed vaccines in chickens. *Avian Dis.*, **34**: 473-478.

Droual, R., Bickford, A.A and Cutler, G.J. (1993). Local reaction and serologic response in commercial layer chickens injected intramuscularly in the leg with oil-adjuvanted *Mycoplasma gallisepticum* bacterin. *Avian Dis.*, **37**: 1001-1008.

Dybvig, K. and Voelker, L.L. (1996). Molecular biology of Mycoplasmas. *Annu. Rev. Microbiol.,* **50**: 25-57.

Ellison, J.S., Olson, L.D. and Barile, M.F. (1992). Immunity and vaccine development. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 491-504. American Society for Microbiology, Washington, D.C..

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

Fadiel, A., Lithwick, S. and Naftolin, F. (2005). The influence of environmental adaptation on bacterial genome structure. *Lett. Appl. Microbiol,* **40**: 12-18.

Ferraz, P.N. and Danelli, M.d.G.M. (2003). Phenotypic and antigenic variation of *Mycoplasma gallisepticum* vaccine strains. *Braz. J. Microbiol.*, **34**: 238-241.

Freundt, E.A. (1973). Principles of Mycoplasma classification. In: Maramorosch, K. (ed), *Mycoplasma and Mycoplasma-like agents of human, animal and plant diseases,* pp. 7-13. N.Y. Acad. Sci., New York, USA.

Gerber, A. (2005). Vraag na volstruis in SA 5 keer groter ná verbod. *Die Burger,* 7 Nov: 14.

Glaser, G., Hyman, H.C. and Razin, S. (1992). Ribosomes. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 169-177. American Society for Microbiology, Washington, D.C..

Goh, M.S., Gorton, T.S., Forsyth, M.H., Troy, K.E. and Geary, S.J. (1998). Molecular and biochemical analysis of a 105 kDa *Mycoplasma gallisepticum* cytadhesin (GapA). *Microbiology,* **144**: 2971-2978.

Harley, E.H. (1998). DNA and Protein Sequence Alignment DAPSA. Computer Program Distributed as Shareware by the Author. University of Cape Town, Cape Town, South Africa.

Henrich, B., Feldman, R-C. and Hadding, U. (1993). Cytoadhesins of *Mycoplasma hominis*. *Infect. Immun.*, **61**(7): 2945-2951.

Henrich, B., Hopfe, M., Kitzerow, A. and Hadding, U. (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**(16): 4873-4878.

Henrich, B., Kitzerow, A., Feldmann, R-C., Schaal, H. and Hadding, U. (1996). Repetitive elements of the *Mycoplasma hominis* adhesin p50 can be differentiated by monoclonal antibodies. *Infect. Immun.*, **64**(10): 4027-4034.

Henrich, B., Lang, K., Kitzerow, A., MacKenzie, C. and Hadding, U. (1998). Truncation as a novel form of variation of the p50 gene in *Mycoplasma hominis*. *Microbiology*, **144**: 2979-2985.

Herrmann, R. (1992). Genome structure and organization. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 157-168. American Society for Microbiology, Washington, D.C..

Huchzermeyer, F.W. (1994). Transmissible diseases. In: Huchzermeyer, F.W., *Ostrich diseases,* pp. 7-45. Agricultural Research Council, South Africa.

Huchzermeyer, F.W. (1998a). The ostrich and other ratites. In: Huchzermeyer, F.W., *Diseases of Ostriches and other Ratites,* pp. 26-76. Agricultural Research Council, South Africa.

Huchzermeyer, F.W. (1998b). Transmissible diseases. In: Huchzermeyer, F.W., *Diseases of Ostriches and other Ratites,* pp. 149-195. Agricultural Research Council, South Africa.

Huchzermeyer, F.W. (1999). Veterinary problems. In: Deeming, D.C. (ed), *The Ostrich: Biology, production and health,* pp. 293-320. CAB International, Cambridge.

Huchzermeyer, F.W. (2002). Diseases of farmed crocodiles and ostriches. *Rev. sci. tech. Off. Int. Epiz.,* **21**(2): 265-276.

Hudson, L. and Hay, F.C. (1980). Initial preparations. In: Hudson, L. and Hay, F.C. (eds), *Practical Immunology, 2nd edition,* pp. 1-23. Blackwell Scientific Publications, Oxford, UK.

Jan, G., Le Henaff, M., Fontenelle, C. and Wroblewski, H. (2001). Biochemical and antigenic characterisation of *Mycoplasma gallisepticum* membrane proteins P52 and P67 (pMGA). *Arch. Microbiol.,* **177**: 81-90.

Jordan, F.T.W. (1979). Avian Mycoplasmas. In: Tully, J.G. and Whitcomb, R.F. (eds), *The Mycoplasmas, Vol 2, Human and animal mycoplasmas,* pp. 1-48. Academic Press Inc., New York.

Jordan, F.T.W. (1990a). Avian Mycoplasmoses. In: Jordan, F.T.W. (ed), *Poultry Diseases, 3rd edition,* pp. 74-85. Bailliere Tindall, Great Britain.

Pectora roborant cultus recti

Jordan, F.T.W. (1990b). Vaccines and Vaccination. In: Jordan, F.T.W. (ed), *Poultry Diseases, 3rd edition,* pp. 418-429. Bailliere Tindall, Great Britain.

Jordan, F.T.W. (1996). Avian Mycoplasmosis. In: Jordan, F.T.W. and Pattison, M. (eds), *Poultry Diseases, 4th Edition,* pp. 81-93. W.B. Saunders Company Ltd., London.

Karaca, K. and Lam, K.M. (1987). Efficacy of commercial *Mycoplasma gallisepticum* bacterin (MG-Bac) in preventing air-sac lesions in chickens. *Avian Dis.*, **31**: 202-203.

Kenny, G.E. (1992). Serodiagnosis. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 505-512. American Society for Microbiology, Washington, D.C..

Kimminau, K.M. (1993). Introducing the Ostrich. *Vet. Technician,* **14**(8): 459-467.

Klainer, A.S. and Pollack, J.D. (1973). Scanning electron microscopy techniques in the study of the surface structure of mycoplasmas. In: Maramorosch, K. (ed), *Mycoplasma and Mycoplasma-like agents of human, animal and plant diseases,* pp. 236-245. N.Y. Acad. Sci., New York, USA.

Kleven, S.H. (1997). *Mycoplasma synoviae* infection. In: Calnek, B.W. (ed), *Diseases of Poultry, 10th Edition,* pp. 220-228. The Iowa State University Press, Iowa, USA.

Kleven, S.H. and Baxter-Jones, C. (1997). *Mycoplasma iowae* infection. In: Calnek, B.W. (ed), *Diseases of Poultry, 10th Edition,* pp. 228-232. The Iowa State University Press, Iowa, USA.

Krause, D.C. (1998). *Mycoplasma pneumoniae* cytadherence: organization and assembly of the attachment organelle. *Trends Microbiol.,* **6**(1): 15-18.

Krause, D.C. and Balish, M.F. (2001). Structure, function, and assembly of the terminal organelle of *Mycoplasma pneumoniae*. *FEMS Microbiol. Lett.,* **198**: 1-7.

Layh-Schmitt, G. and Harkenthal, M. (1999). The 40- and 90-kDa membrane proteins (ORF6 gene product) of *Mycoplasma pneumoniae* are responsible for the tip structure formation and P1 (adhesin) association with the Triton shell. *FEMS Microbiol. Lett.*, **174**:143-149.

Layh-Schmitt, G., Podtelejnikov, A. and Mann, M. (2000). Proteins complexed to the P1 adhesin of *Mycoplasma pneumoniae*. *Microbiology*, **146**:71-747.

Levisohn, S. and Kleven, S.H. (2000). Avian mycoplasmosis (*Mycoplasma gallisepticum*). *Rev. sci. tech. Off. Int. Epiz.,* **19**(2): 425-442.

Ley, D.H. and Yoder, H.W. Jr. (1997). *Mycoplasma gallisepticum* infection. In: Calnek, B.W. (ed), *Diseases of Poultry, 10th Edition,* pp. 194-205. The Iowa State University Press, Iowa, USA.

Ley, E.C., Morishita, T.Y., Harr, B.S., Mohan, R. and Brisker, T. (2000). Serologic survey of slaughter-age ostriches (*Struthio camelus*) for antibodies to selected avian pathogens. *Avian Dis.*, **44**: 989-992.

Lin, M.Y. and Kleven, S.H. (1984). Evaluation of attenuated strains of *Mycoplasma gallisepticum* as vaccines in young chickens. *Avian Dis.*, **28**: 88-99.

Liu, T., Garcia, M., Levisohn, S., Yogev, D. and Kleven, S.H. (2001). Molecular variability of the adhesin-encoding gene *pvpA* among *Mycoplasma gallisepticum* strains and its application in diagnosis. *J. Clin. Microbiol.,* **39**(5): 1882-1888.

Maniloff, J. (1992). Phylogeny of mycoplasmas. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 549-559. American Society for Microbiology, Washington, D.C..

Maniloff, J. (1996). The minimal cell genome: "On being the right size". *Proc. Natl. Acad. Sci. USA,* **93**: 10004-10006.

Marin, A. and Oliver, J.L. (2003). GC-Biased mutation pressure and ORF lengthening. *J. Mol. Evol.,* **56**: 371-372.

McAuliffe, L., Ellis, R.J., Ayling, R.D. and Nicholas, R.A.J. (2003). Differentiation of *Mycoplasma* species by 16S Ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. *J. Clin. Microbiol.,* **41**(10): 4844-4847.

McAuliffe, L., Ellis, R.J., Lawes, J.R., Ayling, R.D. and Nicholas, R.A.J. (2005). 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. *J. Med. Microbiol.,* **54**: 731-739.

Morowitz, H.J. and Wallace, D.C. (1973). Genome size and life cycle of the mycoplasma. In: Maramorosch, K. (ed), *Mycoplasma and Mycoplasma-like agents of human, animal and plant diseases,* pp. 62-73. N.Y. Acad. Sci., New York, USA.

Morsy, M.A., Panangala, V.S., Van Santen, V.L. and Bird, R.C. (1993). Cloning and partial sequence analysis of a *Mycoplasma synoviae* DNA fragment encoding epitopes shared with the major adhesin P1 protein of *Mycoplasma pneumoniae*. *Avian Dis.*, **37**: 1105-1112.

Much, P., Winner, F., Stipkovits, L., Rosengarten, R. and Citti, C. (2002). *Mycoplasma gallisepticum*: influence of cell invasiveness on the outcome of experimental infection in chickens. *FEMS Immunol. Med. Microbiol.,* **34**: 181-186.

Mudahi-Orenstein, S., Levisohn, S., Geary, S.J. and Yogev, D. (2003). Cythadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. *Infect. Immun.,* **71**(7): 3812-3820.

Pectora roborant cultus recti

Nicholas, R.A.J., Ayling, R.D., Bidewell, C.A. and Daykin, J.M. (2002). Mycoplasmas affecting the eyes of poultry. *Vet. Rec.,* **150**(4): 119-120.

Nicolson, G.L., Nasralla, M.Y. and Nisolson, N.L. (1999). The pathogenesis and treatment of Mycoplasmal infections. *Antimicrob. Infect. Dis. News.,* **17**(11): 81-88.

Noormohammadi, A.H., Browning, G.F., Cowling, P.J., O'Rourke, D., Whithear, K.G. and Markham, P.F. (2002a). Detection of antibodies to *Mycoplasma gallisepticum* vaccine ts-11 by an autologous pMGA enzyme-linked immunosorbent assay. *Avian Dis.*, **46**: 405-411.

Noormohammadi, A.H., Browning, G.F., Jones, J. and Whithear, K.G. (2002b). Improved detection of antibodies to *Mycoplasma synoviae* vaccine MS-H using an autologous recombinant MSPB enzyme-linked immunosorbent assay. *Avian Pathol.*, **31**: 611-617.

Olson, N.O. (1984). *Mycoplasma synoviae* infection. In: Hofstad, M.S. (ed), *Diseases of Poultry, 8th Edition,* pp. 212-220. The Iowa State University Press, Iowa, USA.

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

Panigrahy, B., Grumbles, L.C. and Hall, C.F. (1981). Immunogenic potency of oil-emulsified *Mycoplasma gallisepticum* bacterins. *Avian Dis.*, **25**: 821-826.

Papazisi, L., Frasca, S., Gladd, M., Jr., Liao, X., Yogev, D. and Geary, S.J. (2002a). GapA and CrmA coexpression is essential for *Mycoplasma gallisepticum* cytadherence and virulence. *Infect. Immun.*, **70**(12): 6839-6845.

Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Nguyen, D.K., Swartzell, S., Madan, A., Mahairas, G. and Geary, S.J. (2003). The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain R_{low}. *Microbiology*, **149**: 2307-2316.

Papazisi, L., Silbart, L.K., Frasca, S. Jr., Rood, D., Liao, X., Gladd, M., Javed, M.A. and Geary, S.J. (2002b). A modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease. *Vaccine*, **20**: 3709-3719.

Papazisi, L., Troy, K.E., Gorton, T.S., Liao, X. and Geary, S.J. (2000). Analysis of cytadherence-deficient, GapA-negative *Mycoplasma gallisepticum* strain R. *Infect. Immun.,* **68**(12): 6643-6649.

Pattison, M. and Cook, J.K.A. (1996). Vaccines and Vaccination. In: Jordan, F.T.W. and Pattison, M. (eds), *Poultry Diseases, 4th Edition,* pp. 469-483. W.B. Saunders Company Ltd., London.

Perelman, B. (1999). Health management and veterinary procedures. In: Deeming, D.C. (ed), *The Ostrich: Biology, production and health,* pp. 321-346. CAB International, Cambridge.

Pectora roborant cultus recti

Pettersson, B., Tully, J.G., Bolske, G. and Johansson, K-E. (2000). Updated phylogenetic description of the *Mycoplasma hominis* cluster (Weisburg et al. 1989) based on 16S rDNA sequences. *Int. J. Syst. Evol. Microbiol.,* **50**: 291-301.

Razin, S. (1978). The Mycoplasmas. *Microbiol. Rev.,* **42**(2): 414-470.

Razin, S. (1992). Mycoplasma taxonomy and ecology. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 3-22. American Society for Microbiology, Washington, D.C..

Razin, S. (1999). Adherence of pathogenic Mycoplasmas to host cells. *Biosci. Rep.,* **19**(5): 367-372.

Razin, S. and Freundt, E.A. (1984). The Mycoplasmas. In: Krieg, N.R (ed), *Bergey's Manual of Systematic Bacteriology (Vol 1),* pp. 740-770. Williams & Wilkins, Baltimore, USA.

Razin, S. and Jacobs, E. (1992). Mycoplasma adhesion. *J. Gen. Microbiol.,* **138**(3): 407-422.

Razin, S., Yogev, D. and Naot, Y. (1998). Molecular biology and pathogenicity of Mycoplasmas. *Microbiol. Mol. Biol. Rev.,* **62**(4): 1094-1156.

Roberts, M.C. (1992). Antibiotic resistance. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 513-523. American Society for Microbiology, Washington, D.C..

Rocha, E.P.C. and Blanchard, A. (2002). Genomic repeats, genome plasticity and the dynamics of *Mycoplasma* evolution. *Nucleic Acids Res.,* **30**(9): 2031-2042.

Rottem, S. (2002). Sterols and acylated proteins in Mycoplasmas. *Biochem. Biophys. Res. Commun.,* **292**: 1289-1292.

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

Rottem, S. and Barile, M.F. (1993). Beware of mycoplasmas. *Trends Biotechnol.,* **11**: 143-151.

Rottem, S. and Naot, Y. (1998). Subversion and exploitation of host cells by mycoplasmas. *Trends Microbiol.,* **6**(11): 436-440.

Ruland, K., Himmelreich, R. and Herrmann, R. (1994). Sequence divergence in the ORF6 gene of *Mycoplasma pneumoniae. J. Bacteriol.*, **176**(17): 5202-5209.

Shivaprasad, H.L. (1993). Neonatal mortality in ostriches: An overview of possible causes. Proceedings of the Annual Conference of the Association of Avian Veterinarians, Nashville, 31 Aug - 4 Sept, pp. 282-293.

Simecka, J.W. (2005). Immune responses following mycoplasma infection. In: Blanchard, A. and Browning, G. (eds), *Mycoplasmas Molecular Biology Pathogenicity and Strategies for Control*, pp. 485-534. Horizon Bioscience, Norfolk, UK.

Simecka, J.W., Davis, J.K., Davidson, M.K., Ross, S.E., Städtlander, C.T.K.-H. and Cassell, G.H. (1992). Mycoplasma diseases of animals. In: Maniloff, J., McElhaney, E.D., Finch, L.R. and Baseman, J.B. (eds), *Mycoplasmas Molecular Biology and Pathogenesis,* pp. 391-415. American Society for Microbiology, Washington, D.C..

Skadhauge, E. and Dawson, A. (1999). Physiology. In: Deeming, D.C. (ed), *The Ostrich: Biology, production and health,* pp. 51-82. CAB International, Cambridge.

Trachtenberg, S. (1998). Mollicutes - Wall-less bacteria with internal cytoskeletons. *J. Struct. Biol.,* **124**: 244-256.

Van der Vyfer, A. (1992). Viewpoint: The world ostrich industry: will South Africa maintain its domination?. *Agrekon.,* **31**(1): 47-49.

Vasconcelos A.T.R., Ferreira, H.B., Bizarro, C.V., Bonatto, S.L., Carvalho, M.O., Pinto, P.M., Almeida, D.F., Almeida, L.G.P., Almeida, R., Alves-Filho, L., Assuncao, E.N., Azevedo, V.A.C., Bogo, M.R., Brigido, M.M., Brocchi, M., Burity, H.A., Camargo, A.A., Camargo, S.S., Carepo, M.S., Carraro, D.M., de Mattos Cascardo, J.C., Castro, L.A., Cavalcanti, G., Chemale, G., Collevatti, R.G., Cunha, C.W., Dallagiovanna, B., Dambros, B.P., Dellagostin O.A., Falcao, C., Fantinatti-Garboggini, F., Felipe, M.S.S., Fiorentin, L., Franco, G.R., Freitas, N.S.A., Frias, D., Grangeiro, T.B., Grisard, E.C., Guimaraes, C.T., Hungria, M., Jardim, S.N., Krieger, M.A., Laurino, J.P., Lima, L.F.A., Lopes, M.I., Loreto, E.L.S., Madeira, H.M.F., Manfio, G.P., Maranhao, A.Q., Martinkovics, C.T., Medeiros, S.R.B., Moreira, M.A.M., Neiva, M., Ramalho-Neto, C.E., Nicolas, M.F., Oliveira, S.C., Paixao, R.F.C., Pedrosa, F.O., Pena, S.D.J., Pereira, M., Pereira-Ferrari, L., Piffer, I., Pinto, L.S., Potrich, D.P., Salim, A.C.M., Santos, F.R., Schmitt, R., Schneider, M.P.C., Schrank, A., Schrank, I.S., Schuck, A.F., Seuanez, H.N., Silva, D.W., Silva, R., Silva, S.C., Soares, C.M.A., Souza, K.R.L., Souza, R.C., Staats, C.C., Steffens, M.B.R., Teixeira, S.M.R., Urmenyi, T.P., Vainstein, M.H., Zuccherato, L.W., Simpson, A.J.G. and Zaha, A. (2005). Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae. J. Bacteriol.*, **187**(16): 5568-5577.

Verwoerd, D.J. (2000). Ostrich diseases. *Rev. sci. tech. Off. Int. Epiz.,* **19**(2): 638-661.

Webster, R.G. (1998). DNA Vaccination: a Potential Future Strategy. In: Nichlolson, K.G., Webster, R.G. and Hay, A.J (eds), *Textbook of Influenza,* pp. 410-421. Blackwell Science.

Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., Maniloff, J. and Woese, R. (1989). A phylogenetic analysis of Mycoplasmas: basis for their classification. *J. Bacteriol.,* **171**(12): 6455-6467.

Whithear, K.G. (1996). Control of avian mycoplasmoses by vaccination. *Rev. sci. tech. Off. Int. Epiz.,* **15**(4): 1527-1553.

Winner, F., Markova, I., Much, P., Lugmair, A., Siebert-Gulle, K., Vogl, G., Rosengarten, R. and Citti, C. (2003). Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. *Infect. Immun.,* **71**(3): 1265-1273.

Winner, F., Rosengarten, R. and Citti, C. (2000). In vitro cell invasion of *Mycoplasma gallisepticum*. *Infect. Immun.,* **68**(7): 4238-4244.

Wise, K.S. (1993). Adaptive surface variation in mycoplasmas. *Trends Microbiol.,* **1**(2): 59-63.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Direct gene transfer into mouse muscle in vivo. *Science,* **247**: 1465-1468.

Wolgemuth, C.W., Igoshin, O. and Oster, G. (2003). The motility of Mollicutes. *Biophys. J.,* **85**: 828-842.

www.mgc.ac.cn/cgibin/VFs/vfs.cgi?Genus=Mycoplasma&Keyword=Adherence, Identified virulence factors of Mycoplasma: Adherence. (Accessed on 6 October 2005).

www.promega.com/vectors (Accessed on 22 July 2004).

www.saobc.co.za/modules.php?name=News&file=article&sid=19, The South African ostrich industry (9 May 2004). (Accessed on 19 December 2005).

www.saobc.co.za/modules.php?name=News&file=article&sid=31, Voëlgriep lei tot verbod op uitvoer (9 Augustus 2004). (Accessed on 19 December 2005).

www.saobc.co.za/modules.php?name=News&file=article&sid=32, Verbod deur Europese Unie (12 Augustus 2004). (Accessed on 19 December 2005).

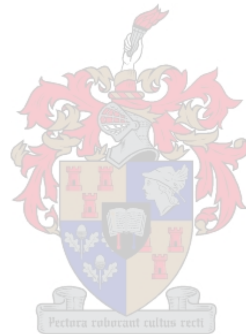
www.saobc.co.za/modules.php?name=News&file=article&sid=51, The European Union (EU) approved the resumption of imports (3 November 2005). (Accessed on 19 December 2005).

Yamamoto, R. and Ghazikhanian, G.Y. (1997). *Mycoplasma meleagridis* infection. In: Calnek, B.W. (ed), *Diseases of Poultry, 10th Edition,* pp. 208-219. The Iowa State University Press, Iowa, USA.

Yoder, H.W. Jr. (1984). *Mycoplasma gallisepticum* infection. In: Hofstad, M.S. (ed), *Diseases of Poultry, 8th Edition,* pp. 190-201. The Iowa State University Press, Iowa, USA.

Pectora roborant cultus recti

Zaki, M.M., Ferguson, N., Leiting, V. and Kleven, S.H. (2004). Safety of *Mycoplasma gallisepticum* vaccine strain 6/85 after backpassage in turkeys. *Avian Dis.*, **48**: 642-646.



Appendix A Nucleotide sequence alignment of domain B

Appendix A Nucleotide sequence alignment of the domain B region of mycoplasma cytadhesin as well as cytadhesin-related molecules. The computer program DAPSA was used for manual multiple sequence alignment of the nucleic acid sequences of *M. gallisepticum* GapA and CrmA (GapA and crmA), *M. pneumoniae* P1 and ORF6 (pneuP1 and orf6), *M. genitalium* MgpB and MgpC (mgpB and mgpC) and *M. pirum* P1-like (pirumP1). Primers for primer approach 2 were developed in the conserved areas which are highlighted in the alignment.

```
                                                                     60
GapA        ---------- ---------- ---------- ---------- ---------- ----------
pneuP1      ---------- ---------- ---------- ---------- ---------- ----------
mgpB        ---------- ---------- ---------- ---------- ---------- ----------
pirumP1     ---------- ---------- ---------- ---------- ---------- ----------
crmA        ---------- ---------- ----GATTTC TGAGGAACAA TCCAATTCAA ACCCGATGAG
orf6        ---------- ---------- ---------- ---------G ATATTTGGGG CAGAGTGGAT
mgpC        ---------- ---------- ---------- ---------G ATGCATGGGG TAAAGTTGAG

                                                                    120
GapA        ---------- ---------- ---------- ---------- ---------- ----------
pneuP1      ---------- ---------- ---------- ---------- ---------- ----------
mgpB        ---------- ---------- ---------- ---------- ---------- ----------
pirumP1     ---------- ---------- ---------- ---------- ---------- ----------
crmA        -----TACTT AATTCAAAAT GGGTTCACTA GTCAAGTGGC TAGAAAC-TT CGTTACAAAC
orf6        TTTGCTGCCA ACAGTGTTTT GCAAGCGCGT AACCTCACTG ATAAAACGGT TGATGAGGTG
mgpC        TTTGCTGATA ACAGTGTATT GCAAGCAAGA AACCTAGTTG ATAAAACTGT TGATGAGATC

                                                                    180
GapA        ---------- ---------- ---------- ---------- ---------- ----------
pneuP1      ---------- ---------- ---------- ---------- ---------- ----------
mgpB        ---------- ---------- ---------- ---------- ---------- ----------
pirumP1     ---------- ---------- ---------- ---------- ---------- ----------
crmA        CAAAGCTTCT TAAACAGTTT AGTTGACTTC ACTCCTGCTA ATGCTGGTAC TAACTACCGT
orf6p       ATCAATAACC CCGATATCCT CCAAAGCTTC TTTAAGTTTA CCCCAGCCTT TGATAACCAA
mgpC        ATCAATACCC CTGAAATCTT AAACTCCTTC TTTAGATTCA CCCCTGCTTT TGAAGATCAA

                                                                    240
GapA        ---------- ---------- ---------- ---------- ---------- ----------
pneuP1      ---------- ---------- ---------- ---------- ---------- ----------
mgpB        ---------- ---------- ---------- ---------- ---------- ----------
pirumP1     ---------- ---------- ---------- ---------- ---------- ----------
crmA        GTAGTGGTTG ATCCTGATGG TAATTTAACA AACCAAAACC TACCTCTAAA AGTTCAGATC
orf6        AGAGCAATGC TAGTGGGGGA AAAGACATCG GATACTACCT TAACGGTTAA ACCGAAGATT
mgpC        AAAGCTACCC TTGTTGCTAC TAAGCAAAGT GATACATCAC TTAGTGTCTC ACCAAGGATC

                                                                    300
GapA        ---------- ---------- ---------- ---------- ---------- ----------
pneuP1      ---------- ---------- ---------- ---------- ---------- ----------
mgpB        ---------- ---------- ---------- ---------- ---------- ----------
pirumP1     ---------- ---------- ---------- ---------- ---------- ----------
crmA        CAATACTTAG ATGGTAAGTA TTATGATGCT AAAT------ -TA------- ----------
orf6        GAGTACTTGG ATGGTAACTT CTATGGTGAG GATTCCAAGA TTGCTGGAAT TCCGCTCAAC
mgpC        CAGTTCTTAG ATGGTAATTT CTATGATCTT AACTCTACCA TCGCTGGGGT ACCTTTAAAC
```

Pectora roborant cultus recti

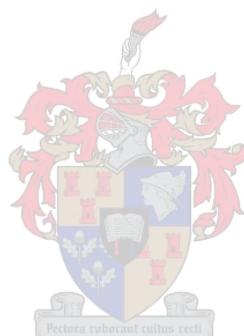
```
                                                                             360
GapA          ---------- ---------- ---------- ---------- ---------- --------CA
pneuP1        ---------- ---------- ---------- ---------- ---------- ----------
mgpB          ---------- ---------- ---------- ---------- ---------G GTCCCCAAAC
pirumP1       ---------- ---------- ---------- -------AAA ATTAATGTTA TAAATAATTC
crmA          ---------- ---------- ---------- ---------- ---------- ----------
orf6          ATTGATTTC- ---------- ---------- ---------- ---------T TCCCTTCC.G
mgpC          ATTGGTTTC- ---------- ---------- ---------- ---------- ----------

                                                                             420
GapA          AGAATTTACT GGTTTTGATG CGCTTCCAGG TTATGTATTA CCAGTAGCGA TCTCGATTCC
pneuP1        ---------- ---...A.CC A.TGA..T.A ...C..G..G ..GT...... ..A.TG.A..
mgpB          T.TC...CAA CCC...A.CC A.TGGG...A C.....C... ..TT.GATTG .AA.TG....
pirumP1       TATT...G.. ..A...AG.. .AA.GG.T.A ..GAA.TC.T ..T....TT. .TG.T.....
crmA          --T..AACAA C--....C.. .TT.A..TTC A.GA...G.G ..TAC...A. .TGGT.G.A.
orf6          GATT...G.. ..C....C.. .TT.A..GTC C.GG..CA.T ..G...T.AG ..GGTTCAT.
mgpC          -------G.. ..G....CA. .A..C..T.C A.GG..GA.C ..T...T.AG .AGGTTC.T.

                                                                             480
GapA          GATCATCATA ATTG--CCTT -GGCATTAG- CTTTAGGTCT -AGG-TATTG GTATTCCAAT
pneuP1        T..TG.TG.G ....T-G..C -A.TG...C- ...-...A.. TGCC--.... .A..C.....
mgpB          T..AG.AG.G ...AT-.... -A.TG...-- ....G..A-. T.ACG-.... .A........
pirumP1       C..TG.AT.. G...--.A.. AATA...G.- -.......--. TGCAG..... .G........
crmA          AT.AGGT..T C...-CAA.. A-TG..-CAT ...-...A-. T..C-...C. .......TT.
orf6          .G.GGG...T C-.CTTAA.C -CTGC.-CAT ...-...C.. TG.--..... .A........
mgpC          AG.TGGG..- C...TTTA.C -TTG-....T ...-...A.. TG..--.... .G..C.....

                                                                             540
GapA          GTCTCAAAAC CGTAAGATGT TGAAACAAGG ATTTGCGATT TCAAACAAAA AAGTTGATAT
pneuP1        .CACA.G... AAAC..GCC. ....GGCT.. G.....C.A ......C... .G......G.
mgpB          .CACAG.... AAA...GCA. .AC..GC... G....ATC.. ..T....... .G......G.
pirumP1       .G..A..C.T AAA..AGCTA .T...GTT.. T....AAT.G CA.C..G.T. ......GA.C
crmA          AAGAGCTC.A A.A..AT.AC AAG.CA.... G..CAAA.CA A..TT..... .........C
orf6          ..A.A.GGT. ..C...C.TC AAG.CTCCA. C....TTGA. GTGTTT.... .G..G....C
mgpC          ..ACAGGGTA A.A..AC.CC AAG.TGC.TC G....TT.A. GTCTTT.... .G.......C

                                                                             600
GapA          TCTGACAACA GCCGTTGGTA GTGTGTTCAA ACAAATTATT AATCGAAC-- -------ATC
pneuP1        GT....C.A. ..G....... ....C..T.. GG....C... ..C..C..-- -------.GG
mgpB          CT....C.A. ..A....... ....C..T.. .G.G..C... ..CA....-- -------.GG
pirumP1       AT....TT.. ..T......G ....T..... .A........ G.CAAT..AA ATTCTAATAA
crmA          CT....TG.T ..T......T CA..T.A... GA.G...... .CC.A...-- -------TG.
orf6          GT.......C ..T..G.... .C....A... GA.G.....C .CC.A...-- -------GAG
mgpC          A..C.....T ..T..C.... ......A... .A.G...... .CC.A...-- -------TGG

                                                                             660
GapA          TGTGA--CAA ATATT----A A-------GA AGA---CY-- CCACAAA--T GCTTC-AAGC
pneuP1        .A.C.GT.-. .---GCGCC. .AACGCTT.. .A----.AAA ...G-----. ..GG.T..A.
mgpB          GA.C--T.T. .C--GCTCCT .AGAAGTTA. .A----.AAG .T..CCC--A A.C---..A.
pirumP1       ....----.. ..------CT ---------- -------AAA .......--. ....-A.G..
crmA          .AACGTTA.G .A.AAA-CCT ---------- ---------- -----GC--. ....T-.G.-
orf6          .....-T... .A.AGCTCCT .GTGCGTT.. .AGCTG.TAA -T.ACGC--. ...C.T..A-
mgpC          ....------ ---------- ---------- ---------- ---------- ----------
```

```
                                                                    720
GapA        CAACAAGAAA GATGGAGCAT C--TT---CA CCAAGCAAGC CATCAGCTCC AGCTGCTAAG
pneuP1      ..-------- ---......C .--CCGCC.. ..----.GTA .--..C.-AA ...--.AGG.
mgpB        ....TCCT.. A.-------- -----CCC.. ....AACCT. ..G------- -----TA..A
pirumP1     TGCAGCT... A.ACC.AATA .AG..--C.. ..TGCT.GAT .TCA.TTAA. .AA..A.TCT
crmA        TGCTGGT... TC...T.ATA AGAAA----- ..-TCTGCTG .TG.TAAA.. T.....---
orf6        -GCA------ ---------- ---------- ...GTT..A. ..G.T..... .A.A...CCA
mgpC        ---------- ---------- ---------- ---------- ---------- ----------

                                                                    780
GapA        AAACCAGCAG GACCAACTAA ACCATCT--G CTCCAGGGGC AAAACCAACA GCACCAGCTA
pneuP1      GCT..TAAGC C....GTGC. ....C..--A AAAA.CCC.. T--------- ----------
mgpB        C..------- ---------- ---------- ---------- ---------- ----------
pirumP1     GTTT.TAG.C CCA.TC.ACC .T.....--- ---------- ---------- ----------
crmA        ---------- --...G.... .......--. .A...AAA.. T.GCT..C.. ..TAA.C.A-
orf6        .G....---- --...GTCC. ....C..--- --AA.AA... T--------- ----------
mgpC        ---------- ---------- ---------- ---------- ---------- ----------


GapA        AACCAAAAGC TCCAGCACCA ACTAAGAAAA TTGAA
pneuP1      ---------- ---------- ---------- -----
mgpB        ---------- ---------- ---------- -----
pirumP1     ---------- ---------- ---------- -----
crmA        ---------- ---------- ---------- -----
orf6        ---------- ---------- ---------- -----
mgpC        ---------- ---------- ---------- -----
```

Appendix B Vaccine trial in ostriches: ELISA results

Farm 1: 3 month old ostrich chicks

Treatment	Time	Response
1	0	0.081
1	0	0.022
1	0	0.061
1	0	0.073
1	0	0.027
1	0	0.042
1	0	0.101
1	0	0.001
1	0	0.125
1	0	0.002
1	7	0.081
1	7	0.043
1	7	0.034
1	7	0.043
1	7	0.023
1	7	0.020
1	7	0.099
1	7	0.011
1	7	0.055
1	7	0.006
1	14	0.087
1	14	0.110
1	14	0.029
1	14	1.143
1	14	0.013
1	14	0.077
1	14	0.060
1	14	0.655
1	14	0.034
1	14	0.718
1	21	0.099
1	21	0.363
1	21	0.055
1	21	1.673
1	21	0.228
1	21	0.162
1	21	0.087
1	21	2.615
1	21	0.088
1	21	0.445
2	0	0.072
2	0	0.000
2	0	0.031
2	0	0.027
2	0	0.004



Farm 1: 3 month old ostrich chicks

Treatment	Time	Response
2	0	0.094
2	0	0.071
2	0	0.110
2	0	0.011
2	0	0.151
2	7	-
2	7	0.036
2	7	0.137
2	7	0.035
2	7	0.000
2	7	0.193
2	7	0.028
2	7	0.102
2	7	0.006
2	7	0.035
2	14	-
2	14	0.538
2	14	0.596
2	14	0.524
2	14	0.070
2	14	0.433
2	14	0.133
2	14	0.611
2	14	1.773
2	14	0.463
2	21	-
2	21	0.390
2	21	1.483
2	21	2.159
2	21	0.333
2	21	0.295
2	21	0.336
2	21	0.287
2	21	3.226
2	21	0.279
3	0	0.129
3	0	0.066
3	0	0.002
3	0	0.079
3	0	0.141
3	0	0.008
3	0	0.078
3	0	0.005
3	0	0.057
3	0	0.056

Farm 1: 3 month old ostrich chicks

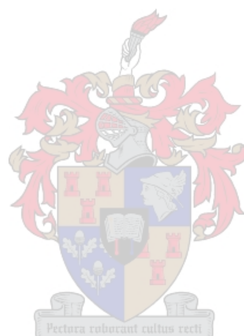
Treatment	Time	Response
3	7	0.118
3	7	0.027
3	7	0.000
3	7	0.069
3	7	0.054
3	7	0.045
3	7	0.026
3	7	0.001
3	7	0.075
3	7	0.041
3	14	0.056
3	14	0.024
3	14	0.017
3	14	0.150
3	14	0.115
3	14	0.031
3	14	0.048
3	14	0.027
3	14	0.019
3	14	0.045
3	21	0.071
3	21	0.097
3	21	0.128
3	21	0.228
3	21	0.070
3	21	0.160
3	21	0.044
3	21	0.048
3	21	0.079
3	21	0.068

Pectora roborant cultus recti

KEY
1 = Group A (*M. synoviae* vaccine)
2 = Group B (*M. gallisepticum* vaccine)
3 = Control (no vaccine)

Farm 2: 4-5 month old ostrich chicks

Treatment	Time	Response
1	0	0.054
1	0	0.040
1	0	0.071
1	0	0.073
1	0	0.124
1	0	0.131
1	0	0.080
1	0	0.105
1	0	0.093
1	0	0.120
1	7	0.119
1	7	0.073
1	7	0.102
1	7	0.117
1	7	0.099
1	7	0.113
1	7	0.079
1	7	0.116
1	7	0.074
1	7	0.100
1	14	0.408
1	14	0.119
1	14	0.100
1	14	0.117
1	14	0.081
1	14	0.369
1	14	0.114
1	14	0.797
1	14	0.104
1	14	0.234
1	21	1.098
1	21	0.234
1	21	0.141
1	21	0.355
1	21	0.649
1	21	2.160
1	21	0.127
1	21	2.724
1	21	1.361
1	21	0.425
2	0	0.081
2	0	0.064
2	0	0.093
2	0	0.083
2	0	0.086



Farm 2: 4-5 month old ostrich chicks

Treatment	Time	Response
2	0	0.063
2	0	0.093
2	0	0.047
2	0	0.065
2	0	0.068
2	7	0.086
2	7	0.089
2	7	0.086
2	7	0.095
2	7	0.092
2	7	0.077
2	7	0.100
2	7	0.083
2	7	0.059
2	7	0.083
2	14	0.351
2	14	0.142
2	14	0.368
2	14	0.835
2	14	0.061
2	14	0.117
2	14	0.341
2	14	0.144
2	14	0.414
2	14	0.120
2	21	1.224
2	21	0.215
2	21	0.301
2	21	1.875
2	21	0.210
2	21	0.292
2	21	0.572
2	21	0.541
2	21	0.739
2	21	1.186
3	0	0.069
3	0	0.055
3	0	0.166
3	0	0.048
3	0	0.083
3	0	0.080
3	0	0.074
3	0	0.054
3	0	0.102
3	0	0.043

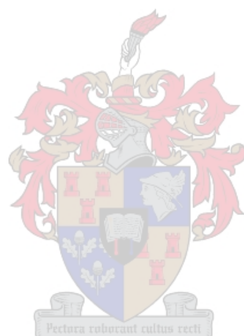
Farm 2: 4-5 month old ostrich chicks

Treatment	Time	Response
3	7	0.120
3	7	0.108
3	7	0.252
3	7	0.107
3	7	0.184
3	7	0.116
3	7	0.125
3	7	0.083
3	7	0.085
3	7	0.052
3	14	0.183
3	14	0.340
3	14	0.204
3	14	0.053
3	14	0.165
3	14	0.148
3	14	0.163
3	14	0.067
3	14	0.167
3	14	0.118
3	21	0.023
3	21	0.045
3	21	0.114
3	21	0.049
3	21	0.127
3	21	-
3	21	0.115
3	21	0.038
3	21	0.088
3	21	0.026

KEY
1 = Group A (*M. synoviae* vaccine)
2 = Group B (*M. gallisepticum* vaccine)
3 = Control (no vaccine)

Farm 3: 6-7 month old ostrich chicks

Treatment	Time	Response
1	0	0.080
1	0	0.004
1	0	0.052
1	0	0.054
1	0	0.070
1	0	0.063
1	0	0.147
1	0	0.133
1	0	0.094
1	0	0.088
1	0	0.068
1	0	0.015
1	0	0.102
1	0	0.068
1	0	0.090
1	0	0.052
1	0	0.067
1	0	0.082
1	0	0.101
1	0	0.106
1	7	0.120
1	7	0.042
1	7	0.077
1	7	0.115
1	7	0.176
1	7	0.128
1	7	0.112
1	7	0.236
1	7	0.102
1	7	0.040
1	7	0.046
1	7	0.081
1	7	0.030
1	7	0.068
1	7	0.144
1	7	0.060
1	7	0.168
1	7	0.035
1	7	0.042
1	7	0.065
1	14	0.169
1	14	0.104
1	14	0.249
1	14	0.422
1	14	0.328
1	14	0.367
1	14	0.231

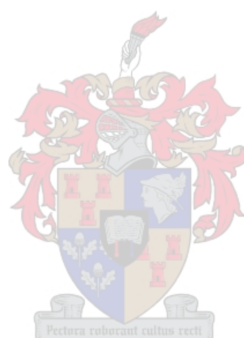


Farm 3: 6-7 month old ostrich chicks

Treatment	**Time**	**Response**
1	14	0.191
1	14	0.108
1	14	0.660
1	14	0.080
1	14	0.043
1	14	0.149
1	14	0.095
1	14	0.322
1	14	0.054
1	14	0.598
1	14	0.043
1	14	0.028
1	14	0.323
1	21	0.117
1	21	0.045
1	21	0.064
1	21	0.412
1	21	0.425
1	21	0.337
1	21	0.319
1	21	0.282
1	21	0.094
1	21	1.140
1	21	0.064
1	21	0.064
1	21	0.298
1	21	0.076
1	21	0.335
1	21	0.084
1	21	0.381
1	21	0.066
1	21	0.049
1	21	0.143
2	0	0.024
2	0	0.066
2	0	0.093
2	0	0.116
2	0	0.068
2	0	0.086
2	0	0.080
2	0	0.085
2	0	0.079
2	0	0.063
2	0	0.058
2	0	0.079
2	0	0.105
2	0	0.074

Farm 3: 6-7 month old ostrich chicks

Treatment	Time	Response
2	0	0.092
2	0	0.074
2	0	0.068
2	0	0.095
2	0	0.057
2	0	0.087
2	7	0.054
2	7	0.045
2	7	0.741
2	7	0.089
2	7	0.186
2	7	0.045
2	7	0.114
2	7	0.096
2	7	0.022
2	7	0.126
2	7	0.000
2	7	0.047
2	7	0.148
2	7	0.079
2	7	0.352
2	7	0.168
2	7	0.106
2	7	0.055
2	7	0.000
2	7	0.081
2	14	0.100
2	14	0.223
2	14	1.216
2	14	0.235
2	14	0.109
2	14	0.189
2	14	0.412
2	14	0.471
2	14	0.124
2	14	0.118
2	14	0.915
2	14	0.216
2	14	1.068
2	14	0.721
2	14	0.374
2	14	0.292
2	14	0.674
2	14	0.115
2	14	0.057
2	14	0.332

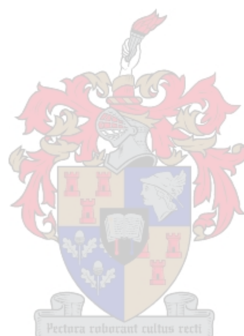


Farm 3: 6-7 month old ostrich chicks

Treatment	Time	Response
2	21	0.071
2	21	0.326
2	21	0.774
2	21	0.354
2	21	0.058
2	21	0.118
2	21	0.444
2	21	1.221
2	21	0.096
2	21	0.250
2	21	1.056
2	21	0.097
2	21	0.975
2	21	0.835
2	21	0.528
2	21	0.200
2	21	0.625
2	21	0.229
2	21	0.031
2	21	0.254
3	0	0.123
3	0	0.137
3	0	0.058
3	0	0.006
3	0	0.028
3	0	0.062
3	0	0.064
3	0	0.058
3	0	0.075
3	0	0.118
3	0	0.027
3	0	0.038
3	0	0.023
3	0	0.011
3	0	0.030
3	0	0.096
3	0	0.120
3	0	0.132
3	0	0.050
3	0	0.078
3	7	0.038
3	7	0.100
3	7	0.052
3	7	0.042
3	7	0.037
3	7	0.074
3	7	0.052

Farm 3: 6-7 month old ostrich chicks

Treatment	Time	Response
3	7	0.083
3	7	0.079
3	7	0.141
3	7	0.000
3	7	0.012
3	7	0.029
3	7	0.043
3	7	0.040
3	7	0.048
3	7	0.063
3	7	0.162
3	7	0.012
3	7	0.000
3	14	0.083
3	14	0.165
3	14	0.095
3	14	0.075
3	14	0.081
3	14	0.184
3	14	0.122
3	14	0.055
3	14	0.090
3	14	0.108
3	14	0.066
3	14	0.025
3	14	0.108
3	14	0.061
3	14	0.093
3	14	-
3	14	0.181
3	14	0.190
3	14	0.126
3	14	0.129
3	21	0.033
3	21	0.207
3	21	0.072
3	21	0.045
3	21	0.084
3	21	0.138
3	21	0.092
3	21	0.142
3	21	0.147
3	21	0.093
3	21	0.055
3	21	0.035
3	21	0.040
3	21	0.069



Farm 3: 6-7 month old ostrich chicks

Treatment	Time	Response
3	21	0.042
3	21	-
3	21	0.149
3	21	0.202
3	21	0.102
3	21	0.305

KEY

1 = Group A (*M. synoviae* vaccine)
2 = Group B (*M. gallisepticum* vaccine)
3 = Control (no vaccine)

Appendix C Statistical analysis of ELISA results using SAS

Farm 1: 3 month old ostrich chicks

The SAS System
General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	3	1 2 3
TIME	4	0 7 14 21

Number of observations in data set = 120
Due to missing values, only 117 observations can be used in this analysis

Dependent variable: RESP

Source	DF	Sum of squares	Mean Square	F Value	Pr > F
Model	11	9.65591576	0.87781052	4.72	<.0001
Error	105	19.5137618	0.18584535		
Corrected Total	116	29.16967756			

R-Square		Coeff Var	Root MSE	RESP Mean	
0.331026		184.3174	0.431098	0.23389	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	2.25637725	1.12818862	6.07	0.0032
TIME	3	4.77109337	1.59036446	8.56	<.0001
TRT*TIME	6	2.62844513	0.43807419	2.36	0.0355

Input data: see Appendix B, Farm 1

Farm 2: 4-5 month old ostrich chicks

The SAS System
General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	3	1 2 3
TIME	4	0 7 14 21

Number of observations in data set = 120
Due to missing values, only 119 observations can be used in this analysis

Dependent variable: RESP

Source	DF	Sum of squares	Mean Square	F Value	Pr > F
Model	11	8.66967587	0.78815235	7.55	<.0001
Error	107	11.16406752	0.10433708		
Corrected Total	118	19.83374339			

R-Square		Coeff Var	Root MSE	RESP Mean	
0.437117		130.3884	0.323013	0.247731	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	1.16945611	0.58472806	5.60	0.0048
TIME	3	4.74995194	1.58331731	15.18	<.0001
TRT*TIME	6	2.75026782	0.45837797	4.39	0.0005

Input data: see Appendix B, Farm 2

Farm 3: 6-7 month old ostrich chicks

The SAS System
General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	3	1 2 3
TIME	4	0 7 14 21

Number of observations in data set = 240
Due to missing values, only 238 observations can be used in this analysis

Dependent variable: RESP

Source	DF	Sum of squares	Mean Square	F Value	Pr > F
Model	11	3.64548919	0.33140811	10.04	<.0001
Error	226	7.45659051	0.03299376		
Corrected Total	237	11.10207970			

R-Square		Coeff Var	Root MSE	RESP Mean	
0.328361		108.2989	0.181642	0.167723	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	1.20135840	0.60067920	18.21	<.0001
TIME	3	1.72300577	0.57433526	17.41	<.0001
TRT*TIME	6	0.72112501	0.12018750	3.64	0.0018

Input data: see Appendix B, Farm 3

Farm 1, 2 & 3

The SAS System
General Linear Models Procedure
Class Level Information

Class	Levels	Values
Farm	3	1 2 3
TRT	3	1 2 3
TIME	4	0 7 14 21

Number of observations in data set = 480
Due to missing values, only 474 observations can be used in this analysis

Dependent variable: RESP

Source	DF	Sum of squares	Mean Square	F Value	Pr > F
Model	13	17.49878953	1.34606073	14.32	<.0001
Error	460	43.25201800	0.09402613		
Corrected Total	473	60.75080753			

R-Square		Coeff Var	Root MSE	RESP Mean	
0.288042		150.2081	0.306637	0.204141	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
FARM	2	0.64530688	0.32265344	3.43	0.0332
TRT	2	3.80547711	1.90273855	20.24	<.0001
TIME	3	9.03725809	3.01241936	32.04	<.0001
TRT*TIME	6	4.01074745	0.66845791	7.11	<.0001