Assessment of vaccine delivery systems and their impact on the enhancement of immunogenicity, potency and safety of specific livestock vaccines used in South Africa

by

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Submitted in partial fulfilment of the requirements of the degree
Philosophiae Doctor
in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

August 2011

DECLARATION

I declare that the dissertation, which I hereby submit for the degree PhD (Microbiology) at the University of Pretoria, is my own work and had not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …….. Date: ……..

ACKNOWLEDGEMENTS

The completion of this work would not have been possible without the collective efforts by different people and institutions.

I extend my sincere appreciations to my promoter, Professor Jacques Theron, of the University of Pretoria, for his guidance and tireless advice in completing this work.

I am indebted to the personnel of Onderstepoort Biological Products, who made it possible to conduct most of the work covered in this thesis. More specifically, I would like to thank Mr. Benny Brett, for his extensive work on oil evaluation, emulsifier's analyses and evaluation as well as different vaccine assessments. I am thankful to the personnel of the Bacterial vaccine production department, for all the manufacturing activities around the project, to the personnel in the Bacterial R&D department, Mr. Raynard Macdonald, for assisting with the Coryza vaccine work; to the personnel in the Quality Control department, under the guidance of Mr. Ben van Biljoen, for assisting with laboratory evaluations of the different emulsions and vaccines.

Special thanks to Professor Robert Bragg, of the University of the Free State, for participating in the work on the Coryza vaccine, including all the chicken work conducted at his laboratory and the Glen Agriculture Centre.

Special thanks also to the scientists at the firm SEPPIC, for supplying some of the emulsifiers used in the study, and contributing to the work on Coryza vaccine, as well as advises on other formulations conducted.

Lastly but not least, I would like to thank my family, including my parents, for their continued support, love, and understanding during the long hours often spent on this project, making me unavailable to a number of family activities.

SUMMARY

Assessment of vaccine delivery systems and their impact on the enhancement of immunogenicity, potency and safety of specific livestock vaccines used in South Africa

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Since its establishment in 1908, the Onderstepoort Veterinary complex has developed more than 50 veterinary vaccines that have been used over the period in the control of animal viral and bacterial diseases in Southern Africa and elsewhere around the world. These vaccines have been either live attenuated or inactivated. The inactivated vaccines are generally formulated with an aqueous or an oil adjuvant. Onderstepoort oil-adjuvanted vaccines have been formulated with the old Freund's formulation, using surfactants that are not always compliant with current regulatory requirements. Other disadvantages associated with use of the traditional Freund's formulation include safety in vaccinated animals, unavailability of certain reagents or components, as well as their impact to the cost of the final vaccine.

The present study was designed to assess four oil-adjuvanted Onderstepoort vaccines, *i.e.* the *E. coli*, the enterotoxaemia or Pulpy kidney, the *Vibrio* or *Campylobacter fetus* and the Infectious coryza vaccines. The first three vaccines are formulated with a surfactant that is no longer readily available and is not registered by regulatory authorities, while the surfactant used in the Infectious coryza vaccine tends to result in adverse reactions in vaccinated chickens. The traditional liquid paraffin Marcol was compared to two new liquid paraffin white oils (PFP8 and PFP14), while the traditional emulsifiers Cirrasol EN-MP® and Arlacel® were compared to two new products, Montanide 103™ and Simulsol-P2®. In addition, Cirrasol EN-MP® was compared to Arlacel®, given the fact that the latter is an approved emulsifier for use in animal vaccines. Two ready-to-use emulsifiers, *i.e.* Montanide™ ISA 70 VG and Montanide™ ISA 206, were also tested with the Infectious coryza vaccine.

The three liquid paraffins and four surfactants were evaluated for their physical characteristics, as well as their ability to generate a safe and effective vaccine when formulated with the above four vaccine antigens. The safety and efficacy of the different formulations were evaluated in both laboratory animals and target animals, *i.e.* sheep, cattle and chicken. The results obtained can be summarized as follows. (i) The two new liquid paraffins had similar physical characteristics as the traditional Marcol, and they also did not cause adverse reactions in vaccinated animals; (ii) Simulsol-P2® displayed a poor ability to form a stable emulsion for the above vaccines; (iii) Montanide 103™ was stable and was safe in most of the vaccines, but caused persistent local reactions in the *E. coli* vaccine; and (iv) the Arlacel® formulations were stable, safe and showed better immunogenicity profiles as compared to the other formulations.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
AHS	African horsesickness
APS	antigen presenting cells
BSE	bovine spongiform encephalitis
BT	bluetongue
C	carbon
CBPP	contagious bovine pleuropneumonia
CFA	complete Freund's adjuvant
cm	centimetre
CNMR	carbon nuclear magnetic resonance
CSF	classical swine fever
CTL	cytolytic T cell
DC	dendritic cells
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
e.g.	for example
FAO	Food and Agriculture Organization of the United Nations
Fig.	figure
FMD	foot-and-mouth disease
g	gram
GDP	gross domestic product
h	hour
HB	Hepatitis B
HLB	hydrophilic-lipophilic balance
HNMR	hydrogen nuclear magnetic resonance
HVT	herpesvirus of turkeys
ID	identity
i.e.	that is
IC	infectious coryza
IFA	incomplete Freund's adjuvant
IFN	interferon

Ig	immunoglobulin
IL	interleukin
I.M.	intra-muscular
i.v.	intravenous
L	litre
L+	mouse neutralization test
LD_{50}	lethal dose, 50%
LPS	lipopolysaccharide
MHC	major histocompatability complex
min	minute
ml	millilitre
mPa/s	millipascal per second
NMR	nuclear magnetic resonance
No.	number
O/W	oil-in-water
OBP	Onderstepoort Biological Products
OVI	Onderstepoort Veterinary Institute
PK	Pulpy kidney
PV	post-vaccination
rpm	revolutions per minute
RT	room temperature
RVF	Rift Valley fever
SAT	serum agglutination test
s/c	sub-cutaneous
TCR	T cell receptors
TGF-β	Transforming growth factor-β
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
v/v	volume per volume
wks	weeks
W/O	water-in-oil
W/O/W	water-in-oil-in-water

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CHAPTER ONE

LITERATURE REVIEW

1.1 ECONOMIC IMPACT OF ANIMAL DISEASES AND THEIR CONTROL IN LIVESTOCK PRODUCTION: ROLE OF VACCINES

Animal production serves multiple purposes worldwide. Livestock production and rearing is an important part of the economy of many countries and is considered to be the greatest contributor to the livelihood of the majority of the poor around the world (Perry *et al.*, 2002). Animal products for human consumption are also a major contributor to the quality of human nutrition. Animal protein is of higher biological value than plant proteins and it supplies many essential nutrients, such as vitamin A and iron, which are often inadequate in rural diets. Moreover, milk and dairy products are recognized as the most balanced food for humans and, in some rural areas, access to quality proteins in the diet is only by means of ingesting animal products (Perry *et al.*, 2002).

The role of livestock in the economy can be illustrated in the South African context. The country is water scarce with only 13% of the land arable and suitable for crop production. The rest is distributed between human settlement and animal farming (intensive and extensive, representing 69% of the total land). Animal farming occupies almost 80% of the agricultural land utilization, and livestock products account for between 37% and 41% of the total value of agricultural output (Anon., 2005). Although primary agriculture products (*e.g.* milk, meat, wool and crops) constitute 4-5% of the gross domestic product (GDP), a very large secondary industry exists, which, for primary animal products, include the distribution and marketing of primary products, processing industry, clothing industry (*e.g.* wool and leather), biopharmaceutical industry and entertainment (horse racing).

The steady development in animal production and subsequent food security has been for centuries hampered by health conditions and diseases, of which infectious diseases have played a major role. Over the past ten years, a number of infectious diseases outbreaks have resulted in serious financial losses that have impacted on national, regional and global economies (Anon., 2002), as illustrated in Tables 1.1 and 1.2.

Table 1.1: Overall costs of some animal disease outbreaks

Disease	Monetary cost
African swine fever, Cote d'Ivoire, 1996	US$ 13-32 million
Contagious bovine pleuropneumonia, Botswana, 1995	US$ 100-400 million
Rift Valley fever, Egypt, 1977 and 1993	200 000 human cases with 600 deaths
FMD, Chinese Province of Taiwan, 1997	US$ 6.6 billion
FMD, United Kingdom, 2001	US$ 9.9 billion
Avian influenza (HPAI), 2004-2006	209 million poultry culled or killed Cost to Asia alone US$ 130 billion up to 2005
BSE, Canada, one case in 2003	US$ 3 billion spent by Government in compensation up to March 2005

Compiled from: FAO Animal Production and Health Paper No. 153

Table 1.2: Cost of recent animal disease outbreaks (US$ million) and impact on specific sectors

	BSE United Kingdom, 1996-1997	**FMD Chinese Province of Taiwan, 1997**	**CSF Netherlands, 1997-1998**	**FMD Uruguay, 2000 and 2001**	**FMD United Kingdom, 2001**	**FMD Republic of Korea, 2000**	**FMD Japan, 2000**
Direct costs							
Compensation	2 433	188	1 183		2 223	377	0.5
Control measures		66	138	20	1 335	66	14.5
Sub-total	2 433	254	1 321	20	3 558	433	15
Indirect costs							
Agricultural sector		2 202	423		489		
Related industries		3 212	596	60	267		
Other		949			4 890		
Sub-total	1 395	6 363	1 019	60	5 646	[#]NA	NA
Total cost	3 828	6 617	2 340	80	9 204	433	15
Impact on GDP	-0.4%	-0.64%	-0.75%	NA	-0.2%	NA	NA

Compiled from: Measuring the Impact of Animal Diseases on Meat Trade: A Case Study Approach (http://www.fao.org/UNFAO/Bodies/ccp/me/02/default.html)

[#] NA; not available

To date, vaccination continues to be the most effective way of controlling animal diseases, either through preventing mortality or reducing morbidity (Rogan and Babiuk, 2005; Meeusen *et al.*, 2007). A large number of animal diseases cannot be controlled unless prevented through vaccination. Animal vaccines are used for all forms of infectious ailments, including viral, bacterial and parasitic or protozoal diseases. In recent years, vaccines have also been developed against helminth diseases and toxicoses (Knox, 2000; Vercruysse *et al.*, 2007). Indeed, vaccines have been the only tools capable of eradicating infectious diseases, either globally or in specific areas. The case of smallpox and poliomyelitis can be mentioned for humans (André, 2003), while Rinderpest in cattle is almost eradicated from the planet through concerted vaccination efforts (Roeder *et al.*, 2004). At regional level, there have been a number of success stories with the elimination of diseases such as Foot-and-Mouth-Disease (FMD) in South America (Rweyemamu and Astudillo, 2002).

Initial forms of vaccination are reported to have been practiced in China and India before 200 BC. In China, the process consisted of inoculation of patients with a powdery substance prepared by picking off pieces from drying pustules of a person suffering from a mild case of smallpox. The powder was then inserted into the patient's nose in order to immunize them (Fenner *et al.*, 1997). During the smallpox outbreaks of the 18th century, different approaches were tried to control or prevent the deadly disease. Edward Jenner, having observed some form of immunity in milkmaids who seemed to be protected from smallpox following mild infections with cowpox, thought of a cross-protection approach. Jenner took infectious fluid from the hand of milkmaid Sarah Nelmes and inserted this fluid, by scratching or injection, into the arm of a healthy local eight year-old boy, James Phipps. Phipps subsequently showed symptoms of cowpox infection. Forty-eight days later, after Phipps had fully recovered from cowpox, Jenner injected some smallpox-infected matter into Phipps, but Phipps did not show signs of smallpox infection (Fenner *et al.*, 1997).

Louis Pasteur and other scientists later perpetuated and strengthened the work on vaccines and vaccination. Pasteur's initial work on anthrax and chicken cholera was aimed at confirming his firm belief in the germ theory of infectious diseases. Subsequently, he demonstrated that inoculation with attenuated microbes allowed protection against virulent forms of the

microorganisms. To honour the pioneering work by Jenner, Pasteur suggested the terms "vaccination" and "vaccine" during the 1881 International Congress of Hygiene in London (Fenner *et al.*, 1997). The term "vaccine" thus derives from Edward Jenner's use of cowpox ("vacca" means cow in Latin), which, when administered to humans, provided them protection against smallpox.

While vaccination and the therapeutic or prophylactic use of drugs play an important role in animal disease control, vaccination is increasingly being viewed as the more sustainable option for controlling animal diseases with devastating economic impact. In veterinary medicine, vaccines have played an enormous role in the development of the modern livestock industry through the efficacious and cost-effective control of especially viral diseases, against which antibiotics have no therapeutic effect (Meeusen *et al.*, 2007). In wildlife management the use of bait vaccination programs has been instrumental in controlling diseases among wildlife populations. This, in turn, has led to a dramatic decline in the transmission of these diseases to humans and domesticated animals (Rupprecht *et al.*, 2006).

In many instances, vaccination has been the only way to sustain economic activities and support the livelihood of affected communities. One such illustration is the compulsory vaccination against FMD in areas surrounding the Kruger National Park, which together with strict control measures, has been the only means allowing South Africa to be recognized as an FMD-free country, and therefore capable of export to the first world countries (Hunter, 1999; Thomson, 2009). Another example is African horsesickness (AHS) in South Africa. Since the recognition of the disease after the introduction of horses into South Africa around 1719, it was established that it would be almost impossible to maintain them in South Africa given the widespread occurrence of AHS. The 1854-1855 outbreak of AHS was estimated to have cost the Cape Colony £ 525 000 and resulted in the death of 40% of the horse population (Coetzer and Guthrie, 2004). The development and use of a vaccine from the turn of the 20th century made it possible to introduce and maintain horses all over Southern Africa.

1.2 HISTORY OF VACCINES AT ONDERSTEPOORT

The onset of veterinary research in South Africa was linked with the need to eradicate or control the worst animal disease experienced by the country at that time, namely the Rinderpest outbreak of 1896. This devastating disease killed 1 002 297 of the 1 556 760 cattle, thus decimating 66.5% of the cattle population of the country, as well as large numbers of susceptible game animals (Rossiter, 2004). The authorities in all parts of the country got involved in attempts to address the problem. The Cape Colony recruited the world-famous Robert Koch, one of the fathers of modern microbiology, to address the problem, while the Natal Province hired Watkins Pitchford, who later established the Allerton Veterinary Laboratory. The Zuid-Afrikaansche Republiek (ZAR) appointed Arnold Theiler as its first State Veterinarian, with the main mandate to combat Rinderpest. Working first at the field laboratory of Morico, and later in the Waterval laboratory, north of Pretoria, Arnold Theiler, together with visiting French microbiologists, developed the first form of immunization against Rinderpest, namely a serum-based immunization procedure (Rossiter, 2004). The concerted effort of these different scientists in different parts of the country not only led to the eradication of Rinderpest in 1898, but also provided an excellent opportunity to establish the economic importance of animal diseases and therefore the need to invest in their control. Decision makers thus became more receptive and approved the building and establishment of the Veterinary Center at Onderstepoort in 1908. This marked the beginning of worldwide success and excellence in the field of veterinary vaccinology.

The first crude vaccines developed at the Veterinary Center at Onderstepoort, after its establishment, were the African horsesickness (AHS) and Bluetongue (BT) vaccines. The AHS serum-virus immunization method was introduced in 1905 (Theiler, 1908, 1909) and involved the injection of a virulent virus strain combined with immune serum obtained from horses by hyperimmunization. The immunity induced in mules was fairly good, and failures in immunity as a result of exposure to natural infection were about 2% and mortality was as low as 0.4% (Theiler, 1930). However, there were instances of severe disease and mortality in immunized mules. For horses, the procedure involved the simultaneous injection of one strain of virus with hyperimmune serum, followed three days later with a second virus strain and a further dose of the same hyperimmune serum. Mortality varied from 3-10% and in recovering horses, convalescence was protracted. On exposure to natural infection, 6-30% of horses developed

AHS (Theiler, 1921). In efforts to find a safer vaccine, the use of a formalized infective horse spleen as vaccine was investigated in Kenya (Whitworth, 1929; Walker, 1931) and at Onderstepoort (du Toit and Alexander, 1930; Du Toit *et al.*, 1933a; 1933b). This formalized vaccine provided good immunity to challenge with virulent homologous virus, but the immunity was transient and the margin of safety small. To ensure durable immunity, virulent blood was administered after the vaccine.

When the first vaccine against Bluetongue (BT) was developed by Theiler in 1906, it consisted of a virus strain, later shown to be serotype 4, which was "attenuated" by passage in sheep until it lost its virulence (Theiler, 1908). It was used as a blood vaccine, consisting of blood obtained from "vaccinated" sheep. The vaccine produced mild BT in recipient animals and had limited protection ability, as it consisted of a single serotype. It nevertheless was used over almost 40 years and afforded sheep farmers a reasonably effective method of control.

Similar approaches for vaccination were also used following the discovery in 1912 of *Anaplasma centrale* and its subsequent use as a vaccine against anaplasmosis caused by *A. marginale*. A blood vaccine for babesiosis was also developed. These blood vaccines against intracellular parasitic organisms are still in use today (Verwoerd, 2000). On the bacterial front, although a number of vaccines were in use in South Africa and/or developed by other institutions such as the Black quarter vaccine, prepared at the Grahamstown Laboratory in the Cape Province in 1887 or the Pasteur Anthrax vaccine developed in 1881 (Kriek and Odendaal, 2004), Onderstepoort was behind some of the greatest successes in bacterial vaccines during the early 20th century. Examples include the Viljoen's spore Anthrax vaccine in 1928, the formol toxoid vaccine against Black quarter in 1929, and the Mason and Sterne's formol toxoid vaccine against botulism that is still used today worldwide (Verwoerd, 2000). Other bacterial vaccines developed at Onderstepoort include the Sterne spore vaccine against Anthrax in 1948, which is currently used worldwide, the enterotoxaemia or Pulpy kidney vaccine in 1948 and the Rev-1 vaccine against brucellosis in rams (Verwoerd, 2000).

The ability to attenuate viruses and other microorganisms through serial passage in heterologous systems, *e.g.* mouse brain or in embryonated eggs, led to the development of new generation

vaccines at Onderstepoort. With AHS, it was discovered that the virus could be attenuated by approximately 100 serial passages intracerebrally in mice. This made it possible to produce an attenuated vaccine containing multiple antigenically different strains that could be used for immunizing large numbers of animals (Alexander and van der Vyver, 1935; Alexander *et al.*, 1936). Immunization with these neurotropic vaccines, which initially comprised four serotypes, was introduced in 1934 and used successfully in South Africa to immunize horses for decades (Erasmus, 1963). By 1949, eight serotypes were incorporated and the vaccine was found to be safe and to induce a wide polyvalent immunity from a single injection (Mcintosh, 1958).

An embryonated egg-attenuated quadrivalent BT vaccine was developed by Alexander in 1940, as the plurality of BT serotypes became evident and concerns regarding the safety of the first generation vaccine grew (Neitz, 1948). The potency of each strain included in this lyophilized vaccine was 250 LD_{50} in embryonated-eggs (Alexander, 1947). Subsequently, isolates of BT were attenuated by 100 passages on eggs by Howell (1969) and these showed a reduction in the severity of temperature reactions and incidence of clinical disease in sheep. Immunogenicity of these isolates was further improved by using plaque selection or purification to select strains at lower egg-passage levels. Ten plaques were selected at random and screened in sheep for low pathogenicity and good immunogenicity. Seventeen of the then known 20 serotypes of BT were shown to be present in South Africa. Since it was also known that several serotypes could be involved in an outbreak, the use of a polyvalent vaccine was imperative. A single vaccine containing 14 serotypes was subsequently developed and used for a time (Verwoerd, 2009). With the development of cell culture technology, Onderstepoort was at the forefront of research and this technology was used to adapt the BT virus to cell culture. The isolation and adaptation of many other viruses allowed the development of vaccines for different diseases, including Lumpy skin disease and Rift Valley fever (Verwoerd, 2000).

Even though vaccine production was limited and at laboratory scale, Onderstepoort was able to cover most of the vaccines required for disease control in South Africa (Tables 1.3 and 1.4). The establishment of a vaccine factory in 1968 led to further progress in large-scale production of veterinary vaccines and in terms of vaccine technology. These included the adaptation of many bacterial vaccines to fermentation culture instead of flasks, the use of large-scale freeze-dryers

Table 1.3: Vaccine production at Onderstepoort for the period July 1957 - June 1958 (Anon., 1961)

Vaccine	**Doses issued**
Anaplasmosis	417 900
Anthrax	8 366 800
Black quarter	2 233 800
Bluetongue	22 587 400
Contagious abortion (*Brucella* S19)	541 700
Botilinus	3 107 300
Distemper	24 200
Enterotoxaemia	9 776 000
Fowl pox	3 974 900
African horsesickness	150 700
Lamb dysentery	385 800
Newcastle disease	192 000
Paratyphoid	375 700
Rabies	47 000
Redwater	45 000
Rift Valley fever	168 400
Total	67 943 700

Table 1.4: Annual vaccine production from over the first 50 years of Onderstepoort (Anon., 1961)

Year	**Total doses issued**
1908	112 700
1918	2 462 800
1928	5 815 400
1938	9 977 700
1948	13 060 300
1958	67 943 700

and developments in adjuvant technology. By 1981, 47 different vaccines were being produced at Onderstepoort, of which 26 were bacterial vaccines, 17 were viral vaccines, two were rickettsial and two were protozoal vaccines. During the financial year 1981-1982, 163 million doses of these vaccines were produced (Onderstepoort Annual Report, 1982-1983).

Despite the fact that as early as the mid-1960s pioneering work was conducted at Onderstepoort in the field of molecular biology and DNA technology, including remarkable work on third generation vaccines, none of these vaccines to date has reached the commercialization stage. These include work on recombinant and subunit AHS and BT vaccines (Huismans *et al.*, 1987; Van Dijk, 1993; Scanlen *et al.*, 2002), and the development of a Lumpy skin virus vector expressing foreign genes from Rift Valley fever virus and Bovine ephemeral fever virus (Wallace and Viljoen, 2005).

1.3 VETERINARY VACCINOLOGY AND ADVANCES IN VACCINE TECHNOLOGY

1.3.1 General veterinary vaccinology

Vaccine research has evolved over time to become very complex and to include aspects such as the study of different vaccine types, advances in technology for their development, vaccine formulation and delivery mechanisms, as well as vaccine testing and registration (Sesardic and Dobbelaar, 2004). Vaccines are designed to provide protection against a disease by administering pathogens, their components or toxins in such a way as to trigger an immune response, without inducing clinical disease symptoms or spreading the infection to other animals. Ideally, a vaccine should provide quick and effective protection to all vaccinated animals. This is, however, impossible to achieve due to a combination of factors, as well as the complexity of the host-pathogen interaction and environmental factors. According to Rogan and Babiuk (2005) the "perfect vaccine" should have the following characteristics:

- multivalent
- induce specific immunity against protective antigens
- protect against disease and possibly infection

- protect newborn animals (irrespective of maternal antibodies)
- induce long duration of immunity
- would not require booster doses
- be free of adverse reactions
- be administered easily (without needles)
- be safe and efficacious

Achieving the "perfect vaccine" is still a dream, but significant progress has been made in the field of veterinary vaccine development with regards to aspects such as a better understanding of the immune response, protection mechanisms and effectors, as well as progress in vaccine adjuvant and delivery systems (Lombard *et al.*, 2007; Mutwiri *et al.*, 2007). The progress in vaccinology has also been occurring at a time where there is increasing pressure from regulatory authorities for safer vaccines, thus requiring adherence to very strict production processes and quality standards, as well as compliant facilities and equipment.

1.3.2 Conventional vaccines

1.3.2.1 Live or attenuated vaccines

Live or attenuated vaccines are derived from either mutant strains or artificially attenuated pathogenic organisms that have reduced virulence in the target host, while maintaining immunogenicity (Meeusen *et al.*, 2007). The main source strategies for live attenuated vaccines include serial passage of the pathogenic organism in either heterologous host animals, *e.g.* rabbits or hens' eggs, or in cell culture. Alternatively, attenuated strains can be sourced through different approaches. These include the use of a related organism in a different host, the use of virulent organisms given by an unnatural route or the use of wild-type pathogens with natural deletions or insertions, resulting in reduced or absence of virulence. One good example has been the use of cowpox virus for protection in humans against smallpox. Another example is the immunization of poultry with Herpesvirus of turkeys (HVT), which is a ubiquitous virus in turkey herds, to protect chickens from Marek's Disease (Okazaki *et al.*, 1970). More recently, an avirulent Rift Valley fever (RVF) virus isolated from a non-fatal case of RVF in the Central African Republic was identified and selected as a good candidate vaccine. After adaptation to

cell culture through limited passage in mice and Vero cells, and then plaque-purification in order to study the homogeneity of virus subpopulations, a clone, designated 13, did not react with specific monoclonal antibodies and was further investigated. It was found to be avirulent in mice, yet immunogenic. The attenuation appears to be the result of a large internal deletion in the NSs gene (Muller *et al.*, 1995). It has since been successfully tested in sheep and cattle. In the bacterial world, *Brucella suis* strain S2, naturally attenuated and developed in China, has been developed into a vaccine capable of oral administration (Xin, 1986). Other bacterial vaccines include vaccine strains for *Salmonella dublin* and *S. cholera suis*, which are avirulent rough variants of the smooth virulent strains of the same organisms (OIE, 2005).

Live or attenuated vaccines have the advantage of being capable of generating early and long-lasting immunity in vaccinated animals. They therefore usually do not require frequent vaccination. Induced mucosal immunity is also very good as compared to inactivated vaccines (Kagnoff, 1996). Their costs are generally low due to the fact that a large amount of organisms can be produced in specific culturing systems per batch, resulting in low production costs. Disadvantages of live attenuated vaccines include the potential risk of residual virulence, linked to the complex nature of the vaccine strain, such as the case of Contagious bovine pleuropneumonia (CBPP) vaccines (Mbulu *et al.*, 2004), or to the susceptibility of a subpopulation of the host being vaccinated, as seen with the BT vaccine used in Europe (Berry *et al.*, 1982). There is also a potential risk of release into new environments of new isolates (Ferrari *et al.*, 2005; Batten *et al.*, 2008).

1.3.2.2 Inactivated vaccines

Inactivated vaccines can result from the inactivation or killing of the pathogens by chemical or physical means. Generally, they are formulated with an adjuvant or immuno-stimulant (Rogan and Babiuk, 2005). Inactivated bacterial vaccines are generally obtained by formalin inactivation of the whole bacterial cell and/or their metabolic products or purified fraction (Meeusen *et al.*, 2007). In the case of viruses, inactivated vaccines can be produced from native viral subunits. Lipid solvents have typically been used in the case of enveloped viruses, such as influenza virus, herpesvirus and coronavirus, to solubilize the virion and release virion components (Huckriede *et al.*, 2005). Inactivated viral vaccines can also be produced from purified native viral proteins.

This was the case with earliest Hepatitis B (HB) vaccines, which were purified HB antigens from the blood of chronically infected carrier individuals (Anon., 2004).

Advantages of inactivated vaccines are mainly concerned with safety, as they are non-replicating and subsequently have no residual virulence risk or risk of release into the environment. They are, however, expensive to produce due to additional downstream processing requirements, which include formulation with an adjuvant and the need for a high payload in the vaccine dose (van Oirschot, 1997).

1.3.2.3 Toxoid vaccines

Toxoid vaccines are based on toxins secreted from an organism, which are altered in such a way as to reduce inherent levels of toxicity, usually through chemical treatment with substances such as formaldehyde (Walker, 1992). Toxoid vaccines are therefore the result of alterations to the toxins secreted by pathogenic microorganisms in order to minimize or suppress their toxicity. Examples of toxoid vaccines include toxin-producing clostridial species such as tetanus and botulism (Walker, 1997). Developments in fermenter technology have made it possible to increase the yield in toxin production, to optimize suitable culture media and select suitable strains that give optimal toxin production (Walker, 1992). Bacterial toxins are generally not produced in significant quantities in synthetic media. They usually require a source of peptides, most of which are natural products such as meat, casein or soybean (Walker, 1997). The use of meat-based products has become problematic since the emergence of Bovine spongiform encephalitis (BSE), thus complicating the registration and acceptability of certain toxoid vaccines (Cashman, 2001).

While toxoid vaccines are usually effective, there are examples of breakdown in the field, usually as a result of overwhelming challenge (Walker, 1997). This has led to the use of anacultures in the production of these vaccines. These are whole cultures where the bacterial cells are not removed from the culture. The potential problem resulting from this approach is the inclusion of unwanted peptides or toxins in the final vaccine.

1.3.3 New vaccine technology

With the advent of biotechnology, molecular biology and recombinant gene technology, a better understanding of immune mechanisms linked to protection against diseases has enabled remarkable improvements in the design, production and use of immunological products. The role of vaccination has also evolved over the years to more than just being used to prevent diseases or infection. As a surveillance and control tool, vaccines are expected to be used as markers in order to discriminate between infected and vaccinated animals (Doel, 2003; Vannier *et al.*, 2007). In certain instances, vaccines are expected not only to prevent diseases, but also to prevent infections. There has also been work done on therapeutic vaccines. These advances are being achieved through different approaches, including generating modified live vaccines by deleting virulence-related genes through the manipulation of the genetic material of target pathogens, or inserting into a non-virulent vector genes coding for specific immunizing antigens from a disease-causing organism (Rogan and Babiuk, 2005; Meeusen *et al.*, 2007). New generation vaccines are either live (virus- or bacterial-vectored) or non-replicating (subunit) or DNA-based (Table 1.5).

1.3.3.1 Subunit vaccines

Over the past two decades, there has been increasing pressure applied by the regulatory authorities, both human and veterinary, to specifically define the protective antigens and produce vaccines that would be free from pathogen-associated toxins, extraneous agents and immunosuppressive components (Castle, 2005). Subunit vaccines based on recombinant protein immunogens, DNA immunogens, and non-pathogenic vectors are currently the most cost-effective methods of producing antigens free from the exogenous material that is characteristic of conventional vaccines (Rogan and Babiuk, 2005).

Initially, subunit vaccines were produced by purifying the specific antigens from cultures of the pathogenic bacteria or viruses. Besides being expensive due to the large-scale production required, this approach has the potential risk of generating an antigen that may still contain residues of unwanted material or virulence. However, the advent of molecular biology and genetic engineering has impacted tremendously on vaccine development by providing the tools and techniques to produce a single protein in a prokaryotic or eukaryotic expression system.

Table 1.5: Vaccine types

Vaccine type	Form	Description
Conventional live	Live organism	Derived from mutant or attenuated strains of the pathogen; display reduced virulence in the host, while maintaining immunogenicity
Conventional inactivated	Inactivated organism	Contain highly virulent pathogenic strains; inactivated utilizing chemical or physical methods
Toxoids	Biochemical substances	Based on toxins secreted from an organism, which are chemically altered to reduce inherent toxicity
Gene-deleted	Live or inactivated organisms	Specific genes encoding virulence or the capability for replication are deleted, providing enhanced safety
Marker	Live or inactivated organisms	Specific deletions or the addition of a microbial protein code into the genome of the pathogen enable differentiation between vaccinated and naturally infected animals
Vector	Live organisms	Genes encoding antigenicity of a pathogen are inserted into the genome of a viral or bacterial carrier, known as a "vector"
DNA	Genetic material	Plasmid DNA responsible for the expression of an antigenic protein is inoculated into the host animal to stimulate an immune response
Subunit	Antigenic particles	Based on purified protein or glycoprotein components of a pathogen, which induce an immune response
Synthetic	Antigenic particles	Contain synthetically engineered particles, usually peptides, which induce an immune response
Edible	Antigenic particles	Based on genetically modified plant material that expresses desired antigens and can be delivered via animal feed

Compiled from: Veterinary Vaccines, Animal Pharm Report 2005

Specific proteins from pathogenic organisms responsible for triggering immune responses in host animals can be expressed in a host system. The gene that encodes the protective antigen is cloned into a secondary, preferably non-pathogenic organism that is capable of expressing the immunogen in its native form or with minimal alteration. This protein can then be expressed and harvested using traditional bacterial antigen production methods, or delivered by a live non-pathogenic vector. If the protein is produced in prokaryotic systems, it can be tailored in such a way that the protein of interest is expressed on the surface of the bacteria, in the periplasm, as insoluble inclusion bodies or secreted into the media (Rogan and Babiuk, 2005; Wesley, 2005).

To date different prokaryotic and eukaryotic expression systems have been tried and used to produce commercial veterinary vaccines. Bacterial expression systems are excellent candidates for the production of non-glycosylated proteins. Bacteria such as *Escherichia coli*, *Bacillus brevis* (Ichikawa *et al.*, 1993; Udaka and Yamagata, 1993; Nagahama *et al.*, 1996; Shiga *et al.*, 2000; Yokomizo *et al.*, 2002; Kashima and Udaka, 2004) and *Salmonella typhimurium* (Husseiny *et al.*, 2005; Yang *et al.*, 2005; Salam *et al.*, 2006; Hanna *et al.*, 2008; Zekarias *et al.*, 2008) have been used extensively for the expression of a wide variety of foreign genes and, as a result, many production, stabilization and optimization strategies have been described. Although prokaryotic expression is efficient and affordable for the production of a broad range of immunogens, including a few natively glycosylated proteins, production of many viral glycoproteins in prokaryotic systems does not result in immunologically protective proteins due to the lack of glycosylation, despite producing significant immune responses (Wesley, 2005).

Eukaryotic expression systems include yeast, insect cells, plants and mammalian cells. These expression systems are more suitable for the expression of glycoproteins and other modified proteins (Rogan and Babiuk, 1995). The most commonly used yeast expression systems are *Saccharomyces cerevisiae* and *Pichia pastoris*. Notably, *S. cerevisiae* was used to produce the first-ever subunit vaccine for Hepatitis B, which was licensed and commercialized in 1986 (Anon., 2004). Insect cell expression is based on infection of cultured caterpillar or lepidopteran cells with a recombinant baculovirus designed to express the gene product under the control of the strong polyhedron promoter, typically resulting in a high yield of immunologically active protein (O'Reilly *et al.*, 1992; Beljelarskaya, 2011). Proteins are expressed glycosylated. The

major limitation of insect cell expression has been the inability to achieve high densities due to the requirement of high dissolved oxygen levels, which cannot be achieved with traditional fermentation techniques due to the cells' sensitivity to shear forces (Radford *et al.*, 1997; Maranga *et al.*, 2002; Ikonomou *et al.*, 2003).

In addition to the above expression systems, virus expression is also used. The most commonly used virus expression systems in veterinary vaccines have included baculovirus in insect cells and vaccinia virus expression (Moss and Flexner, 1987; Beljelarskaya, 2011). At Onderstepoort, extensive research has been conducted with these systems to express viral proteins of BTV and AHSV. The expression and purification of most AHSV proteins have been difficult due to insolubility problems and limited immunogenicity (Du Plessis *et al.*, 1998), although the work by Scanlen *et al.* (2002) showed that increased immunogenicity is associated to the correct choice of adjuvant.

1.3.3.2 Vector vaccines

Genetically modified bacteria or viruses can act as carriers, known as vectors, when genetic material responsible for the stimulation of an immune response is cloned into them. The vectors act therefore as vaccine delivery vehicles. Administration of vector material triggers a natural immune response, extending to include a response to the pathogen from which genetic material has been taken from. They thus combine the benefits of a modified live vaccine with those of a subunit vaccine (Rogan and Babiuk, 2005).

Bacterial vectors, based mainly on Gram-negative bacteria such as *E. coli*, *S. typhimurium* and *Vibrio cholera* (Garmory *et al.*, 2003; Daudel *et al.*, 2007), have been extensively studied. Gram-negative bacteria can elicit strong and long-lasting immune responses to foreign antigens by establishing limited infections, which resemble mild forms of the natural infection. Moreover, the lipopolysaccharide (LPS)-rich cell walls of Gram-negative bacteria act as natural adjuvants (Rogan and Babiuk, 2005).

Initial work regarding virus vector vaccines involved the use of vaccinia virus to generate vaccines such as the rabies vaccines, where the glycoprotein of the virus is expressed in a

vaccinia vector. This vaccine was used extensively in Europe to stop the spread of rabies in wild animals (Pastoret and Brochier, 1999). A number of viruses have to date been transformed into vectors and successfully tested for the expression of foreign genes. The use at Onderstepoort of the Neethling strain of the Lumpy skin virus as a vector for the expression of Rift Valley fever and Bovine ephemeral fever glycoproteins can be mentioned (Wallace and Viljoen, 2005). Poxviruses, given their large and relatively basic genome, have also extensively been studied as virus vectors, as they can accommodate relatively large inserts (Paoletti *et al.*, 1996). The need for species-specific expression, as requested by most regulators fearing the potential of expression in non-target animals, has led to the use of vectors such as canarypox, which does not further replicate in mammalian hosts after initial expression (Poulet *et al.*, 2007). A number of canarypox-based veterinary vaccines have been commercialized and more are being developed (Boone *et al.*, 2007).

1.3.3.3 Marker vaccines

The principle of a marker vaccine is to allow serological differentiation between infected and vaccinated individuals. This differentiation is based on the absence of one or more proteins in the vaccine that are present in the wild-type microorganism (Doel, 2003). The use of companion tests that detect antibodies against the protein(s) lacking in the vaccine strain can detect infected individuals in vaccinated populations. Infection in a vaccinated herd can thus be monitored (Vannier *et al.*, 2007). Marker vaccines, either live, inactivated or recombinant are generated through one of the following strategies.

- Naturally occurring mutants or variants, but sharing with the target pathogen common highly immunogenic antigens. Examples include the Avian influenza vaccines against the highly pathogenic H5N2, in which case vaccines have been developed using H5N1 or H5NX (Capua *et al.*, 2003; Capua *et al.*, 2004).
- Purification of vaccine antigen during production processes, thus excluding in the vaccine a protein or antigen that is present during natural infection and highly immunogenic. Examples include the Foot-and-Mouth Disease (FMD) virus non-structural proteins (Paton *et al.*, 2006; Foord *et al.*, 2007).

1.3.3.4 DNA vaccines

The basic concept of DNA vaccines is to deliver plasmid DNA encoding for protective proteins into the cells of the host animal where they can direct transcription and translation, effectively transforming the vaccinate into a mammalian bioreactor for the production of its own vaccine (Danko and Wolff, 1994; Donnelly *et al.*, 1997; Rogan and Babiuk, 2005). The principle resulting in the transcription of genes into proteins in muscle cells was first described by Wolff *et al.* (1990) who conducted their groundbreaking experiments in mice. The transcription seems to work better when the plasmid DNA is transferred into muscle, using different strategies such as injection or the use of a biolistic or gene gun (Williams *et al.*, 1991).

From its earliest days, the use of DNA-based immunization appealed to the scientific community since it theoretically presented a number of advantages, including:

- Low risk of local reactions at the injection site, as DNA vaccines do not require a chemical adjuvant.
- Safety advantage over live vaccines, as a result of consisting solely of nucleic acid and therefore not being capable of replicating and resulting in possible residual virulence.
- Ability to generate both cellular and humoral immunity. Despite the fact that the antigen is produced by the host's cellular machinery, the immune system recognizes the protein as being foreign and mounts both a cellular and humoral immune response very similar to ones induced by live vaccines or in animals recovered from natural infection (Van Drunen Little-van den Hurk *et al.*, 1998; Dunham, 2002).
- Allow the production of vaccines against organisms that are difficult or dangerous to culture in the laboratory.
- Experimental DNA vaccines have shown the ability to circumvent maternal antibody interference when injected into neonates (Rogan and Babiuk, 2005).
- The production of DNA vaccines can be scaled up fairly easily. The bacterial cells are produced by fermentation in defined media, followed by the extraction and purification of plasmid DNA from bacterial cells containing the desired gene in the plasmid (Przybylowski *et al.*, 2007).

A number of challenges have, however, made it difficult to fast-track the release of DNA vaccines for widespread use. These range from regulatory aspects, perceived safety risks and limited efficacy. Despite these challenges the veterinary sector has been capable of registering the first DNA vaccine, which is the West Nile vaccine for horses (CDC, 2005). Some of the main problems associated with the use of DNA vaccines are:

- Identification of the most suitable delivery system. Since the DNA has to be delivered into cells, and preferably muscle cells, different routes have been studied with different results. DNA vaccines have been administered intradermally, intramuscularly, as naked DNA or in association with different formulations aimed at facilitating transmembrane passage (Vannier and Martignat, 2005).
- Risk of possible integration of plasmid DNA into the host genome and potential risk of malignancy, and integration of foreign genes into germ cells could potentially lead to vertical transmission (Vannier and Martignat, 2005).
- The potential exists for the development of antibodies against the DNA vaccine itself, with the putative consequence of an autoimmune disease developing (Vannier and Martignat, 2005).

1.3.3.5 Edible vaccines

The oral route appears to be the ideal means of delivering prophylactic and therapeutic vaccines, offering significant advantages over systemic delivery. Most notably, oral delivery can be associated with simple administration and theoretically improved safety, as it does not involve any invasive procedure (Loza-Rubio and Rojas-Anaya, 2010). The prospect of expressing vaccine antigens in cereal and oilseed crops presents the possibility of the vaccine immunogen being stable at ambient temperatures, thereby minimizing the need for the cold chain. In addition, unlike systemic immunization, oral delivery can induce mucosal immune responses (Berinstein *et al.*, 2005; Hefferon, 2010). However, the oral route of vaccine delivery is the most difficult because of the numerous barriers posed by the gastrointestinal tract. To facilitate effective immunization with peptide and protein vaccines, antigens must be protected, their uptake must be enhanced and the innate immune response must be activated. Numerous delivery

systems and adjuvants have been evaluated for oral vaccine delivery, including live vectors, inert particles and bacterial toxins (Loza-Rubio and Rojas-Anaya, 2010).

The most advanced expression systems to date for veterinary oral vaccines are plant-based. Academic research plant expression is often conducted in tobacco plants, due to their relative ease of transformation. In contrast, at industry-level the focus has been on cereals, as they have a number of advantageous properties. Not only are they cost-effective, but they also offer long-term stability of the expressed protein. Moreover, they rapidly produce large volumes of the desired product and can be easily processed into a deliverable form for oral vaccination (Streatfield, 2005).

There are, however, several challenges for the widespread use of oral vaccination. Most notably, oral immunization appears to require 100- to 1000-fold more antigen to be presented than is required for parenteral delivery (Streatfield *et al.*, 2003). For ruminants, the requirement is that the encapsulating cell remains intact during passage through the rumen so as not to expose the immunogen to ruminal proteases and subsequent inactivation; the lysis should preferably take place in the abomasums or proximal duodenum (Rogan and Babiuk, 2005; Streatfield, 2005). Furthermore, a major concern regarding the use of direct-fed plant vaccines is the risk to the human food chain due to inadvertent cross-contamination, and potential issues of immunogenicity and tolerance to oral vaccines (Arntzen, 1997).

1.4 VACCINE DELIVERY AND VACCINE ADJUVANTS

Key considerations for an effective vaccine generally include efficacy, safety, ease of administration and cost-effectiveness. Put in different terms, a vaccine should ideally protect by generating a quick, solid and durable immunity without causing disease or serious side-effects (Meeusen *et al.*, 2007). Thus, vaccines aim at prophylactically inducing effector molecules and cells that are capable of eliminating a pathogen as quickly as possible. It is now well recognized that a range of primary immune responses is required by the host for this elimination to be successful (Shijns, 2001; Mutwiri *et al.*, 2007). The different types and classes of effectors that

contribute to the immune response leading to the elimination of pathogenic microorganisms are summarized in Table 1.6.

Table 1.6: Classification of adaptative effector immune responses (Shijns, 2001)

Immunity	**Effector response**	**Effectors**	**Action**	**T helper (Th) activity**	**Cytokines**
Cell-mediated	DTH (delayed type hypersensitivity)	$CD4^+$	Macrophage activation	Th1-associated	IFN-γ
	CTL (cytolytic T cell activity)	$CD4^+$ $CD8^+$			
Humoral	Lysis	IgG2a	Opsonizing		
			Complement binding		
	Neutralizing	IgG1	Neutralizing (receptor blockage)	Th2-associated	IL-4
		IgE	Allergy helminths		
		IgA	Mucosal	Th3-associated	TGF-β

The most efficient protection against a variety of bacterial, viral and parasitic infections is provided by long-lived, antigen-specific neutralizing antibodies (Bachmann *et al.*, 1999). Cellular immunity, on the other hand, is critical for the control of certain intracellular pathogens (Shijns, 2001). One major disadvantage of inactivated and subunit vaccines is the fact that they do not replicate in the host and, as such, have a limited ability to mimic the immune response of the pathogen they are expected to generate protection against. It is therefore critical that delivery techniques and vaccine formulation be designed in such a way that the suitable immune response is generated.

In 1925, Ramon demonstrated that it was possible to artificially augment antigen-specific antibodies to diphtheria and tetanus toxoids when substances such as agar, metallic salts, lecithin and saponin were administered prior to vaccination (Aucouturier *et al.*, 2001). The augmenting

substance, called adjuvant (from the Latin "*adjuvare*" meaning to help), has since been shown to be capable of significantly increasing the levels of protection afforded by a vaccine. To date a large variety of adjuvants have been developed, most of them targeting a very specific type of immunity and suitable for specific types of organisms. Nevertheless, still little is known regarding the key immunological events that lead to vaccination-induced immunity. A review covering some of the current knowledge regarding adjuvant activity is provided in the following sections.

1.4.1 Development of immune response to vaccines and adjuvants

In order for the immune system to be activated, it requires appropriate presentation and recognition of an antigen. In mammals, immune activation is initiated in the lymph nodes that drain the non-lymphoid tissue in which antigen has been either newly expressed or introduced via the afferent lymph vessels (Schijns, 2001). Naïve lymphocytes, capable of recognizing the antigen, circulate continuously between these lymph nodes and the spleen. Activation of these cells, which carry the cognate receptor for the antigen, requires delivery of the antigen into the lymph node. Most often, it is antigen-loaded dendritic cells (DCs) that serve as transport vehicles (Schijns, 2001). Immature DCs act as sentinels in peripheral tissues where they capture incoming antigens, self-antigens in cell debris, and vaccine components by fluid-phase pinocytosis or receptor-mediated uptake. They are called "professional" antigen presenting cells (APCs), "nature's adjuvant", because of their unique ability to activate the scarce number of naïve T helper (Th) cells present in the lymph node (Schijns, 2001). DCs and APCs, in general, process captured antigen and present antigen-peptide fragments in association with major histocompatibility complex (MHC) class II or MHC class I molecules, depending on whether the pathway of presentation to the T helper and cytotoxic T cells (CTL) is exogenous or endogenous, respectively (see Fig. 1.1). Naïve T cells express T cell receptors (TCR), which recognize antigens presented by APC and are therefore activated. The strength and intensity of the activation are dependent on additional co-stimulatory molecules, and the intensity of the mobilization of DCs. Adjuvants play a role in increasing the mobilization and influx of DC toward a foreign antigen, such as a vaccine antigen, and therefore assist in an improved priming of T cells and their clonal expansion, as well as memory and effector cell formation (Schijns, 2001).

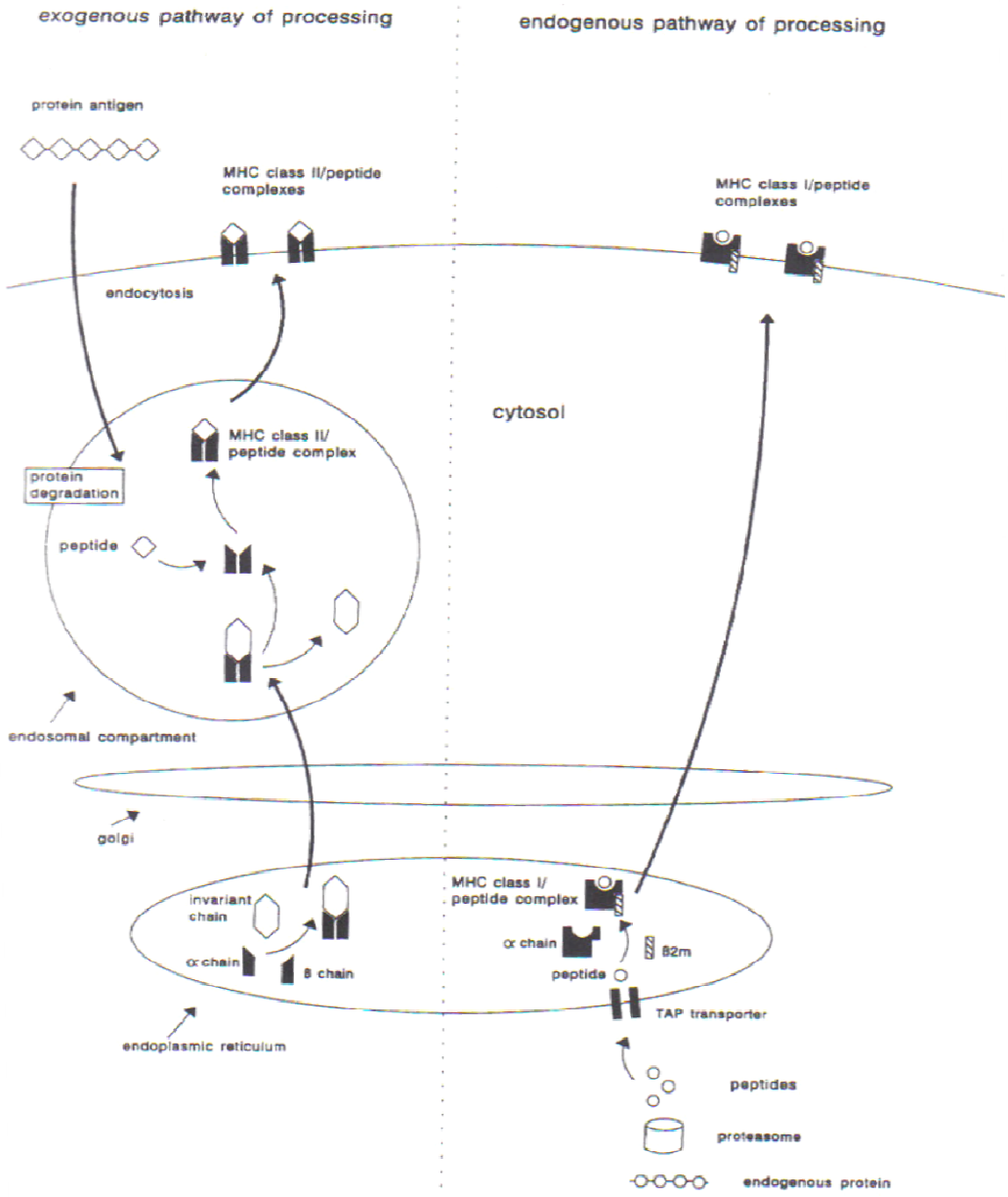

Fig. 1.1: Intracellular pathways of MHC class I- and II-restricted processing and presentation (Rimmelzwaan and Ostherhaus, 1997). An exogenous and an endogenous pathway have been identified for the processing and presentation of antigens to T helper (Th) cells and cytotoxic T cell lymphocytes (CTL). In the exogenous pathway, antigens are taken up into lysosomes where the proteins are degraded into peptides and associated to MHC class II, and then recognized by $CD4^+$. In the endogenous pathway, proteins are degraded in the cytosol into peptides, transported by transporter proteins and associated to MHC class I molecules.

1.4.2 Mechanism of action and effect of adjuvants

Different authors have defined the mode of action of adjuvants differently, either based on their nature or based on their specific activities in specific systems (animal or human). Below is a summary of some of the known mechanisms of adjuvant action, as summarized by O'Hagan (2000).

- Stabilizes epitope conformation.
- Generates a depot at the site of inoculation with slow release of antigen.
- Targets the antigen to antigen-presenting cells (APCs) by formation of multimolecular aggregates, or by binding antigen to a cell-surface receptor on APCs.
- Directs antigen presentation by MHC class I or MHC class II pathways by means of fusion or disruption of cell membranes, or by direct peptide exchange on surface MHC molecules.
- Preferentially stimulates Th1 or Th2 $CD4^+$ T helper cells or $CD8^+$ cytotoxic T lymphocytes by modulation of the cytokine network in the local microenvironment.

The beneficial effects of vaccine adjuvants can include the following:

- Increase in the potency of antigenically weak peptides. One local example is the baculovirus-expressed VP2 of African horsesickness virus (AHSV) with the use of a specific saponin adjuvant (Scanlen *et al.*, 2002).
- They can enhance the speed, vigour and persistence of the immune response to stronger antigens. Good examples include the effect of different adjuvants with the FMD antigen, used for the production of vaccines needed either in an emergency situation or in a normal control program (Barnett *et al.*, 1996).
- They can modulate antibody avidity, specificity, quantity, isotype and subclass. Water-in-oil emulsions have shown in a number of studies with different types of antigens that they help to induce higher IgG2a antibody levels (Aucouturier *et al.*, 2001). Vaccines formulated with aluminum predominantly stimulate the Th2-like immune response, inducing subclass IgG1 and IgE antibodies (Bowersock *et al.*, 1999).
- They can select for or modulate humoral or cell-mediated immunity. This can be achieved in many ways. Firstly, by modulating antigen processing, thus eliciting both T helper cells and the cytotoxic T cell response. Secondly, the immune response can be modulated in

favour of MHC class I or MHC class II, depending on the type of adjuvant. Thirdly, adjuvants can modulate the immune response by preferentially stimulating Th1 or Th2 $CD4^+$ T helper cells (Gupta, 1998).

- Adjuvants can increase the immune response to vaccines in immunologically immature, suppressed or senescent individuals.
- Vaccine adjuvants can allow the decrease in the amount of antigen required, and in so doing reduce the cost of the vaccine. The reduction in the amount of antigen also contributes to reducing the likelihood of antigen competition in combination vaccines.

In addition to the above, Cox and Coulter (1997) identified five modes-of-action for adjuvants, which are summarized in Table 1.7 below.

Table 1.7: Modes of adjuvant action (Cox and Coulter, 1997)

Action	Adjuvant type	Benefit
1. Immunomodulation	Generally small molecules or proteins that modify the cytokine network	Up-regulation of immune response and selection of Th1 or Th2
2. Presentation	Generally amphipathic molecules or complexes that interact with immunogen in its native conformation	Increased neutralizing antibody response and greater duration of response
3. CTL induction	Particles that can bind or enclose immunogen and that can fuse with or disrupt cell membranes	Cytosolic processing of protein, yielding correct class 1 restricted peptides
	Water-in-oil emulsions for direct attachment of peptide to cell surface MHC-1	Simple process if promiscuous peptide is known
4. Targeting	Particulate adjuvants that bind immunogen, and adjuvants that saturate Kupffer cells	Efficient use of adjuvant and immunogen
	Carbohydrate adjuvants that target lectin receptors on macrophages and DCs	As above, but may also determine type of response if targeting is selective
5. Depot generation	Water-in-oil emulsions for short term	Efficient
	Microspheres or nanospheres for long term	Potential for single-dose vaccine

1.4.3 Types of adjuvants

With developments in vaccinology and immunology, many compounds are described as having adjuvant properties, and different methods of classification can be identified. According to Cox and Coulter (1997), adjuvants can be classified into particulate and non-particulate compounds. The former exist in a microscopic particle form, whereas non-particulate adjuvants are not dependent upon any particulate or multimeric nature for activity. They are generally immunomodulators, although some improve targeting. Based on their origin, adjuvants can also be classified as suggested by Audibert and Lise (1993) into:

- Plant or vegetable products, *e.g.* saponin, glucan extract and vegetable oils.
- Bacterial products, *e.g.* monophosphosphoryl lipid A, cholera toxin, lipopolysaccharide (LPS) mainly from Gram-negative bacteria, and muramyl dipeptide (MDP) from mycobacteria that is used in complete Freund's adjuvant (CFA).
- Chemical compounds, *e.g.* aluminum-based adjuvants, surfactants, emulsions, nanoparticles and polymeric microspheres.
- Cytokines such as IFN-γ or hormones like dehydroepiandrosterone (DHEA).

1.5 OIL ADJUVANTS AND THEIR USE IN VACCINES

The most commonly used adjuvants in veterinary vaccines are aluminum salts and oil emulsions. The role of oil emulsions as adjuvant has been known since the first description in 1916 by Le Moignic and Pinoy of an increased immune response when a suspension of killed *S. typhimurium* was administered in mineral oil. Oil emulsions as adjuvants received more attention following the work by Freund in the 1930s, demonstrating increased antibody responses when using paraffin (mineral) oil mixed with killed mycobacteria. This resulted in the well known complete Freund's adjuvant (CFA). Later he found a similar effect in immunization of protein antigens with the same paraffin-based emulsion, but without the killed *Mycobacterium tuberculosis* incorporated; this has been called the incomplete Freund's adjuvant, generally referred to as IFA (Chang *et al.*, 1998; Jansen *et al.*, 2005).

Oil vaccines are emulsions consisting of the antigen(s), oil and surfactant. An emulsion is a heterogenous system or a dispersion of a liquid, called the dispersed phase, in a second liquid, called the continuous phase. The dispersed phase is not normally miscible with the continuous phase. In the case of vaccines, the two phases consist of water (the antigenic medium) and oil. In order to stabilize the emulsion, surfactants are added (Aucouturier *et al.*, 2001). Surfactants are usually organic compounds that are amphipathic, *i.e.* they contain both non-polar hydrophobic groups and polar hydrophilic groups. Therefore, they are typically sparingly soluble in both organic solvents and water (Ascarateil and Dupuis, 2006).

As for most adjuvants the mechanisms of actions of emulsions are not yet clearly elucidated. It is, however, agreed that they rely on a combination of different mechanisms. Three modes of action are generally considered to be involved with oil adjuvants. These are (i) depot effect and slow release through the establishment of a repository antigen-containing locus at the site of injection, allowing a gradual and continuous release of the antigen; (ii) provision of a vehicle capable of transporting emulsified antigen through the lymphatic system to distant sites such as draining lymph nodes and the spleen, creating additional foci of antibody formation; and (iii) interaction with mononuclear cells, such as phagocytic cells and antigen presenting cells (Lindblad, 2000).

The kinetics of release of the antigen will vary with the type of emulsion. The work by Aikawa *et al.* (1998), using an inverted dialysis tube method to assess the *in vitro* kinetic release of a protein from an emulsion clearly shows these differences (Fig. 1.2). Whereas the protein without adjuvant is immediately released, oil-in-water (O/W) emulsions allow a slight delay, but the protein is quickly released. Water-in-oil (W/O) emulsions induce no or very little release of the protein. This is correlated with the stability of the emulsion and as soon as the emulsion breaks, large amounts of protein are released, but slower than O/W emulsions. Water-in-oil-in-water (W/O/W) emulsions have a combined action in that they have an early release effect that lasts longer than the O/W emulsions.

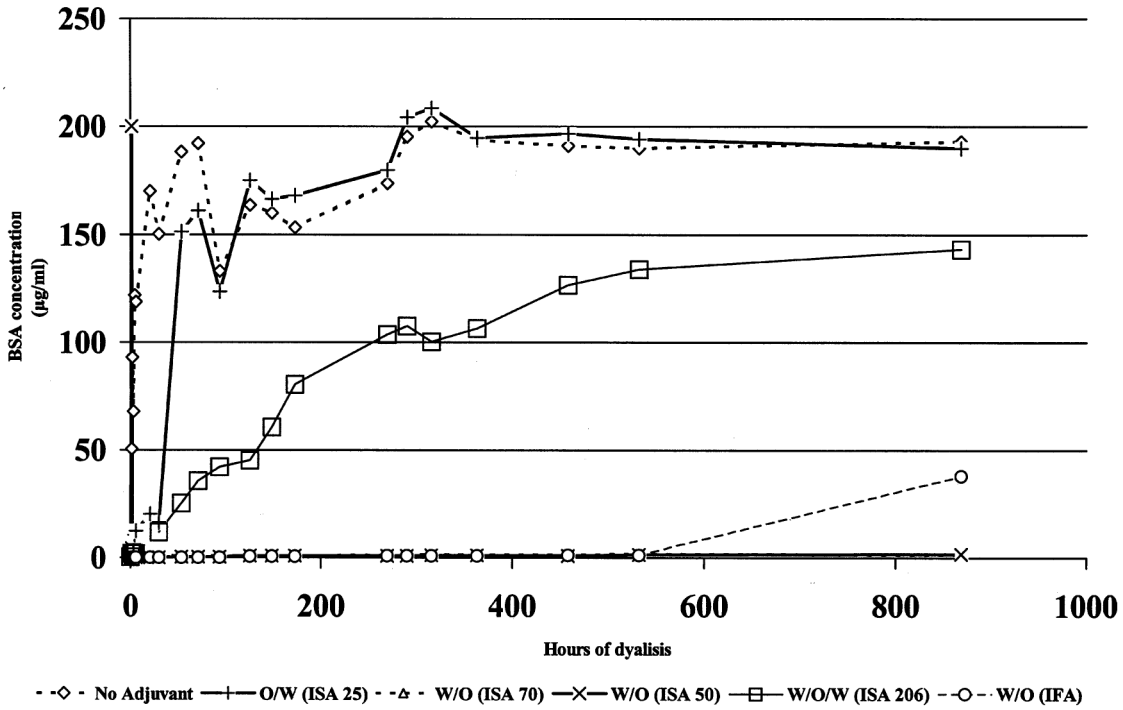

Fig. 1.2: *In vitro* kinetic release of bovine serum albumin (BSA) formulated in various emulsions (Aucouturier *et al.*, 2001). The inverted dialysis tube method employed to assess the *in vitro* kinetic release of the BSA clearly show differences according to the type of emulsion. The protein without adjuvant is immediately released, whereas O/W emulsions allow a slight delay, but the protein is quickly released. W/O emulsions induce no or very low release of antigen. W/O/W emulsions have an intermediate behaviour.

1.6 OIL VACCINES USED IN THIS STUDY

The present study was designed to evaluate four oil-based vaccines produced by Onderstepoort Biological Products (OBP), namely *E. coli*, Pulpy kidney, *Vibrio* and Infectious coryza vaccines, which are described below. It became critical to review and possibly improve the formulation of these vaccines for regulatory compliance, and also for improved safety and efficacy.

1.6.1 Pulpy kidney vaccine

Pulpy kidney, or enterotoxaemia, is an economically important disease of sheep in South Africa, affecting mainly young animals aged 4 to 6 months, and in good body condition. Pulpy kidney manifests itself in peracute, acute or occasionally chronic forms. It is caused by systemic effects of the epsilon toxin produced by the anaerobic bacterium *Clostridium perfringens* type D (Kriek and Odendaal, 2004). *C. perfringens* forms resistant spores that persist in the soil. Rich rations such as lush and green pasture or abrupt changes in feeding regimes are believed to precipitate the occurrence of the disease. The protein-rich material stimulates the organisms in the intestines to multiply rapidly and to produce lethal toxins. Due to the almost certain death in affected animals, there is almost no form of treatment. The only practical means of controlling the occurrence of Pulpy kidney is to immunize animals. The vaccine is produced by growing the highly toxigenic strain of *C. perfringens* type D in enriched broth, resulting in the production of high concentrations of prototoxins, which are clarified and treated with trypsin. The latter treatment leads to the conversion of the prototoxin into toxins, which are treated with formalin to produce the innocuous toxoids. The toxoids are formulated into an alum-precipitated vaccine or into an oil-emulsion vaccine. With the two OBP vaccines, the immunization is conducted in lambs from 4 to 5 months of age or even sooner if they are weaned at an early age. Optimal immunization is obtained by first vaccinating animals with an oil-emulsion vaccine, followed by a second injection of the alum-precipitated vaccine 4 to 6 weeks later.

1.6.2 *Vibrio* (*Campylobacter fetus*) vaccine

Vibriosis of cattle is a typical venereal disease and is spread from one animal to another during mating. The etiological agent, of which there are a number of subspecies, is *Campylobacter fetus*, formerly called *Vibrio fetus* (Irons *et al.*, 2004). When infected bulls mate with susceptible heifers or cows the infection is transmitted and an inflammatory reaction results in the female genital organs. Conception is usually prevented and the embryo is resorbed or aborted at a very early age. This may result in cows coming on heat repeatedly and subsequent low calving percentage in the herd. The current oil-emulsion OBP vaccine was developed according to the methods described by Clark *et al.* (1972) and Cameron (1982). It is an oil emulsion of formalin-inactivated subspecies of *C. fetus*, namely *C. fetus venerealis* and *C. fetus fetus.* Two biotypes of

C. fetus venerealis are included in the vaccine, given the high occurrence of this subspecies in Southern Africa (Irons *et al.*, 2004). It is recommended to vaccinate both heifers and bulls 8 weeks before the breeding season. A single annual vaccination is thereupon recommended to be given 4 weeks prior to each ensuing breeding season.

1.6.3 *Escherichia coli* vaccine

E. coli infection in livestock is either in an enteric or extra-intestinal form. The disease affects young animals and occurs during the first few days of life. The most common clinical manifestation of the enteric infection is a severe diarrhoea. The disease occurs worldwide and in Southern Africa the enteric colibacillosis occurs most commonly in piglets, and less frequently in calves and lambs (Gyles *et al.*, 2004). Different strains of the causative microorganism are involved in the occurrence of the disease. Given the widespread distribution of non-pathogenic strains, it is important to determine the virulence factors of isolates during the diagnostic process. Enterotoxigenic *E. coli* (ETEC), which produces one or more enterotoxins and colonizes the small intestine in a species-specific manner by means of pili, are the most frequent causes of enteric colibacillosis in calves (Gyles *et al.*, 2004). Vaccination of pregnant cows and ewes is effective in controlling *E. coli* diarrhea in neonate animals. The OBP *E. coli* vaccine is a formalized whole-cell bacterin from smooth strains emulsified in oil. The strains included in the vaccine are reviewed periodically in order to include the most current occurring pathogenic strains. Serotypes including antigen K99 pili and K88 pili are important and included in the vaccine for cattle and sheep.

1.6.4 Infectious coryza vaccine

Infectious coryza (IC) is an acute respiratory disease of chickens, and the cause of serious economic losses as a result of an increased number of culls and a drop in egg production in laying flocks (Droual *et al*., 1990). The causative agent, *Avibacterium paragallinarum*, is a bacterium that can be either NAD (or V-factor) -dependent or -independent (Mouahid *et al.*, 1992; Bragg *et al*., 1993) for growth *in vitro*. Three serogroups are recognized, A, B and C (Page, 1962), with up to four serovars within serogroup A and C (Kume *et al.*, 1983; Blackall *et. al.*, 1990). Inactivated multivalent vaccines, mainly formulated with an oil emulsion, are used

worldwide for the control of IC, most of them comprising serovars of serogroups A, B and C (Blackall, 1999). The vaccine produced by OBP was introduced in the mid-1970s, and seemed to decrease the incidence of the disease, although studies conducted by Bragg *et al.* (1996) demonstrated a significant shift in the incidence of the serovars occurring in the country over a 30-year period.

1.7 REGULATORY LANDSCAPE FOR VETERINARY VACCINES

While considerable progress has been made in terms of vaccine technology, as described above, there has been also considerable evolution in regulatory requirements for the registration or marketing authorization of animal vaccines. Manufacturers of vaccines have to comply with increasingly stringent requirements in terms of efficacy, and more in terms of safety. Different countries, especially the large veterinary product markets, have different registration requirements, making it even more complex for a vaccine manufacturer to comply (Jones *et al.*, 2007). The OIE and other international bodies have initiated processes of harmonization of regulations (Holmes *et al.*, 2007), which is still far from easing this process. In addition, for food animal products there are now strict guidelines to comply with in terms of residue limits of veterinary medicinal products in foodstuffs of animal origin; the rationale being that the use of veterinary medicinal products in food-producing animals may result in the presence of residues of foodstuffs obtained from treated animals.

The regulations in place in most developed countries requires that veterinary vaccine manufacturers produce the vaccine in facilities that comply with strict requirements such as Good Manufacturing Practices (GMP), laboratory activities related to these vaccines comply with Good laboratory Practices (GLP) and clinical work for the development or release of batches comply with Good Clinical Practices (GCP). These requirements have subsequently increased the cost of development and production of veterinary products and vaccines, with some of the consequences being that only commercial production of vaccines can be sustained (no longer in government laboratories), lack of interest by manufacturers to develop and produce vaccines destined to small markets such as vaccines for minor species, *e.g.* rabbits and goats, or vaccines for neglected animal diseases (de Foucault, 2004).

The increasing concern for human safety has also lead regulatory authorities to formulate strict requirements on food animal veterinary products in terms of residue limit for foodstuffs obtained from treated or vaccinated animals. For veterinary vaccines it has become imperative in all developed countries to use starting materials, including components of vaccine adjuvants that are included in specific directives, with known maximum residue limit in food products derived from these animals. As a result of scientific and technical progress it is possible to detect the presence of residues of veterinary medicines in foodstuffs at ever lower levels. It is therefore necessary to establish maximum residue limits for pharmacologically active substances, which are used in veterinary medicinal products in respect of all the various foodstuffs of animal origin, including meat, fish, milk, eggs and honey.

Veterinary medicinal products in South Africa are currently registered under two Acts and are administered by two separate regulatory authorities in two different governmental Departments:

- The Medicines and Related Substances Control Act, 1965 (Act No. 101 of 1965) (Act 101), administered by the Directorate Medicines Control within the National Department of Health. These products are called Veterinary Medicines.
- The Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No. 36 of 1947) (Act 36), administered by a Register appointed within the National Department of Agriculture. These products are called Stock Remedies.

A vaccine manufacturer or distributor in South Africa has to register any immunological product destined for animal use to one of these two Acts. The two acts address Adverse Drug Reactions, which should be dealt with when registering the product (by providing information and data showing the innocuity of the product) and through reporting of any adverse reaction in the use of the product. The latter is addressed in terms of products failing to meet the reported withdrawal period or reactions occurring in people following use of veterinary drugs (either accidental or intentional).

South African manufacturers have therefore to monitor the safety profile of different components used in the preparation and formulation veterinary vaccine. The European Pharmacopeia has issued a directive that is extensively used globally as a basis for the establishment of maximum

residue limits of veterinary medicinal products in foodstuffs of animal origin: Directive 2377/90 of 26 June 1990 (Anon., 1990). This directive provides a list of substances that can be used, with the maximum residue limit. The use of products not included in the directive would require the production of data through target animal experiments to demonstrate the maximum residue limits that could be acceptable for human consumers. A product such as Cirrasol EN-MP, for example, is not included in this directive, and would therefore require extensive evaluation in order to determine its innocuity when used in food animals.

1.8 PURPOSE OF THE THESIS

A large number of veterinary vaccines have been developed at Onderstepoort over the past 100 years for the control of infectious diseases of livestock in Southern Africa (Verwoerd, 2000; Thomson *et al.*, 2003; Dungu *et al.*, 2004). While being generally successful in controlling a number of tropical and production diseases endemic to Southern Africa, most of these vaccines have undergone improvements of different sorts, based on either the need to improve their efficacy or their safety (Anon., 1961; Thomson *et al.*, 2003; Dungu *et al.*, 2004). Despite extensive progress in the field of vaccinology over the last 20 years (Aucouturier *et al.*, 2001; Swayne, 2002; Singh and O'Hagan, 2003), very little improvement has taken place with the Onderstepoort range of vaccines, which are still being produced by Onderstepoort Biological Products (OBP).

While it has not always been easy to assess the actual contribution to the control of the corresponding disease for a number of these vaccines, due to a lack of effective evaluation tools (such as appropriate measurement of the protection level, or whether the lack of disease is the result of lack of challenge or the consequence of an effective protection), it is quite clear for some vaccines that the level of protection generated or the immunogenicity has been limited (Bragg *et al.*, 1996; Hunter and Wallace, 2001; Thomson *et al.*, 2003). Additionally, the evolution and changes in the epidemiology and pathogenicity of some of the pathogens occurring in the country, has rendered some of the vaccines irrelevant or limited in their broad protection ability.

There has been a strong drive in recent years toward safety and stringent compliance to international standards for biological products. Efficacy has been the main focus of vaccine quality in the past over certain current considerations of safety, hence a number of traditional vaccines are now considered non-compliant with modern safety criteria. There is therefore a need to initiate improvements on a number of current OBP vaccines in order to support their continued use in the control of endemic diseases in Southern Africa and for the export market. It is also imperative that the improvements to be considered maintain the cost-effectiveness of the products for the manufacturer, as well as for the end-user.

In order to improve the immunogenicity of most vaccines, especially for non-replicating vaccine antigens (inactivated or subunit vaccines), they are formulated with adjuvants. For many years the selection of adjuvants has not been based on their suitability for the specific antigen and target animal, but rather by their individual generic characteristics. One such example is the fact that very little attention was given to the type of emulsion in oil-adjuvanted vaccines, whether it was a water-in-oil, oil-in-water or double water-in-oil-in-water emulsion. It is now well established, for example, that the continuous phase in an oil emulsion will affect the early or late presentation of the antigen to the host immune system for a long or short period (Aucouturier *et al.*, 2001). Moreover, it has been shown in recent years that the suitability of the adjuvant depends on the nature of the protective immune response, the physiology of the target animal, the suitable efficacy/safety ratio or adjuvanticity/toxicity ratio, and other characteristics such as safety and compliance with international quality standards (Sesardic and Dobbelaer, 2004; Lombard *et al.*, 2007).

The present study is thus designed to address the imperative need to improve the efficacy, safety and regulatory compliance of a number of OBP vaccines, focusing mainly on oil-adjuvanted vaccines.

1.9 SPECIFIC OBJECTIVES OF THIS STUDY

The first overall objective was to re-assess the formulation of a number of oil-adjuvanted vaccines for their efficacy, safety, physical characteristics (viscosity) and compliance with regulation.

The second objective was the re-design of specific vaccines and their evaluation *in vitro*, in laboratory animals and in field trials in target animals.

The following are specific objectives of the study:

- Analysis of the oil adjuvant used in the formulation of specific vaccine emulsions.
- Evaluation of emulsions and subsequent vaccines made with in-house and pre-formulated commercial oil adjuvants.
- Laboratory trials of different vaccine formulations on laboratory and target animals.
- Evaluation of final redesigned vaccines during field trials in target animals.

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CHAPTER TWO

STUDY OF MINERAL OILS USED IN THE FORMULATION OF THE ONDERSTEPOORT BIOLOGICAL PRODUCTS (OBP) INFECTIOUS CORYZA AND PULPY KIDNEY OIL VACCINES

2.1 INTRODUCTION

Oil vaccines are heterogeneous emulsions that consist of the dispersed or liquid phase, oil and surfactant(s). In other words, an emulsion is a dispersion of a liquid, called the dispersed phase, in a second liquid, called the continuous phase. The surfactant is used to allow the dispersed phase to become miscible with the continuous phase. In the case of vaccines, the two phases consist of water (the antigenic medium) and oil, and surfactants are added to stabilize the emulsion (Aucouturier *et al.*, 2001).

The oil used in vaccine emulsions can be either mineral or non-mineral oil. Generally, they may induce local or systemic reactions such as granuloma, abscesses or fever, depending on the nature, chemical structure and origin of the oil (Gupta *et al.*, 1993; Jansen *et al.*, 2005). Mineral oils, such as liquid paraffin, have been used since Freund's work with complete and incomplete Freund's adjuvants (Chang *et al.*, 1998). Mineral oils tend to remain at the site of injection, creating a depot effect, and are eliminated progressively by macrophages (Lindblad, 2000). They can also be partially metabolized into fatty acids, triglycerides, phospholipids or sterols (Aucouturier *et al.*, 2001). Bollinger (1970) demonstrated that 30% of the mineral oil disappears during the first month, and the majority of the oil migrating outside the injection site end up in the liver and fatty tissues in the form of phospholipids and fatty acids. Mineral oils are a mix of several hydrocarbons with different lengths of carbon (C) chains. Work by some research groups has demonstrated the direct impact of the length of the C chain on the safety of adjuvants (Lindblad, 2000): shorter chains (less than 14 C) are most efficient but induce local lesions, while longer chains are safer but less efficient. These differences appear to be linked to the solubilizing and detergent properties of short C chains.

Various non-mineral oils have been tested for their adjuvant characteristics, including many plant oils such as peanut and soybean oils (Eghafona, 1996; Stone, 1997). While safer than mineral oils due to their easily metabolizable nature, they tend to be less efficacious as they fail

to show the benefit from the depot effect. Extensive studies were conducted by Hilleman *et al.* (1972) during the 1960s with an adjuvant named "Adjuvant 65". It consists of 86% peanut oil and 10% Arlacel A (an ester mannide monooleate), which also acts as a surfactant (Gupta *et al.*, 1993). Peanut oil has the advantage of containing metabolizable triglycerides that can be broken down into glycerol and free fatty acid by the body's lipases and thus be cleared. Results obtained through years of trials of the adjuvant with an influenza vaccine in humans proved that while being safe and potent, the formulated vaccine had less adjuvanticity than incomplete Freund's adjuvant (IFA) (Stuart-Harris, 1969). Similar attempts in chickens with Newcastle disease vaccines showed the same trend, with mineral oil-based vaccines showing a better adjuvant effect than metabolizable oil vaccines, including peanut oil (Brugh *et al.*, 1983). However, more recent work suggests that similar adjuvanticity can be obtained with animal, vegetable or other synthetic oils, using organic surfactants (Stone, 1997).

The choice of a suitable oil to be incorporated into an adjuvant is therefore critical, and should take into account a number of safety and efficacy characteristics. These will depend on the target animals for the vaccine to be formulated and the type of immunity required. While vaccine emulsions can be water-in-oil, oil-in-water or water-in-oil-in-water (Aucouturier *et al.*, 2001; Yang *et al.*, 2005), most of the oil vaccines produced by Onderstepoort Biological Products (OBP) have been water-in-oil emulsions. This is due mainly to historical reasons, being based on the IFA principle (Jansen, 1962). In addition, most OBP vaccines contain two emulsifiers: one used for the water phase and another used in the oil phase.

Oil tends to constitute the largest portion of most OBP water-in-oil vaccines, constituting approximately 60% of the whole vaccine emulsion. Liquid paraffin is the mineral oil used in all instances. Given the inherent variations in the characteristics of the liquid paraffin, depending on its origin, which also impacts the chemical composition and level of purity, it is critical to evaluate and conduct a number of tests on each brand and sample received, since there are several suppliers. The commonly used tests include viscosity assays, safety in laboratory animals, purity by means of nuclear magnetic resonance (NMR) and the ability to form good and stable emulsions (emulsifiability and stability). For a commercial entity such as OBP the cost of

the oil is also of great importance, as it will directly affect the production cost of the final vaccine.

Marcol® is a liquid paraffin or white oil largely used in the manufacture of oil emulsions for vaccines. Given the characteristics of Marcol®, which are standard to other available white oils, and given its high cost the present study was designed with the aim of conducting a comparative evaluation with other cheaper similar white oils. The study was therefore specifically aimed at evaluating three different liquid paraffin white oils, including the standard still in use at OBP, namely Marcol®. The most suitable white oil, based on its safety, efficacy and cost-effectiveness characteristics, was to be used for the next steps of the overall study, which aimed at improving the overall formulation of different OBP oil-adjuvanted vaccines.

2.2 MATERIALS AND METHODS

2.2.1 Liquid paraffins

Three liquid paraffin white oils were tested during the course of this study. These comprised Marcol®, which is currently supplied by Zenex, South Africa, under the name of Marcol 52. Marcol 52 conforms to the FDA21 CFR 172.878 and 178.3620 regulations (Anon., 2006), and its purity level complies with most Pharmacopeias (*e.g.* United States Pharmacopeia XXIV, Japanese Pharmacopoeia XIV and European Pharmacopoeia 2002). The cost of this product was R 25-00 per litre (approximately US$ 3.64). The other two liquid paraffins tested, namely C8 and C14, are distributed by Petroleum Fine Products (PFP), Durban, South Africa. The cost of the C8 liquid paraffin was R8.50 per litre, while that of C14 was R 5.50 per litre (approximately US$ 1.24 and US$ 0.8, respectively).

2.2.2 Viscosity testing

The Brookfield method for determining viscosity was used to assess the viscosity of the three liquid paraffins, together with a standard control sample. The Brookfield LV Viscometer (Brookfield Engineering Labs, Inc.) is a rotational viscometer and it is based on the principle that the force required to turn an object in a fluid can indicate the viscosity of that fluid. The

viscometer determines therefore the required force for rotating a disk or bob in a fluid at known speed. This method is captured in a Standard Operating Procedure used for Viscosity Testing at OBP, with reference SOP number QC-EQ-004. Briefly, 11 ml of the oil, using a syringe, was poured into the equipment's LV-1 cylindrical spindle and the samples were tested at three different rotational speeds, *i.e.* 100 rpm, 50 rpm and 20 rpm, respectively. The values obtained in each of the three analyses were used in the formula indicated below in order to determine the viscosity in millipascal per second (mPa/s). A final value less than 300 mPa/s is considered acceptable for vaccine formulation.

$$\text{mPa/s} = [(100 \text{ rpm reading} \times 20) + (50 \text{ rpm reading} \times 50) + (20 \text{ rpm reading} \times 100)] \div 3$$

2.2.3 Safety in experimental animals

Each of the three liquid paraffins was tested for safety in adult mice. A total of 40 adult female mice, weighing approximately 18 g each, were subdivided into four groups of 10 mice each, corresponding to the three oil treatments and a control group that was injected with a saline solution. Each mouse was injected subcutaneously with 0.25 ml of oil. The animals were kept over a 10-day period. Records of their weights were taken before the treatment, and on day 2, 3, 7 and 10 post-treatment. The mice were observed daily for any local reaction at the injection site or for any systemic clinical signs. On day 10, they were euthanized and evaluated for any signs of peritonitis. This experiment was performed with the approval of the OBP Ethics Committee.

2.2.4 Purity test by means of Nuclear Magnetic Resonance (NMR) spectroscopy

In NMR spectroscopy analysis, a substance is subjected to a magnetic field of varying intensity. The nuclei of the atoms (either hydrogen or carbon, depending on the method) in the molecule absorb energy at varying intensity. The positions and number of peaks observed give an indication of the structure of the molecule (Günther, 1995). In this study, the aim of the NMR spectroscopy analyses, conducted at the University of South Africa, Pretoria, South Africa, was to assess the purity of the liquid paraffin. Since liquid paraffin is white oil, only alkanes should be present and no double bonds or other functional groups should be demonstrated. Consequently, both carbon nuclear magnetic resonance (CNMR) and proton nuclear magnetic resonance (HNMR) spectroscopy analyses were conducted. These spectroscopy analyses were

performed to rule out or detect the following groups or contaminants, *i.e.* aromatic carbons, olefinic carbons, carbonyl carbons and heteroatom-bearing carbons.

2.2.5 Emulsifiability and stability of emulsion produced

The ability of each of the three liquid paraffins to form a stable emulsion was assessed by using them to formulate two types of vaccine at laboratory scale, *i.e.* the Infectious coryza (*Avibacterium paragallinarum*) and the Pulpy kidney (*Clostridium perfringens* type D) vaccines. The standard oil formulation, using Arlacel 83V and Tween-80 as emulsifiers, was used to formulate the Infectious coryza vaccine, while Cirrasol EN-MP® and Tween-80 were used for the Pulpy kidney vaccine. Each of the three white oils under investigation was used to generate three different formulations for each vaccine. The rate of incorporation of the Infectious coryza vaccine is summarized in Table 2.1, while the rate of incorporation of the Pulpy kidney vaccine is indicated in Table 2.2.

Table 2.1: Rate of incorporation of different components used to produce the Infectious coryza emulsion vaccine

	Weight (g)	**Density (g/L)**	**Volume (L)**	**Volume (for 1000 L)**
*White oil	60.0	0.83	72.29	636.15
Arlacel 83V	5.0	0.9	5.56	48.9
Tween-80	1.0	1.08	0.926	8.15
Antigen	35.0	1.0	35.0	308.0
Total	101		113.78	1001.2

* The white oil used was either Marcol 52, PFP C8 or PFP C14

Table 2.2: Rate of incorporation of different components used to produce the Pulpy kidney emulsion Vaccine (Experimental formulations F05026)

	Weight (g)	**Density (g/L)**	**Volume (L)**	**Volume (for 1000 L)**
*White oil	54.0	0.83	65.06	638.0
Arlacel 83V	4.5	0.98	4.59	45.0
Tween-80	0.9	1.08	0.833	8.2
Antigen	31.5	1.0	31.5	308.9
Total	90.9		101.98	1000.1

* The white oil used was either Marcol 52, PFP C8 or PFP C14

The stability of the vaccine formulation is critical, as it affects the storage and handling ability of a vaccine. Different temperatures are usually considered for different time periods. Stability at room temperature is considered to be most important as it corresponds to the handling of the vaccine in the field, whereas tests at 37°C are used for accelerated stability, simulating therefore the long-term storage ability of the vaccine (OIE, 2005). All vaccine samples were therefore tested for stability at room temperature and 37ºC, as follow. Ten vials of each vaccine sample were prepared and allowed to reach room temperature by keeping them for 24 h in a 20ºC ± 1ºC (RT) environment. The vaccine samples were shaken before incubation. Five samples of each vaccine type were incubated at 20ºC ± 1ºC (RT) for 21 days, while the other five samples were incubated at 37ºC ± 1ºC for 21 days. All samples were left stationary, with no movement or shaking for the entire 21-day period. The vaccine samples were monitored weekly for any form of separation in the vial, and observations were recorded. On day 21, the stability of the vaccine samples was assessed. The vaccine is considered unstable if more than two phases can be observed, with the water phase clearly visible at the bottom. In contrast, when the vaccine shows just one or two phases, it is considered stable.

2.3 RESULTS

2.3.1 Viscosity of the liquid paraffins

Since the oils used in vaccine emulsions may affect the viscosity of the formulated emulsion, the viscosity of the different liquid paraffins used in this study was determined and compared (data not shown). The viscosity of the PFP C14 sample (16.3 mPa/s) was somewhat higher than that of Marcol 52 (9.3 mPa/s), while the PFP C8 sample had the lowest viscosity (8.6 mPa/s). Therefore, the PFP C8 sample would be preferable, since its low viscosity may improve the injectability of the vaccine emulsion.

2.3.2 Safety of the liquid paraffins in experimental animals

The results of the safety test conducted in mice are recorded in Tables 2.3a-d for PFP C8, PFP C14, Marcol 52 and the saline control, respectively. Weight values recorded for each mouse over a 10-day period showed no signs of abnormal weight loss, indicating no adverse reaction linked to the white oil. On day 10, all mice were sacrificed and post-mortem examinations conducted.

The results of these examinations indicated a lack of peritonitis and are thus an additional indication of the lack of adverse reactions.

Table 2.3a: Safety test in mice injected with the liquid paraffin PFP C8

Animal ID	Weight of mouse in gram						Post-mortem findings: peritonitis
	Day 0	Day 2	Day 3	Day 5	Day 7	Day 10	
1A	19.5	22.0	23.5	26.3	31.2	34.2	No signs
2A	19.1	21.2	22.6	26.7	30.1	32.5	No signs
3A	18.6	21.9	23.3	23.6	29.9	32.0	No signs
4A	18.3	20.5	21.5	26.7	29.3	30.2	No signs
5A	17.7	20.6	22.3	25.1	28.9	30.8	No signs
6A	18.5	22.0	22.6	24.2	24.5	24.7	No signs
7A	18.8	21.8	23.3	26.0	26.6	29.9	No signs
8A	19.6	25.0	26.2	29.3	30.5	31.3	No signs
9A	19.9	21.4	22.9	26.2	28.3	30.9	No signs
10A	19.2	20.3	21.6	24.6	27.1	29.9	No signs

Table 2.3b: Safety test in mice injected with the liquid paraffin PFP C14

Animal ID	Weight of mouse in gram						Post-mortem findings: peritonitis
	Day 0	Day 2	Day 3	Day 5	Day 7	Day 10	
1B	19.2	22.2	23.3	25.8	29.4	29.9	No signs
2B	19.5	21.7	22.3	24.9	27.5	29.3	No signs
3B	18.9	20.6	21.6	24.5	27.3	29.2	No signs
4B	18.2	20.2	20.9	23.4	26.2	27.9	No signs
5B	17.4	19.3	21.3	24.2	26.6	27.5	No signs
6B	17.6	20.2	21.3	25.2	27.7	31.4	No signs
7B	19.5	18.9	19.2	20.6	21.8	22.2	No signs
8B	18.1	19.9	22.6	27.1	30.5	32.9	No signs
9B	18.5	21.3	22.3	24.0	25.9	25.9	No signs
10B	17.6	19.5	20.9	22.8	25.1	25.3	No signs

Table 2.3c: Safety test in mice injected with the liquid paraffin Marcol 52

Animal ID	Weight of mouse in gram						Post-mortem findings: peritonitis
	Day 0	Day 2	Day 3	Day 5	Day 7	Day 10	
1C	19.4	24.2	25.2	27.2	28.2	28.0	No signs
2C	18.8	24.5	26.5	28.3	28.6	28.9	No signs
3C	19.9	27.4	27.6	29.2	29.8	29.8	No signs
4C	19.1	24.0	26.5	28.5	29.8	30.9	No signs
5C	18.5	24.2	25.2	27.1	27.8	26.1	No signs
6C	18.8	20.5	21.2	22.5	23.6	23.6	No signs
7C	19.8	22.2	21.2	23.1	26.3	26.8	No signs
8C	17.9	21.3	23.7	26.2	28.9	30.4	No signs
9C	19.5	25.0	26.3	28.8	30.6	29.4	No signs
10C	19.2	23.7	24.4	27.0	29.2	29.5	No signs

Table 2.3d: Safety test in mice injected with saline as control

Animal ID	Weight of mouse in gram						Post-mortem findings: peritonitis
	Day 0	Day 2	Day 3	Day 5	Day 7	Day 10	
1D	18.3	22.5	23.4	25.3	26.5	27.3	No signs
2D	19.7	23.0	24.0	26.1	27.3	27.3	No signs
3D	18.6	20.9	22.4	24.0	25.4	26.8	No signs
4D	17.8	20.8	22.3	24.3	26.0	26.8	No signs
5D	19.0	21.1	21.5	21.7	18.1	18.3	No signs
6D	18.1	21.4	22.9	25.4	27.2	29.5	No signs
7D	18.1	21.7	22.3	24.2	25.7	25.3	No signs
8D	17.6	17.0	18.1	20.0	23.9	19.9	No signs
9D	19.3	22.6	23.2	25.2	27.0	26.5	No signs
10D	18.5	21.4	22.1	23.3	24.2	20.6	No signs

2.3.3 Purity of the liquid paraffins

Both HNMR and CNMR spectroscopy analyses were conducted on the two grades of white oil, *i.e.* PFP C8 and PFP C14, and compared to results for Marcol 52, which is the standard oil used in OBP vaccines. For these oils the CNMR scans of the samples excluded the presence of contaminating aromatic carbons, olefinic carbons, carbonyl carbons and heteroatom-bearing carbon (see Fig. 2.1 on CNMR of PFP C8). These results were also confirmed by HNMR analyses. The HNMR results of all three samples ruled out the presence of possible contaminant molecules, as shown in Fig. 2.2. Although the PFP C14 sample showed trace amounts of non-specific impurities, no chemical groups other than alkanes were detected in the PFP C8 sample. This is what is expected of white oil and the results are in agreement with that obtained for the Marcol 52 oil sample.

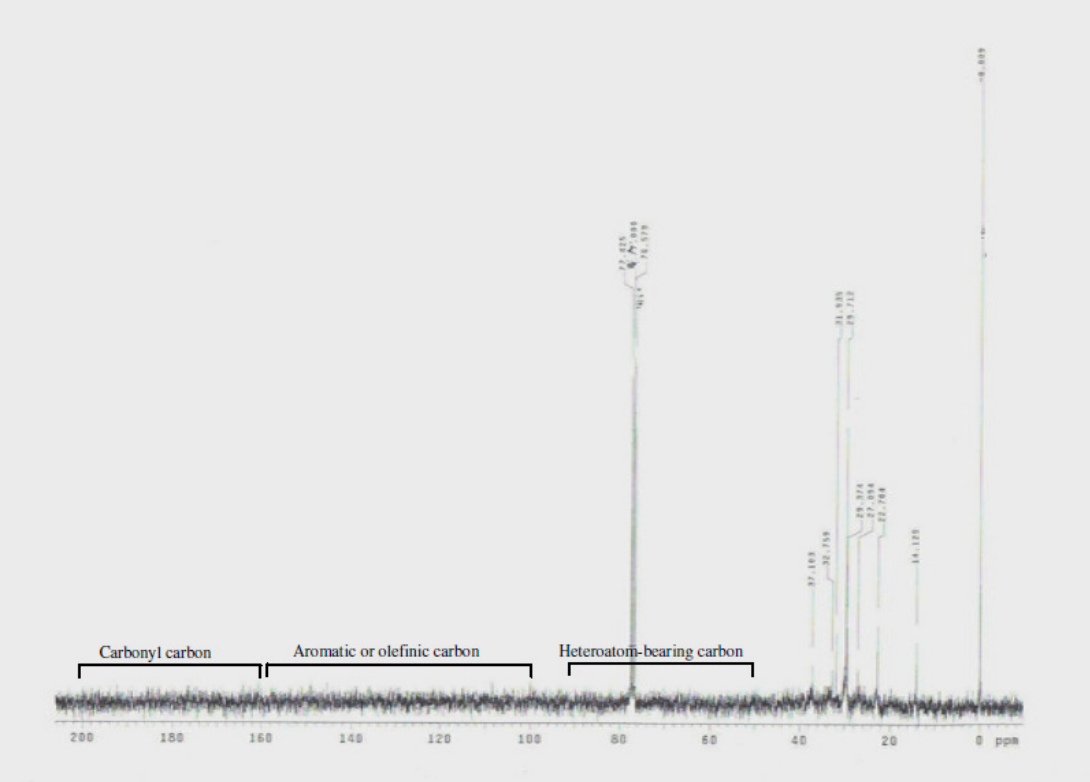

Fig. 2.1: CNMR spectroscopy analysis of the PFP C8 liquid paraffin. The result rules out the presence of carbonyl groups, aromatic or olifenic carbons and heteroatom-bearing carbons, *viz.* NO, OH or a halogen group. Positions where these carbon molecules would be expected are indicated in the scan.

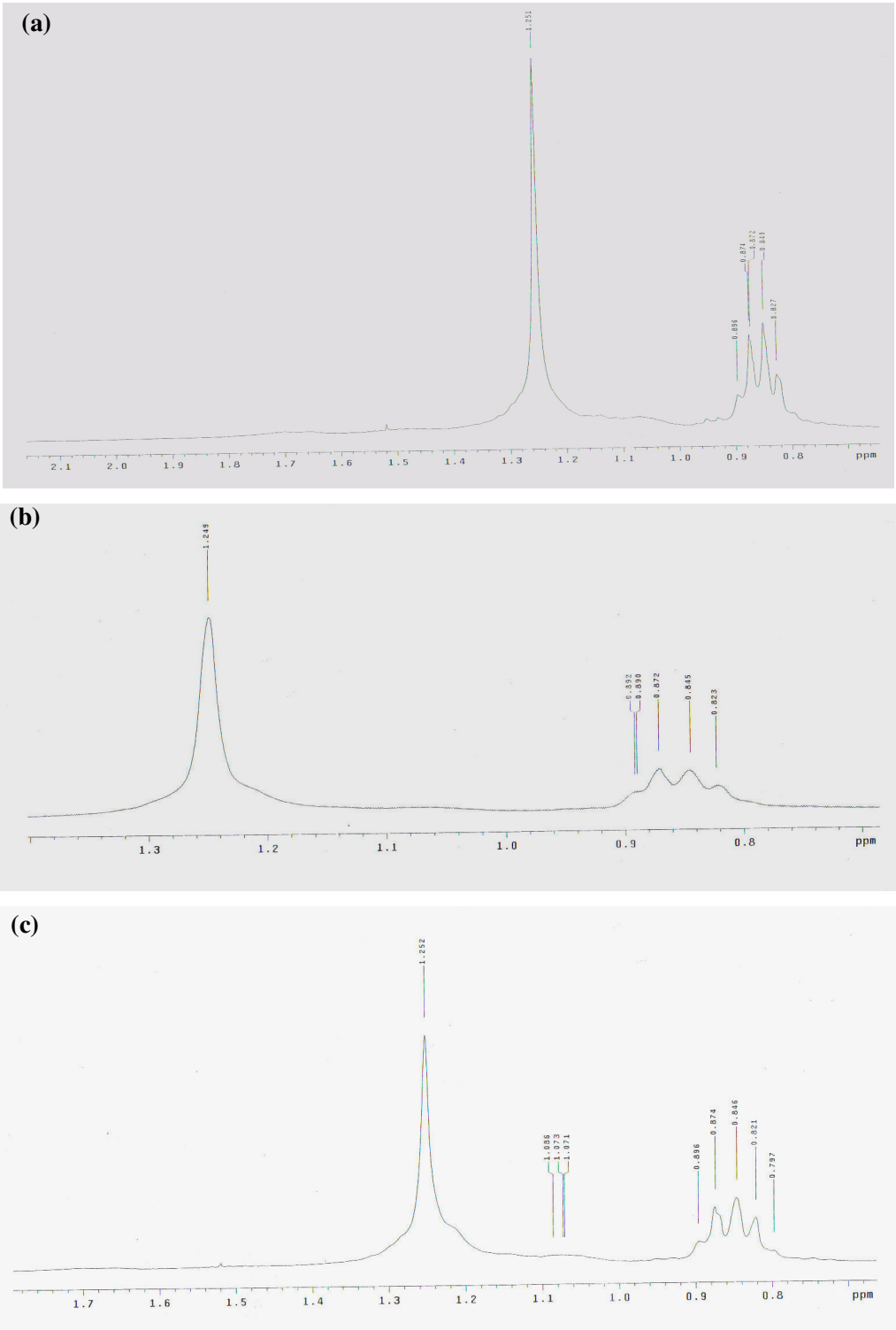

Fig. 2.2: HNMR spectroscopy analysis of the Marcol (a), PFP C8 (b) and PFP C14 (c) liquid paraffins.

2.3.4 Emulsifiability and stability of emulsions produced

The results obtained with the trial vaccine emulsions produced from PFP C8, PFP C14 and Marcol 52 are indicated in Table 2.4. The standard Arlacel 83V formulation was used for the Infectious coryza vaccine, while the Pulpy kidney vaccine was formulated with Cirrasol EN-MP® as surfactant. The vaccine emulsions were stored at room temperature and at 37°C, and their stability was assessed at 21 days post-incubation. The results indicated that all of the vaccine emulsions were stable, irrespective of the liquid paraffin used in their formulation. Although sedimentation was observed following storage of the Pulpy kidney vaccine formulation at 37°C, this is normally observed in Pulpy kidney formulations (with Cirrasol EN-MP®), but does not occur in the Infectious coryza Arlacel 83V formulation.

Table 2.4: Stability results of Infectious coryza and Pulpy kidney vaccines formulated with three different oils, *i.e.* PFP C8, PFP C14 and Marcol 52, following incubation at room temperature (RT) and at 37°C for 21 days

Vaccine type	White oil	Days incubated	Storage temperature	Result
Infectious coryza	PFP C8	21	RT (21°C)	Stable
	PFP C14	21	RT (21°C)	Stable
	Marcol 52	21	RT (21°C)	Stable
	PFP C8	21	37°C	Stable
	PFP C14	21	37°C	Stable
	Marcol 52	21	37°C	Stable
Pulpy kidney	PFP C8	21	RT (21°C)	± Stable
	PFP C14	21	RT (21°C)	± Stable
	Marcol 52	21	RT (21°C)	± Stable
	PFP C8	21	37°C	Sedimentation
	PFP C14	21	37°C	Sedimentation
	Marcol 52	21	37°C	Sedimentation

2.4 DISCUSSION

The viscosity values obtained for the three types of mineral oil were far below the cut-off value of 300 mPa/s. Although this value is used for formulated emulsions, it was, however, important to assess the mineral oil on their own as they constitute an important component of the emulsion, affecting therefore the viscosity. PFP C8 oil was considered to be preferable due to its viscosity value, which is lower than PFP C14. Even though the difference in viscosity between PFP C8 and Marcol 52 was very small, and might be attributed to possible assay variability, the price difference between these two products gives PFP C8 a clear advantage. Low viscosity is critical for vaccine oil emulsions, as it is related to the injectability of the vaccine (Aucouturier *et al.*, 2001). Injectability is defined as the ease of drawing a vaccine into a syringe, as well as the ease of injecting it into the animal. Vaccines with high viscosity make it difficult to achieve these two operations. In addition, such vaccines tend to result in a greater local reaction linked to the trauma they cause at the injection site. Low viscosity, however, can also result in unstable vaccines (Aucouturier *et al.*, 2001). It is therefore critical to conduct appropriate evaluations in order to determine the type of desired emulsion and identify the suitable oil to achieve this. Water-in-oil emulsions tend to result in high viscosity, as is the case with IFA. Factors that can improve the viscosity of water-in-oil emulsions include surfactants with an optimized hydrophilic-lipophilic balance (HLB), and the nature of the oil (Yang *et al.*, 2005). The ratio of water to oil is also critical in improving the viscosity.

The safety evaluation of the oil was conducted by injecting mice subcutaneously, and monitoring them for adverse reactions over a 10-day period. The results indicated that all three oils were safe, as no weight loss or peritonitis occurred. Mice are commonly used to assess safety of oils used in vaccines since adverse reactions can be observed within three days post-inoculation (Fox, 2009). Adverse reactions with paraffin oil would be an indication of possible impurities in the oils. Since all three oils tested were essentially similar in their chemical composition, it could therefore be concluded that the lack of adverse reactions in mice illustrate a good level of purity of the three oils. The purity of the respective oils was subsequently confirmed by NMR spectroscopy analyses, which represents a very accurate approach for assessing the purity of oils (Fox, 2009; Balinovayl *et al.*, 1994).

Two bacterial vaccines, *i.e.* Infectious coryza and Pulpy kidney vaccines, were used to assess the ability of the respective mineral oils to form a stable emulsion. No difference in the emulsifiability of the different oils, formulated with different emulsifiers and antigen, could be seen. The three oils generated stable emulsions that complied with standard criteria used for the release of the respective vaccines. The Infectious coryza vaccine was stable even at 37°C for a period of 21 days. The sedimentation observed with the Pulpy kidney vaccine at 37°C after 21 days was not due to a break-up of the emulsion, but it is a common occurrence with this vaccine and can be reconstituted after vigorous shaking.

In addition to the nature of the oil used, a number of factors can affect the stability of an emulsion. These include the ratio of incorporation of different components of an emulsion, as well as the particle size of the dispersed phase (Liivgren-Bengtsson *et al.*, 1996; Aucoututier *et al.*, 2001). Emulsions with small particles tend to generate more stable emulsions. Antigenic media, more commonly bacterial antigens, often contain proteins which have surfactant properties, as they are constituted of polar and non-polar groups (Aucouturier *et al.*, 2001). This may modify the global HLB, inducing poor stability. In this case, specific HLB adjustments need to be done.

The results of the different evaluations conducted in the present study indicated that the three oils investigated are essentially similar, although PFP C8 has the advantage over PFP C14 of having a better viscosity. The other differentiating criteria, especially between C8 and Marcol 52, remain therefore cost and availability.

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CHAPTER THREE

STUDY OF EMULSIFIERS USED IN THE FORMULATION OF ONDERSTEPOORT BIOLOGICAL PRODUCTS (OBP) VIBRIOSIS, *Escherichia coli* and PULPY KIDNEY OIL VACCINES

3.1 INTRODUCTION

Surfactants or emulsifiers are critical components of oil-based vaccines. Their main role is to stabilize the dispersion of the water phase (*i.e.* the vaccine antigen) into the oil phase (Griffin, 1949; Yang *et al.*, 2005). Surfactants are usually organic amphipathic compounds, containing hydrophobic groups (their "tails") and hydrophilic groups (their "heads"). Therefore, they are typically sparingly soluble in both organic solvents and water. In water-in-oil emulsions, they reduce the interfacial tension between the oil and water by adsorbing at the liquid-liquid interface (Aucouturier *et al.*, 2001; Yang *et al.*, 2005).

Surfactants are often classified into three primary groups, *i.e.* anionic, cationic and non-ionic. The classification is linked to the presence of formally charged groups in the head. A non-ionic surfactant has no charged groups in its head. The head of an ionic surfactant carries a net charge. If the charge is negative, the surfactant is more specifically called anionic, and if the charge is positive, it is called cationic. If a surfactant contains a head with two oppositely charged groups, it is termed zwitterionic (Woodard, 1990; Ascarateil and Dupuis, 2006). The surfactants used in oil vaccine emulsions are generally non-ionic.

Surfactants can also be defined by their hydrophilic-lipophilic balance (HLB) value, which provides information regarding their relative affinity for water and oil phases. According to the HLB value of the surfactant, different kinds of emulsions can be achieved (Aucouturier *et al.*, 2001; Yang *et al.*, 2005). Those with a low HLB value have a high affinity for oily phases and render water-in-oil emulsions. In this case, the antigenic phase is comprised of droplets dispersed into the continuous oily phase. In contrast, those with a high HLB value have a high affinity for the aqueous phase and render oil-in-water emulsions, where the continuous phase is water and the dispersed phase is oil. Finally, with certain specific surfactant systems, when the HLB value is intermediate, water-in-oil-in-water emulsions, also referred to as double emulsions, can be

produced. In this case, the continuous phase is aqueous and the dispersed phase is oil, but inside the oil droplets, an entrapped aqueous phase is found (Yang *et al.*, 2005).

In order to stabilize water-in-oil vaccine emulsions, emulsifiers are incorporated in each of the phases of the emulsion, *i.e.* the water and the oil phases, respectively. The rates of incorporation are determined empirically and vary from different vaccines or different manufacturers. In addition to the assessment of the physicochemical characteristics of an emulsion (*e.g.* conductivity, viscosity, particle size and stability) (Spickler and Roth, 2003), it is critical to determine accurately the rate of incorporation of each phase in the emulsion, and taking into consideration the HLB values. The amount of emulsifier incorporated in a vaccine, while easing the formulation of the vaccine, may have a negative impact on the safety and local reactions induced by the vaccine (Roth, 1999; Spickler and Roth, 2003; Oda *et al.*, 2006).

The surfactant structure and its HLB value also have an impact on the viscosity of the emulsion (Yang *et al.*, 2005). High viscosity in a vaccine renders them difficult to inject and may thus result in local reactions at the injection site due to the high pressure needed to inject them. The characteristics of the emulsifying agent and the proportion of the aqueous phase can be used to improve the viscosity of the emulsion (Hunter *et al.*, 1981). It is therefore important to determine the HLB value of surfactant to be used, and identify the required HLB value for a specific emulsion.

In all the oil vaccines produced by Onderstepoort Biological Products (OBP), two surfactants or emulsifiers are used. One is used with the water phase, and a second with the oil phase. The water-phase emulsifier is invariably polyoxyethylene (20) sorbitan monooleate, commercially known as Tween-80, and is a widely used surfactant known to improve the stability of disperse systems (Hsu *et al.*, 2003). Two types of surfactants have been used traditionally for the oil phase, namely Arlacel® (sorbitan sesquioleate) and Cirrasol EN-MP® (a polyoxyethylene olyl or cetyl ether). The present study was designed to assess surfactants currently used in three oil-based OBP vaccines (*i.e.* the *E. coli*, Pulpy kidney and *Vibrio* vaccines), and to evaluate the suitability of a number of alternative surfactants. More specifically, there was a need to find an alternative to the oil-phase surfactant used in these vaccines, Cirrasol EN-MP®, as its supply was

becoming problematic and about to be discontinued. Moreover, Cirrasol EN-MP® is also not a registered surfactant for use in vaccines. The newly formulated vaccines were subsequently evaluated both in laboratory and target animals (cattle and sheep) for efficacy and safety, respectively.

3.2 MATERIALS AND METHODS

3.2.1 Emulsifiers

Three alternative oil-phase surfactants were evaluated during the course of this study, namely Montanide 103™, Arlacel C and Simulsol-P2®. The properties of these surfactants, in addition to those used currently in OBP oil-based vaccines, are summarized in Table 3.1.

Table 3.1: Characteristics of different surfactants used in the formulation of the Pulpy kidney, *Vibrio* and *E. coli* emulsion vaccines

Emulsifier	Description	HLB value	Phase incorporated
Tween-80	Polyethoxylated sorbitan fatty acid ester Water-phase surfactant used to improve the stability of dispersed systems	15	Water phase
Cirrasol EN-MP®	Non-ionic polyoxyethylene olyl or cetyl ether Emulsifying surfactant produced by SEPPIC	6	Oil phase
Arlacel C	Sorbitan sesquioleate Supplied by Uniqema	3.7	Oil phase
Montanide 103™	Anhydromannitoletheroctadecenoate, belonging to the mannide oleate family Manufactured by SEPPIC	6.5	Oil and water phases
Simulsol-P2®	Polyethoxylated fatty alcohol Manufactured by SEPPIC	6.6	Oil phase

3.2.2 Formulations of emulsions and experimental vaccines

3.2.2.1 Simulsol-P2®

Given that Arlacel® is used in a number of vaccines at OBP and Simulsol-P2® was new, an initial experiment was designed to evaluate its rate of incorporation for one specific vaccine, namely the Pulpy kidney vaccine. This selection was based on the HLB value of Simulsol-P2® being similar to that of the Cirrasol EN-MP® surfactant, which is used in the formulation of the Pulpy kidney vaccine at OBP (Table 3.1). In order to generate data that could be comparable to the Cirrasol EN-MP®-based vaccine, the Simulsol-P2® emulsions were formulated with the Marcol 52 white oil.

The Simulsol-P2® formulation used for the Pulpy kidney vaccine was as follows. For the oil phase, 60 volume parts of Marcol 52 (liquid paraffin) were mixed with 8 volume parts of Cirrasol EN-MP® (HLB 6). For the water phase, 30 volume parts of the vaccine antigen were mixed with 2 volume parts of Tween-80 (HLB 15). The HLB value of the emulsion composed of x% of surfactants of HLB A and y% of surfactants of HLB B was obtained using the formula (Middleton, 1968): HLB (A + B) = (Ax + By) / (x + y). Thus, the HLB for the above Pulpy kidney vaccine formulation was therefore $(0.6 \times 6) + (0.32 \times 15)/1 = 8.8$.

3.2.2.2 Cirrasol EN-MP®, Arlacel C and Montanide 103™

The above three emulsifiers were used in formulations of the *E. coli*, Pulpy kidney and *Vibrio* vaccines. The different formulations made for these three vaccines are summarized in Tables 3.2, 3.3 and 3.4, respectively. Based on the results obtained in Chapter 2, indicating that the white oil PFP C8 may serve as an alternative to Marcol 52, the Arlacel C- and Montanide 103™-based emulsions were formulated with the PFP C8 white oil. It must also be noted that for Montanide 103™ there is no need to include a water-phase surfactant (Tween-80). A diluent (saline) was added in the Arlacel C formulations to make up the volume of the antigen to 30% of water phase, as per the manufacturer's recommendation. The amount of antigen in the water phase, subsequently in a dose of vaccine, was therefore unchanged between the Arlacel C- and the Cirrasol EN-MP®-based formulation.

Table 3.2: Cirrasol EN-MP®, Arlacel C and Montanide 103™ formulations of the *E. coli* vaccine

***E. coli* vaccine**								
Cirrasol EN-MP® formulation F05076			**Arlacel C formulation F05077**			**Montanide 103™ formulation F05078**		
Antigen	300 ml	30% (v/v)	Antigen	315 ml	30.9% (v/v)	Antigen	30 ml	31% (v/v)
Marcol 52	600 ml	60% (v/v)	PFP C8	540 g	63.8% (v/v)	PFP C8	40.74 g	62% (v/v)
Cirrasol	80 ml	8% (v/v)	Arlacel	45.0 g	4.5% (v/v)	Montanide	5.72 g	7% (v/v)
Tween-80	20 ml	2% (v/v)	Tween-80	9.0 g	0.817% (v/v)	Tween-80	———	———

Table 3.3: Cirrasol EN-MP®, Arlacel C and Montanide 103™ formulations of the Pulpy kidney vaccine

Pulpy kidney vaccine								
Cirrasol EN-MP® formulation F04042			**Arlacel C formulation F04040**			**Montanide 103™ formulation F04041**		
Antigen	300 ml	30% (v/v)	Antigen	315 ml	30.9% (v/v)	Antigen	315 ml	31% (v/v)
Marcol 52	600 ml	60% (v/v)	PFP C8	540 g	63.8% (v/v)	PFP C8	618 ml	62% (v/v)
Cirrasol	80 ml	8% (v/v)	Arlacel	45.0 g	4.5% (v/v)	Montanide	74.23 ml	7% (v/v)
Tween-80	20 ml	2% (v/v)	Tween-80	9.0 g	0.817% (v/v)	Tween-80	———	———

Table 3.4: Cirrasol EN-MP®, Arlacel C and Montanide 103™ formulations of the *Vibrio* (*Campylobacter fetus*) vaccine

***Vibrio* vaccine**								
Cirrasol EN-MP® formulation F05025			**Arlacel C formulation F05026**			**Montanide 103™ formulation F05027**		
Antigen	300 ml	30% (v/v)	Antigen	315 ml	30.9% (v/v)	Antigen	315 ml	35% (v/v)
Marcol 52	600 ml	60% (v/v)	PFP C8	540 g	63.8% (v/v)	PFP C8	618.1 ml	57% (v/v)
Cirrasol	80 ml	8% (v/v)	Arlacel	45.0 g	4.5% (v/v)	Montanide	74.23 ml	8% (v/v)
Tween-80	20 ml	2% (v/v)	Tween-80	9.0 g	0.817% (v/v)	Tween-80	———	———

3.2.3 Evaluation of vaccine emulsions

Droplet and conductivity tests were both used to determine the type of emulsion that formed. These tests were conducted on all formulations evaluated. The droplet test was performed by dropping an emulsion droplet into a beaker containing water: a droplet of a water-in-oil emulsion will stay on the surface, given the fact that the continuous phase is oil, while an oil-in-water droplet will spread in the water (Aucouturier *et al.*, 2001). The conductivity of the emulsions was determined using a CDM83 conductivity meter (Radiometer, Copenhagen), and the samples were equilibrated at 25°C before measurement. The conductivity of water-in-oil emulsions is less than 5 microsiemens, whereas that of oil-in-water emulsions is higher than 5 microsiemens (Mowat and Rweyemamu, 1997). The stability of the emulsion was determined at room temperature (RT) and at 37°C over a 21-day period, as previously described (Section 2.2.5, Chapter 2).

3.2.4 Animal experiments

The safety and efficacy of the *E. coli*, Pulpy kidney and *Vibrio* vaccines formulated with each one of the three different formulations, *i.e.* Cirrasol EN-MP® (or the standard formulation), Arlacel C and Montanide 103™, were evaluated in small and large animals. The efficacy of the different formulations was evaluated in small animals, *i.e.* guinea pigs and mice, whereas their safety and innocuity were evaluated in small and large animals (sheep and cattle). This experiment was performed with the approval of the OBP Ethics Committee.

For the efficacy testing the animal experiments were conducted as follows. For the *E. coli* and *Vibrio* vaccines, a total of 36 naïve guinea pigs were selected and subdivided into groups of six animals. Each group of six animals was used for each of the formulations, *i.e.* three groups for the *E. coli* and three groups for the *Vibrio* formulations. The guinea pigs in each group of six were subsequently immunized with each of the formulations. They were then bled at 4 and 6 weeks post-vaccination, their sera prepared and used for the corresponding serum agglutination test (SAT), as described in the following section.

For the Pulpy kidney vaccine, a total of 24 guinea pigs were used and they were subdivided into groups of eight guinea pigs. Each group of eight guinea pigs was immunized with one of the three Pulpy kidney vaccine formulations. Blood samples were collected at 6 weeks post-vaccination, serum samples prepared and used in the toxin neutralization (L+) test, as described in the following section.

The innocuity tests for each vaccine were performed by vaccinating small and large animals with the various vaccines and then observing them daily for a period of two weeks post-vaccination. For the *E. coli* and the Pulpy kidney vaccines, each formulation was used in ten adult mice, eight guinea pigs, two cattle and two sheep. Each formulation was inoculated using different routes and doses, as detailed in Table 3.9. Vaccinated animals were monitored daily over 14 days for any adverse reactions. Rectal temperatures and local reaction at the injection site were monitored and recorded for sheep and cattle. The innocuity of the *Vibrio* vaccine was similarly tested on eight guinea pigs, one bovine and one sheep.

3.2.5 Assays for evaluation of the potency of different vaccines

The tests performed were similar to those used for the standard quality control of the currently equivalent vaccines. The *E. coli* and *Vibrio* vaccines were tested for potency using the serum agglutination test (SAT), whereas the toxin neutralization test, referred to as L+, was used for determining the efficacy of the Pulpy kidney vaccines.

3.2.5.1 Serum agglutination test (SAT)

This test is a simple, quantitative agglutination test whereby an antigen dilution is reacted with a series of standard antiserum dilutions. In this case the test is used to determine the concentration of the antigen. Reaction occurs at the point where the amounts of antigen and antiserum are equivalent. A positive reaction is indicated by visible precipitation of the serum/antigen complex formed. The test is usually read after 24 h, and the result is expressed in terms of the dilution at which the reaction occurs (Ruckerbouer *et al.*, 1971; OIE manual, 2004).

Sera from guinea pigs, immunized with each of the different *E. coli* and *Vibrio* vaccine formulations and bled at 4 and 6 weeks post-vaccination, were prepared for the corresponding SAT, as per OBP Standard Operating Procedure QA-ME-236. Briefly, the vaccine antigen was centrifuged after which an equal volume of 0.1% formalin-PBS was added, mixed properly and stored at 4°C. The guinea pig sera were diluted 1:5 in 1% formalin-PBS and pipetted into the wells of 96-well microtiter plates. The serum mixes were serially diluted in the microtiter wells, and 50 µl of a constant concentration of antigen was pipetted into all wells containing the serially diluted sera. Following incubation of the sealed plates for 24 h, the plates were interpreted: reading from left to right, the last dilution value where agglutination occurred is noted as the result. The presence of a white pellet in the bottom of the wells indicated that no agglutination took place.

3.2.5.2 Toxin neutralization test (L+)

This test is a quantitative toxin neutralization test used in the assay of antitoxin potency (Jansen, 1967; Cameron, 1982). A series of toxin dilutions of known potency is reacted with a series of antitoxin dilutions of unknown potency, obtained from, for example, vaccinated guinea pigs. The series so reacted is then injected into mice, which then die over a period of a few days. The point at which there is no longer sufficient antitoxin to protect the mice indicates the potency of the antitoxin. The dilution at which this occurs is converted into toxin units derived from a known standard toxin, and the result is expressed as the maximum number of toxin units against which the guinea pig antitoxin protects.

Guinea pigs, immunized with the three experimental batches of Pulpy kidney vaccine, were bled at 6 weeks post-vaccination and their sera prepared for the L+ test as follows. The guinea pig sera were prepared into three serum samples, called A, B and AB, where A comprised of four pooled guinea pigs sera, B comprised the pool of the remaining four sera, and for AB the sera of A and B were pooled in an equal volume of each. The test sera were diluted in PBS and serially mixed with the toxin, as shown in Table 3.5. The prepared test samples were incubated at 37°C (± 0.5ºC) for 30 min or at 20°C (± 0.5ºC) for 60 min. The samples (0.2 ml) were injected intravenously into two mice per dilution for Sample A and B, and four mice per dilution for Sample AB. The dilutions were injected into mice within 30 min after preparation. The mice

were observed for 24 h and the results were noted. Animals that died were noted with a (+), and animals that lived were noted with a (L). The lowest Unit/ml value where the animals died is noted as the vaccine Unit/ml value (British Pharmacopeia, 1993). In parallel the potency of a standardized toxin was evaluated, using standardized sera, as a control.

Table 3.5: Pulpy kidney test sample dilution

Units/ml	50	33.3	20	10	5	2.5	1
Saline (ml)	0.6	0.4	0.9	0.8	0.6	0.2	0.8
Serum sample (ml)	0.4 ($^1/_{100}$)	0.6 ($^1/_{100}$)	0.1 ($^1/_{10}$)	0.2 ($^1/_{10}$)	0.4 ($^1/_{10}$)	0.8 ($^1/_{10}$)	0.2 ($^1/_{10}$)
Prepared toxin (ml)	1(L+)	1(L+)	1(L+)	1(L+)	1(L+)	1(L+)	1(L+)

3.3 RESULTS AND DISCUSSION

3.3.1 Evaluation of stability characteristics of Simulsol-P2®

Due to the closeness of the HLB value of Simulsol-P2® to that of Cirrasol EN-MP® (6.6 and 6, respectively), it was evaluated in different formulations of the Pulpy kidney antigen. The final HLB values were calculated and the resulting emulsion evaluated for its stability at room temperature and at 37°C. Three different experimental formulations were used. In the first experimental formulation, Cirrasol EN-MP® was replaced volume-for-volume by Simulsol-P2®, with all the other components kept the same. In the second experimental formulation, the formulation was adjusted to a HLB value equivalent to the Cirrasol EN-MP®-based vaccine (*i.e.* an HLB value of 8.8). In a third experiment, two other formulations were attempted. These were empirical and advised by the supplier of the emulsifiers, based on undisclosed information from similar formulations on non-OBP products. The amounts of emulsifier used were reduced by almost half of those in the previous experiment.

The results obtained from the different experimental formulations indicated the following. Substitution of Cirrasol EN-MP® (HLB of 6) with Simulsol-P2® (HLB of 6.6) yielded an average HLB of 9.28 for the emulsion. This vaccine formulation gave a “translucent” emulsion,

indicating that the HLB was too high for a water-in-oil emulsion to form. Moreover, there was also high conductivity in the continuous phase.

The formulation was then adjusted to give a HLB equivalent to that of the current Cirrasol EN-MP®-based vaccine, *i.e.* an HLB of 8.8. Thus, the formulation consisted of 60% (v/v) of Marcol 52, 8.57% (v/v) of Simulsol-P2®, 1.43% (v/v) of Tween-80 and 30% (v/v) of antigen. In this case, the result was more promising. There was no conductivity in the continuous phase. Although the emulsion was stable at room temperature, the antigen settled to the bottom with pure oil as supernatant. At 37°C, separation and breaking of the emulsion occurred.

In the third experiment, two formulations were prepared. In the first vaccine formulation, consisting of 66.6% (v/v) of Marcol 52, 4.2% (v/v) of Simulsol-P2®, 0.88% (v/v) of Tween-80 and 28.6% (v/v) of antigen, an average HLB value of 9.1 was obtained. The conductivity was in the continuous phase, and the separation of the water and oil phases thus occurred. Since the HLB value was too high, the emulsion turned into oil-in-water. In the second vaccine formulation, which was identical to the first except for the use of 4.76% (v/v) of Simulsol-P2® and 0.44% (v/v) of Tween-80, an average HLB value of 8.85 was obtained. In this case, there was no conductivity in the continuous phase. However, a break-up and separation of the emulsion was observed.

The results obtained in the three experiments above may therefore be summarized as follow. In the first experiment, the high average HLB value of 9.28 forced an oil-in-water emulsion. In the second experiment, the amount of emulsifier was too much and likely forced a large particle size, which caused the precipitation of emulsified particles and later separation. In experiment 3a, the HLB value was again too high and the emulsion tended to oil-in-water, whereas in experiment 3b, the emulsifier was too little, causing separation of the emulsion. Taken together, the results obtained during the evaluation of the physical characteristics of the OBP Pulpy kidney vaccine emulsions containing Simulsol-P2® thus indicated a poor ability to form a stable emulsion. The suitability of an emulsifier is highly empirical due to the complexity of interaction between different components of the mixture (including trace substances in the antigen) and the

possibility of generating unstable emulsions (Aucouturier *et al.*, 2001). Simulsol-P2® was therefore disqualified for further evaluation.

3.3.2 Evaluation of Cirrasol EN-MP® versus Arlacel C and Montanide 103™

In order to determine the suitability of Arlacel C and Montanide 103™ as possible replacements for Cirrasol EN-MP® in the *E. coli*, Pulpy kidney and *Vibrio* vaccines, different formulations of these vaccines were made and compared for their efficacy and safety in experimental and target animals.

In the standard OBP Cirrasol EN-MP®-based vaccines, the oil used, which is the bulk of the vaccine dose, is the white mineral oil or liquid paraffin commercially known as Marcol 52. It conforms to the FDA21 CFR 172.878 and 178.3620 regulations, and its purity level complies with that of the United States Pharmacopeia XXIV, Japanese Pharmacopoeia XIV and European Pharmacopoeia 2002. Two non-ionic emulsifiers are used. The emulsifier in the water or antigen phase, Tween-80, is added to a final composition of 2% of the final vaccine. The second emulsifier, used in the oil phase, Cirrasol EN-MP®, forms 8% of the final vaccine. For Arlacel C, a formulation similar to that used in the OBP Infectious coryza vaccine (Chapter 4), which is also based on Arlacel C, was used. However, PFP C8 was used as the white oil and the antigen was incorporated at the rate of 30%, as is the case with the Cirrasol EN-MP®-based Pulpy kidney, *Vibrio* and *E. coli* vaccines. The final HLB of the formulation was 5.3, and the water-in oil-emulsion thus produced was satisfactory and passed both the conductivity and droplet tests. Thus, in summary, in both vaccines the oil phase forms more than 60% of the final product, while the antigen and water phase is approximately 30% of the final vaccine. These vaccine formulations are also composed of 4 to 8% of non-ionic emulsifier for the oil (*i.e.* Cirrasol EN-MP® or Arlacel C), and approximately 0.8 to 2% of non-ionic emulsifier for the water phase (Tween-80).

Given the fact that Montanide 103™, with an HLB of 6.5, is a commercial vaccine emulsifier used as unique surfactant for the water and oil phases, its incorporation rate was determined by the manufacturer, *i.e.* SEPPIC, and therefore did not require adjustment. Montanide 103™ was incorporated at 8% mass. In order to meet a similar specification as the current Cirrasol EN-

MP®-based vaccine formulation, the antigen had to be incorporated at the rate of 30%. This resulted in a formulation typically consisting of 62% of PFP C8, 7% of Montanide 103™ and 30% of antigen. This formulation yielded a water-in-oil emulsion, which was stable and passed the droplet and conductivity tests.

3.3.2.1 Efficacy of the vaccine formulations

The efficacy for the Pulpy kidney, *E. coli* and *Vibrio* vaccines formulated with the two promising emulsifiers, *i.e.* Arlacel C and Montanide 103™, were compared to the corresponding standard vaccines formulated with Cirrasol EN-MP®. The efficacy studies were conducted in small and large animals, after obtaining approval from the OBP Animal Ethical Committee. Blood samples collected from vaccinated animals were subjected to the serum agglutination test (SAT) for the *E. coli* and *Vibrio* vaccines, whereas the toxin neutralization test or L+ was used for the determination of the efficacy of the Pulpy kidney vaccines.

- ***E. coli* vaccine**

The SAT was used to evaluate the efficacy of the three formulations of the *E. coli* vaccines, following their immunization in guinea pigs. Blood samples were collected at 4 and 6 weeks post-vaccination. The SAT results obtained for the three formulations are provided in Table 3.6.

Table 3.6: Quality control serum agglutination tests (SAT) in guinea pigs for the Cirrasol EN-MP®, Arlacel C and Montanide 103™ formulations of the *E. coli* vaccine

Cirrasol EN-MP® formulation (F05076)			**Arlacel C formulation (F05077)**			**Montanide 103™ formulation (F05078)**		
	SAT titers (K99 antiserum)			**SAT titers (K99 antiserum)**			**SAT titers (K99 antiserum)**	
Animal	**4 wks PV**	**6 wks PV**	**Animal**	**4 wks PV**	**6 wks PV**	**Animal**	**4 wks PV**	**6 wks PV**
1	256	64	1	512	4096	1	4096	2048
2	32	256	2	1024	4096	2	4096	4096
3	64	32	3	1024	4096	3	4096	4096
4	128	128	4	4096	2048	4	1024	2048
5	32	512	5	2048	2048	5	2048	1024
6	32	64	6	1024	4096	6	2048	4096

The potency results obtained for the *E. coli* vaccine indicated that the Arlacel C and Montanide 103™ formulation had significantly higher titres than the Cirrasol EN-MP® formulation. Although the significance of such higher titer at herd- or flock-level can only be studied in large groups of animals over a longer period of time, at least similar or better protection as the registered Cirrasol EN-MP® vaccine can be guaranteed in the target animals vaccinated with the Arlacel C or Montanide 103™ vaccine.

- **Pulpy kidney vaccine**

The toxin neutralization assay or L+ was used to assess the efficacy of the Pulpy kidney vaccine. Three groups of eight guinea pigs were immunized each with one of the three formulations of the Pulpy kidney vaccine. They were bled 6 weeks post-vaccination. The serum samples were then prepared and used in the toxin neutralization (L+) test, as described under Section 3.2.5.2. The results obtained are summarized in Table 3.7.

Table 3.7: Mouse neutralization assay (L+) test of the current and alternative formulations of the Pulpy kidney vaccine 6 weeks post-vaccination

Cirrasol EN-MP® formulation (F04042)								
Sample	**50**	**33.3**	**20**	**10**	**5**	**2.5**	**1**	**Results (Units/ml)**
A	++	++	++	++	LL	LL	LL	10
B	++	++	++	LL	LL	LL	LL	20
AB	++++	++++	++++	++++	LLLL	LLLL	LLLL	10
Arlacel C formulation (F04040)								
Sample	**50**	**33.3**	**20**	**10**	**5**	**2.5**	**1**	**Results (Units/ml)**
A	++	++	++	++	LL	LL	LL	10
B	++	++	++	+L	LL	LL	LL	20
AB	++++	++++	++++	++LL	LLLL	LLLL	LLLL	10-20

Montanide 103™ formulation (F04041)								
Sample	**50**	**33.3**	**20**	**10**	**5**	**2.5**	**1**	**Results (Units/ml)**
A	++	++	++	+L	LL	LL	LL	20
B	++	++	++	++	LL	LL	LL	10
AB	++++	++++	++++	++++	LLLL	LLLL	LLLL	10

The results obtained for the three samples of each one of the three formulations did not show any significant differences. The efficacy results of the two new formulations were therefore similar to those of the standard Cirrasol EN-MP® vaccine.

It was difficult to explain the reason why Pulpy kidney vaccine formulated in Cirrasol EN-MP® demonstrated similar efficacy with the Arlacel C and Montanide 103™ formulations, while the similar formulation of the *Vibrio* and *E. coli* vaccines showed better efficacy than the traditional Cirrasol EN-MP® formulations of these vaccines. It is difficult to attribute the difference to the assay method used, *i.e.* the toxin neutralization, which is reported to be a sensitive method, although it uses indirect approaches through mice. It is also difficult to attribute the lack of difference to the fact that Pulpy kidney vaccine is a toxoid, while the other two are inactivated bacteria. The similarity in the efficacy between the alternative formulations and Cirrasol EN-MP® was rather considered as a very positive outcome, as it provided the opportunity to comfortably consider an alternative, given the challenges with Cirrasol EN-MP®.

- ***Vibrio* (*C. fetus*) vaccine**

The SAT was used to evaluate the efficacy of the three formulations of the *Vibrio* vaccines, following their immunization in guinea pigs. Blood samples were collected at 4 and 6 weeks post-vaccination. The guinea pig sera were subjected to the SAT using two or three standard antigens. The results obtained for the three formulations are indicated in Table 3.8.

Table 3.8: Quality control serum agglutination tests (SAT) in guinea pigs for the Cirrasol EN-MP®, Arlacel C and Montanide 103™ formulations of the *Vibrio* (*C. fetus*) vaccine

Cirrasol EN-MP® formulation (F05025)						
	SAT titers (7572 antiserum)		**SAT titers (68/4 antiserum)**		**SAT titers (873/5 antiserum)**	
Animal	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**
1	320	320	160	320	640	0
2	160	160	160	1280	160	320
3	80	0	40	0	0	0
4	640	40	40	0	320	0
5	320	40	320	0	0	0
6	160	160	0	640	0	160
Arlacel C formulation (F05026)						
	SAT titers (7572 antiserum)		**SAT titers (68/4 antiserum)**		**SAT titers (873/5 antiserum)**	
Animal	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**
1	5120	10240	5120	10240	#ND	ND
2	5120	10240	5120	5120	ND	ND
3	5120	10240	2560	10240	ND	ND
4	2560	10240	5120	5120	ND	ND
5	5120	10240	5120	10240	ND	ND
6	2560	10240	5120	10240	ND	ND
Montanide 103™ formulation (F05027)						
	SAT titers (7572 antiserum)		**SAT titers (68/4 antiserum)**		**SAT titers (873/5 antiserum)**	
Animal	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**	**4 wks PV**	**6 wks PV**
1	5120	10240	5120	10240	ND	ND
2	2560	10240	5120	10240	ND	ND
3	2560	10240	10240	10240	ND	ND
4	2560	10240	10240	1280	ND	ND
5	2560	10240	5120	10240	ND	ND
6	2560	10240	10240	5120	ND	ND

ND; not determined

The serum agglutination test (SAT) for the *Vibrio* (*C. fetus*) vaccine is generally conducted on three different standard antigens, with a minimum of two: antigen 7572, 68/4 and 873/5 have been well characterized and are used in the evaluation of new *Vibrio* vaccine batches. The Cirrasol EN-MP® vaccine was evaluated against all three antisera, while the Arlacel C and Montanide 103™ were not tested against the 873/5 antigen. Due to the limited availability of the standardized antigens, two out of the three are initially used, and the third antigen is assessed in case of inconclusive results. The Arlacel C formulation of the *Vibrio* (*C. fetus*) vaccine displayed titres up to 30-times higher than the Cirrasol EN-MP® formulation. Equally, the titers of the Montanide 103™ vaccine were also higher than those of the Cirrasol EN-MP® vaccine.

3.3.2.2 Innocuity and safety of the vaccine formulations

The quality control procedures in small animals provided an indication of the potency of the vaccines in the target animals. In these procedures either guinea pigs (*E. coli* and *Vibrio*), or guinea pigs and mice (Pulpy kidney) were used. The safety of the vaccines was, however, conducted in the target animals (sheep and/or cattle). The results for the innocuity and safety test performed on the different formulations of the *E. coli*, Pulpy kidney and *Vibrio* vaccines are summarized in Table 3.9.

No adverse reactions were seen at the injection site of the small animals or in the target animals (bovine) vaccinated with the Arlacel C- and Cirrasol EN-MP®-based *E. coli* vaccines. However, the Montanide 103™-based vaccine caused swellings at the injection site for the bovines. In the case of the three Pulpy kidney formulations, no adverse local reactions were seen in the target animals. For the Arlacel C and Montanide 103™ formulations of the *Vibrio* vaccine, no reactions occurred at the injection site. The Cirrasol EN-MP®-based *Vibrio* vaccine, however, resulted in a swelling at the injection site of the vaccinated bovine, starting 4 days after vaccination and measuring approximately 7 cm in diameter. This swelling, however, decreased progressively to 2 cm on day 14. Nevertheless, it was considered as acceptable and not representing any safety problem. The vaccination results in target animals (sheep and cattle) furthermore provided additional safety data for the PFP C8 liquid paraffin, since vaccines formulated with this white oil had similar safety performance as the Marcol 52-based equivalent.

Table 3.9: Innocuity and safety data of different formulations of the *E. coli*, Pulpy kidney (PK) and *Vibrio* (*C. fetus*) vaccines tested in laboratory animals (guinea pigs and adult mice), and in target large animals (sheep and cattle), monitored over a 14-day period

Vaccine	Animals	*Vaccination regime	Temperature reaction over 14 days	#Local reactions/death	
				Day 7	Day 14
E. coli- Cirrasol EN-MP®	10 adult mice	0.2 ml s/c			No death
	8 guinea pigs	0.5 ml I.M.			No death
	2 cattle	2 ml I.M.	Normal	Normal	Normal
	2 sheep	2 ml I.M.	Normal	Normal	Normal
E. coli-Arlacel C	10 adult mice	0.2 ml s/c			No death
	8 guinea pigs	0.5 ml I.M.			No death
	2 cattle	2 ml I.M.	Normal	Normal	Normal
	2 sheep	2 ml I.M.	Normal	Normal	Normal
E. coli- Montanide 103™	10 adult mice	0.2 ml s/c			No death
	8 guinea pigs	0.5 ml I.M.			No death
	2 cattle	2 ml I.M.	Normal		Swelling 12 cm
	2 sheep	2 ml I.M.	Normal	Normal	Normal
PK-Cirrasol EN-MP®	10 adult mice	0.2 ml i.v.			No death
	8 guinea pigs	1 ml s/c			No death
	2 sheep	2 ml s/c	Normal	Normal	Normal
PK-Arlacel C	10 adult mice	0.2 ml i.v.			No death
	8 guinea pigs	1 ml s/c			No death
	2 sheep	2 ml s/c	Normal	Normal	Normal
PK-Montanide 103™	10 adult mice	0.2 ml i.v.			No death
	8 guinea pigs	1 ml s/c			No death
	2 sheep	2 ml s/c	Normal	Normal	Normal
Vibrio-Cirrasol EN-MP®	8 guinea pigs	1 ml s/c	N.A.	-	No death
	1 bovine	2 ml s/c	Normal	7-cm lump	2-cm lump
Vibrio-Arlacel C	8 guinea pigs	1 ml s/c	N.A.		No death
	1 bovine	2 ml s/c	Normal	Normal	Normal
Vibrio-Montanide 103™	8 guinea pigs	1 ml s/c	N.A.		No death
	1 bovine	2 ml s/c	Normal	Normal	Normal

* Vaccines were administered as per normal prescription either intravenously (i.v.), sub-cutaneously (s/c) or intra-muscularly (I.M.)

Local reaction at injection sites were recorded for sheep and cattle, while death was recorded for guinea pigs and adult mice

In conclusion, the Arlacel C and Montanide 103™ formulations of all the vaccines passed all the registered quality control tests. In certain cases the alternative formulations were much more effective than the current formulation, which contained Cirrasol EN-MP®. The Arlacel C formulation is safe for use in the target animals since no adverse reactions were detected in any of the tests. Although the results obtained indicated that the Arlacel C formulation is more immunogenic than the current formulation for some of the vaccines, long-term field trials will be useful to determine whether they are more efficacious than the current vaccines. At least the minimum required level of protection was recorded for all the alternative formulations, making them suitable for replacing the current formulation.

Montanide 103™ showed also very good physical and immunological characteristics when formulated with the three vaccines under evaluation. One of the vaccines, however, the Montanide 103™-based *E. coli* vaccine caused persistent local reactions at the injection site of the vaccinated cattle. Although requiring further investigations, these results, together with the high cost of the Montanide 103™, as compared to Arlacel C, directed the preference for the latter as an alternative for further field evaluation of the three vaccines.

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CHAPTER FOUR

STUDY ON THE EFFICACY AND SAFETY OF DIFFERENT ANTIGENS AND OIL FORMULATIONS OF INFECTIOUS CORYZA VACCINES CONTAINING A NAD-INDEPENDENT STRAIN OF *Avibacterium paragallinarum*

4.1 INTRODUCTION

Infectious coryza (IC) is an acute respiratory disease of chickens, and the cause of serious economic losses as a result of an increased number of culls and a drop in egg production in laying flocks. The impact on broilers has also been shown during outbreaks in the USA (Droual *et al.*, 1990). The causative agent, *Avibacterium paragallinarum* (Blackall *et al.*, 2005), is a bacterial organism that can be either NAD (or V-factor)-dependent or -independent (Bragg *et al.*, 1993; Mouahid *et al.*, 1992) for growth *in vitro*. Three serogroups are recognized, namely A, B and C (Page, 1962), with up to four serovars within serogroup A and C (Blackall *et al.*, 1990; Kume *et al.*, 1983). The role played by NAD-independent serovars has been extensively studied in South Africa (Miflin *et al.*, 1995; Bragg *et al.*, 1996), where they have been shown to be the most dominant serovars in certain provinces such as Kwa-Zulu Natal.

Since first recorded in South Africa in the late 1960s (Buys, 1982), IC has occurred widely in different parts of the country. Despite the introduction of a vaccine in the mid-1970s, which seemed to decrease the incidence of the disease, more outbreaks were recorded in the 1980s. Studies conducted by Bragg *et al.* (1996) demonstrated a significant shift in the incidence of the four serovars occurring in the country over a 30-year period. Serovar C-3 had become predominant, representing 73% of isolated serovars in the 1990s, mainly in the period following the introduction of the vaccine, which did not include C-3. This work and the work by other researchers demonstrated and confirmed the importance of including locally occurring serovars in the vaccine for effective control (Terzolo *et al.*, 1997; Blackall, 1999).

This Chapter has been published:

Dungu, B., Brett, B., MacDonald, R., DeVille, S., DuPuis, L, Theron, J. and Bragg, R.R. (2009). Study on the efficacy and safety of different antigens and oil formulations of infectious coryza vaccines containing an NAD-independent strain of *Avibacterium paragallinarum.* Onderstepoort Journal of Veterinary Research, 76: 299-309.

Inactivated multivalent vaccines are used worldwide for the control of IC, most of them comprising serovars of serogroups A, B and C (Blackall, 1999). As for most inactivated vaccines, the adjuvant used for the formulation of the vaccine plays a critical role in defining the type of protection afforded by the antigen and the duration of immunity. Most IC vaccines are formulated with an oil emulsion. Ideally the vaccine should provide effective protection, while being safe and having the least effect on chicken productivity. A long-lasting immunity is therefore preferred in order to avoid re-vaccinating layers during their production period. Water-in-oil emulsions are thus commonly used, as they are known to induce strong and long-term immunity (Aucouturier *et al.*, 2001). One of their main disadvantages, however, is the possibility of local reactions when used with a crude antigen (Dupuis *et al.*, 2006). Double oil emulsions, consisting of a water-in-oil-in-water emulsion, are usually safer and also suitable for poultry (Dupuis *et al*., 2006). Depending on the type of formulation, type of emulsifying agent and the surfactant used, different responses can be obtained with different formulations that will affect the protection ability of the vaccine, hence the need for *in vivo* evaluations.

An effective combination of a relevant vaccine strain and suitable adjuvant is critical for ensuring that the vaccine provides optimal protection. Since its introduction in the mid-1970s the Onderstepoort Biological Products (OBP) IC vaccine has been adjusted to include the main serovars occurring in the country. The latest vaccine, CoryzaPlus Vaccine, includes a C-3 NAD-independent strain together with locally occurring serovar A and C isolates. The present study was thus designed to evaluate the efficacy and safety of alternative experimental oil vaccines formulated with the same vaccine antigens as the OBP CoryzaPlus IC vaccine, but using different oil adjuvants. The safety and ability of these formulations to provide long-term protection to layers kept throughout a production cycle were compared to those of an international IC commercial vaccine registered in South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Vaccines

The OBP CoryzaPlus vaccine was used in the study, together with two experimental oil vaccines formulated with the same vaccine antigens as the OBP vaccine and a commercial vaccine, referred to as "ComA". The OBP CoryzaPlus vaccine is a water-in-oil emulsion of formalin-inactivated *A. paragallinarum* strains. It is a quadrivalent vaccine containing serotypes A and C (two strains) and a C-3 NAD-independent vaccine strain. The same strains were used to formulate the two experimental vaccines: one with the water-in-oil-in-water double emulsion Montanide™ ISA 206 VG (SEPPIC) and the other with the water-in-oil emulsion, Montanide™ ISA 70 VG (SEPPIC). The commercial vaccine is a water-in-oil emulsion that contains serovar A, B and C strains, but not the NAD-independent strain. The emulsions of the experimental vaccines were evaluated at the SEPPIC Vaccine Department Laboratory in Castres (France) for physical and chemical properties, including particle size and stability at 4°C, room temperature and 37°C up to one month. They were compared to placebo emulsions, made of a saline solution.

4.2.2 Chickens

In order to ensure homogeneity among the chickens to be studied, 750 fertilized eggs were procured and incubated simultaneously at the Agriculture Research Council (ARC) Poultry Research Center at Glen Agriculture College, situated near Bloemfontein in the Free State Province, South Africa. Hatched chicks were kept for 16 weeks before being placed randomly into groups of 70 birds each. Two of the groups, totaling 140 chicks, were used for each vaccine, and the same number for non-vaccinated controls. For the challenge experiment, specific groups of birds were transferred to isolated layer facilities in the Animal House of the University of the Free State (UFS), Bloemfontein. This experiment was performed with the approval of the UFS Ethics Committee under project number 04/04.

4.2.3 Bacterial isolates used for challenge

The challenge strains used in this experiment represented all three recognized serogroups of *A. paragallinarum* and major strains known to occur in South Africa. Serovar C-3 (Tongaat) strain

of *A. paragallinarum*, known to be highly virulent and very prevalent in South Africa was used, in parallel with Strain C-2 (serovar C-2), isolate 0083 (serogroup A), Strain B (serogroup B) and the NAD-independent strain 1750 (serovar C-3). The purity of each of the challenge strains was established by plating out the challenge bacteria onto BTA agar, streaking with *Staphylococcus aureus* and incubating at 37°C (Bragg, 2002a).

4.2.4 Vaccination and challenge experiments

Chickens in the four study groups were vaccinated at 17 weeks of age, and boosted 3 weeks later. The birds were vaccinated by the subcutaneous injection of 0.5 ml of the respective vaccines during both vaccination procedures. All vaccinations were conducted by the same operator. The safety of each vaccine was assessed by evaluating local reactions in vaccinated chickens during the first week post-inoculation. This evaluation was conducted on a number of chickens randomly selected in each group, during the first and the second vaccination. Post-vaccination signs recorded were principally swellings of different sizes. They were graded as "none", when there was no detectable swelling, "mild" for a small lesion and "severe" when there was a prominent swelling, sometimes affecting the general health of the chicken.

Two rounds of challenges were conducted. The first round of challenges with virulent strains of *A. paragallinarum* was conducted when the birds were between 31 and 35 weeks of age (from 15 weeks post-vaccination). The second round of challenge, conducted in the remaining unchallenged vaccinated chickens, took place when they were between 51 and 55 weeks of age. As controls, unvaccinated chickens of similar age were included in all challenge experiments. The challenge method used was according to the "in-contact" challenge model established by Bragg (2002a), in which one bird in a group of ten birds is directly challenged by intra-sinus injection with 0.1 ml of a bacterial suspension. The remaining birds in the group are challenged through a natural in-contact route as the ten birds in each group are in adjoining cages with a communal water supply. The clinical signs were recorded and scored over the 20 days post-challenge observation period, according to the method described by Bragg (2002a), and used to calculate percentage protection according to the above method.

Due to space constraints at the animal laboratory facility of the University of the Free State, each round of vaccination was divided into two phases. For the first round of challenge (Week 31 of age), the first phase involved the Tongaat strain, the C-2 strain and the 0083 strain. The cages were then cleaned, disinfected and left empty for a week before conducting the second phase with the NAD-independent strain and the serogroup B strain. For the second round, the first phase (Week 51 of age) involved the Tongaat and the C-2 strains, while the second phase involved strain 0083 and the NAD-independent strain 1750.

4.3 RESULTS

4.3.1 Stability of the experimental IC oil vaccines

Tables 4.1 and 4.2 summarize the results obtained for the physical characteristic evaluation and stability of the two experimental vaccines formulated with Montanide™ ISA 206 VG and Montanide™ ISA 70 VG, respectively. Each one of the two experimental coryza vaccines had comparable physical characteristics to the equivalent SEPPIC placebo in terms of homogeneity and particle size. They all were also stable for a month at 4°C and room temperature (RT).

Table 4.1: Comparative evaluation of the water-in-oil-in-water (W/O/W) emulsion coryza special vaccine formulated with ISA 206 adjuvant, and a SEPPIC placebo emulsified in the same adjuvant

	Type of emulsion	**KF (%)**	**D (v, 0.5) D (v, 0.9) in µm**	**Stability after 15 days 4°C/RT/37°C**	**Stability after one month 4°C/RT/37°C**
SEPPIC placebo emulsion	W/O/W	47.5	0.26 0.62	Stable/ Stable/ Dep	Stable/ Stable/ Dep
Coryza ISA 206 vaccine	W/O/W	49.0	0.24 0.42	Stable/ Stable/ BR	Stable/ Stable/ BR

Karl Fisher method (KF) defines the emulsion water content, while D (v) is the mean of particles diameter

Abbreviations: W/O/W, water-in-oil; Dep, depots; BR, breaking

Table 4.2: Comparative evaluation of the coryza special vaccine formulated with ISA 70 adjuvant, and a SEPPIC placebo emulsified in the same adjuvant

	Type of emulsion	KF (%)	D (v, 0.5) D (v, 0.9) in µm	Stability after 15 days 4°C/RT/37°C	Stability after one month 4°C/RT/37°C
SEPPIC placebo emulsion	W/O	29.0	0.33 0.84	Stable/ Stable/ Stable	Stable/ Stable/ Stable
Coryza ISA 70 vaccine	W/O	29.8	0.26 0.55	Stable/ Stable/ Stable	Stable/ Stable/ Stable

Karl Fisher method (KF) defines the emulsion water content, while D (v) is the mean of particles diameter

Abbreviations: W/O, water-in-oil

4.3.2 Post-vaccinal safety of the vaccines

Vaccination reactions were recorded during the first week post-vaccination in all groups, following the first and second vaccination. The results of the vaccination reaction observations are given in Table 4.3, and Figs. 4.1 and 4.2.

After the first vaccination, no vaccination reactions were seen in the control group (which were not vaccinated) and in the birds vaccinated with the Montanide™ ISA 206 VG vaccine. The most severe clinical signs were observed in the birds vaccinated with the OBP IC vaccine and the Montanide™ ISA 70 VG formulation.

After the second vaccination, no reactions were observed in the control group or in the birds vaccinated with the Montanide™ ISA 206 VG formulation. The worst vaccination reactions were seen in the group of birds vaccinated with the ComA vaccine, with 20% of the birds showing very severe vaccination reactions, characterized by subcutaneous swelling of varying degree. In some cases, the vaccination reactions were so severe that these birds were removed from the experiment for ethical reasons, as their general health condition was seriously deteriorating.

Table 4.3: Number of birds showing vaccination reactions for each of the different vaccines

Vaccine	First vaccination				Second vaccination			
	No. birds	None	Mild	Severe	No. birds	None	Mild	Severe
Control	60	60 (100%)	0	0	60	60 (100%)	0	0
ComA	50	36 (72%)	12 (24%)	2 (4%)	83	43 (52%)	23 (28%)	17 (20%)
ISA 206	50	46 (92%)	4 (8%)	0 (0%)	78	78 (100%)	0	0
OBP IC	45	23 (51%)	17 (38%)	5 (11%)	122	103 (84%)	4 (3%)	15 (12%)
ISA 70	46	28 (61%)	14 (30%)	4 (9%)	154	149 (97%)	3 (2%)	2 (1%)

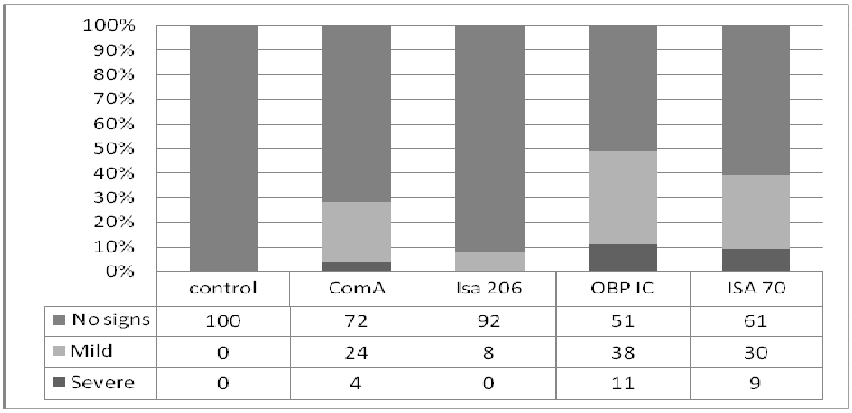


Fig. 4.1: Graphic representation of the percentage of vaccination reactions obtained after the first vaccination with the different vaccines used in this experiment.

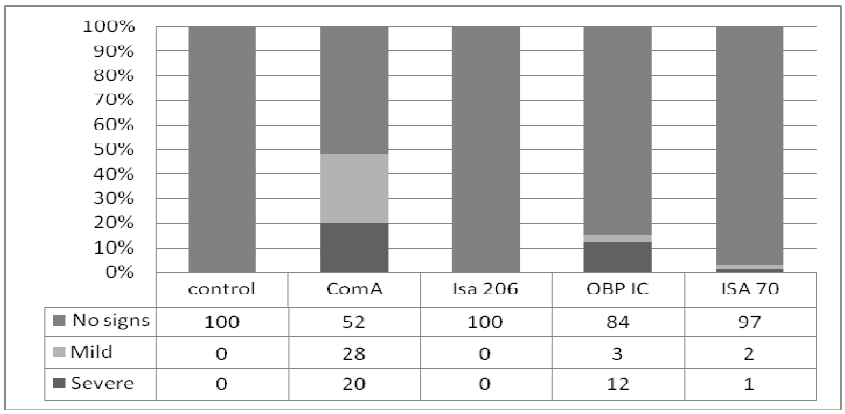


Fig. 4.2: Graphic representation of the vaccination reactions obtained after the second vaccination with the different vaccines used in this experiment.

4.3.3 Efficacy of the vaccine formulations to different serovars after the first challenges

The mean daily disease and protection scores for each of the groups of birds are summarized in Tables 4.4 and 4.5, respectively. No clinical signs occurred in birds challenged with the strain of serovar B, thus the efficacy of the vaccine against serogroup B could not be determined. The percentage protection calculated from the disease challenge and a comparison of the mean disease scores in the first and second rounds can be seen in Table 4.6.

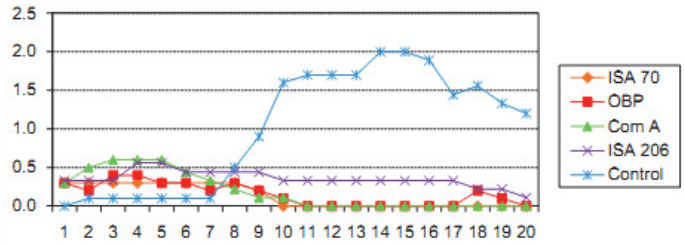
4.4 DISCUSSION

Vaccine reactions of varying degrees occurred in all groups after the first vaccination, irrespective of the type of vaccine. The least severe vaccination reactions were seen in chickens that received the Montanide™ ISA 206 VG formulation, which can be attributed to the fact that this was the only formulation in which the continuous phase is aqueous (water-in-oil-in-water), therefore causing the least inflammatory local reaction as compared to water-in-oil emulsions (Jansen *et al.*, 2006). After the second round of vaccination, the birds vaccinated with the ComA vaccine showed the most severe vaccination reactions: a total of 14 birds were removed from the experiment for ethical reasons based on the severity of their reactions. The two experimental formulations induced vaccination reactions in only 3% of the birds with the Montanide™ ISA 70 VG formulation and none of the birds inoculated with the Montanide™ ISA 206 VG formulation. Overall, the Montanide™ ISA formulations showed the least number of local post-vaccination reactions.

A quadrivalent vaccine needs a potent adjuvant to induce protection. The reactogenic properties of the inactivated bacteria used as antigen in the present vaccines require a safe adjuvant. The acceptable balance between efficacy and safety is obtained through the use of a specific adjuvant formulation. Montanide™ ISA adjuvants are based on a homogenous ready-to-use mix of purified mineral oils and refined oleic esters of anhydrous mannitol of vegetable origin. Emulsifying properties of specific surfactants can only be obtained through strict synthesis parameters, and have a direct impact on the vaccine safety and efficacy (Stone, 1988). Furthermore, surfactants used in Montanide™ formulations are manufactured in dedicated

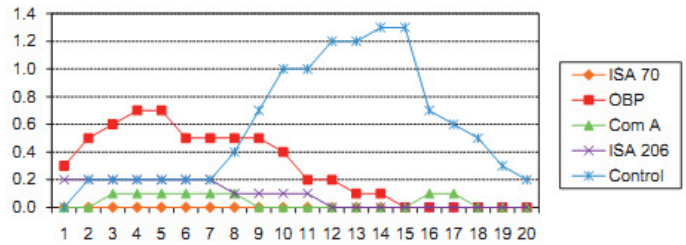
Table 4.4: First vaccination: Graphic representation of the mean daily disease scores obtained from each of the vaccines for chicken challenged with different *A. paragallinarum* challenge strains, and table summarizing the corresponding mean lesion score and percentage protection - the Tongaat (C-3) strain (1); C-2 strain (2); the A-1 isolate 0083 (3); the NAD independent C-3 isolate 1750 (4)

1: Tongaat C3 strain



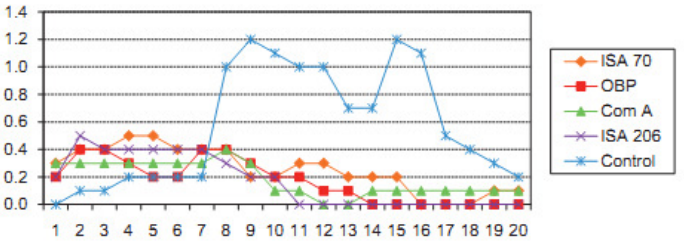
Vaccine	Mean score	% protection
Control	1.010	–
ComA	0.191	81.1
ISA 206	0.360	64.4
OBP	0.150	85.1
ISA 70	0.130	87.1

2: Tongaat C-2 strain



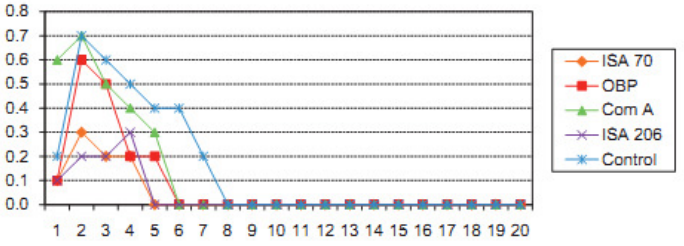
Vaccine	Mean score	% protection
Control	0.58	–
ComA	0.04	93.1
ISA 206	0.09	84.5
OBP	0.29	50.0
ISA 70	0.00	100.0

3: A-1 isolate 0083



Vaccine	Mean score	% protection
Control	0.55	–
ComA	0.19	65.5
ISA 206	0.17	69.1
OBP	0.20	63.6
ISA 70	0.26	52.7

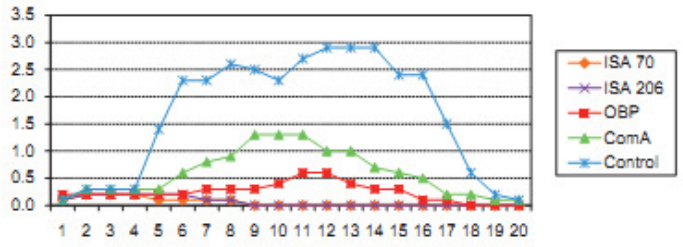
4: NAD-independent C-3 isolate 1750



Vaccine	Mean score	% protection
Control	0.15	–
ComA	0.125	16.7
ISA 206	0.04	73.3
OBP	0.04	73.3
ISA 70	0.08	46.7

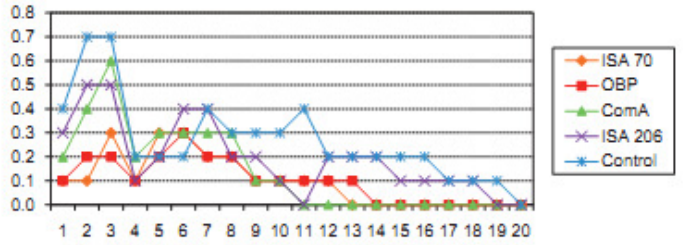
Table 4.5: Second vaccination: Graphic representation of the daily protection scores provided by each of the vaccines for chicken challenged with different *A. paragallinarum* challenge strains, and table summarizing the corresponding mean lesion score and percentage protection - the Tongaat (C-3) strain (1); C-2 strain (2); the A-1 isolate 0083 (3); the NAD independent C-3 isolate 1750 (4)

1: Tongaat C3 strain



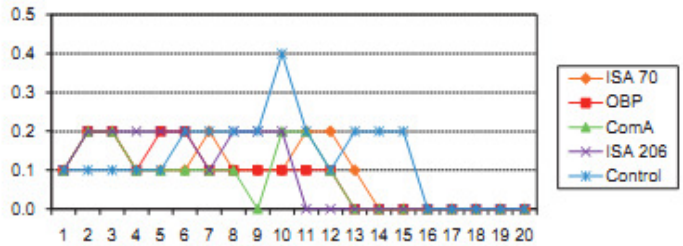
Vaccine	Mean score	% protection
Control	1.65	-
ComA	0.595	64.0
ISA 206	0.065	96.1
OBP	0.245	85.2
ISA 70	0.055	96.7

2: Tongaat C-2 strain



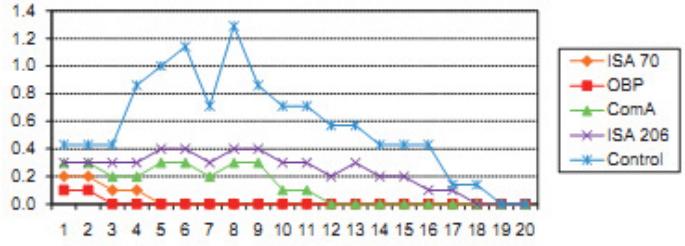
Vaccine	Mean score	% protection
Control	0.27	-
ComA	0.14	48.1
ISA 206	0.195	27.8
OBP	0.10	63.0
ISA 70	0.10	63.0

3: A-1 isolate 0083



Vaccine	Mean score	% protection
Control	0.130	-
ComA	0.075	42.3
ISA 206	0.090	30.8
OBP	0.090	30.8
ISA 70	0.080	38.5

4: NAD-independent C-3 isolate 1750



Vaccine	Mean score	% protection
Control	0.56	-
ComA	0.13	76.8
ISA 206	0.24	57.1
OBP	0.01	98.2
ISA 70	0.03	94.6

Table 4.6: Comparison of all of the vaccines based on percentage protection against the NAD-dependent strains, NAD-independent strain, and for all of the challenge strains used (excluding serovar B-1, which did not result in any clinical signs when used to challenge chickens in this experiment, including the unvaccinated controls). Mean protections obtained for the first and second round of challenges for all of the vaccines and challenged strains used in this experiment are also included

Challenge strain	ComA		OBP		ISA 206		ISA 70	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
C-3	81.1	64.0	85.1	85.2	64.4	96.1	87.1	96.7
C-2	93.1	48.1	50.0	63.0	84.5	27.8	100	63.0
A-1	65.5	42.3	63.6	30.8	69.1	30.8	54.7	38.5
Mean NAD-dependant protection	**79.9**	**51.5**	**66.2**	**59.7**	**72.7**	**51.6**	**80.6**	**66.1**
NAD-independent C-3	16.7	76.8	73.3	98.2	73.3	57.1	46.7	94.6
Mean overall protection (all isolates)	**64.1**	**64.2**	**68**	**78.9**	**72.8**	**54.3**	**72.1**	**80.3**

equipment, which avoids any cross-contamination. The hydrophilic parts of these amphiphile molecules are made of mannitol sugar, known to have better injectability than sorbitol-based surfactants. Adjuvant formulations of this quality do not induce adverse reactions observed when a multipurpose industrial sorbitan oleate-based formulation (Tween/Span adjuvant formulation) is used. The Montanide™ ISA 206 VG renders a water-in-oil-in-water emulsion in a one-step process, giving a very fluid vaccine when containing 50% of adjuvant. The Montanide™ ISA 70 VG renders a water-in-oil emulsion containing 70% of adjuvant. In the latter case, the antigenic media are entrapped in the oily phase. The different antigenic release profiles (Aucouturier *et al.*, 2001) and the ratio of adjuvant to antigenic media can explain the perfect safety profile obtained with Montanide™ ISA 206 VG. Indeed, aqueous-based formulations are quickly eliminated from the injection site, while water-in-oil emulsions ensure a long-lasting release of the antigens (Jansen *et al.*, 2005).

The virulence of different challenge strains could be assessed through the monitoring of clinical scores in the unvaccinated control animals. The Tongaat C-3 strain demonstrated the highest virulence, followed by the C-2 and the A-1 strains. Serovar B did not induce any adverse reactions. The high clinical score recorded with serovar C-3 (1.01 and 1.65 after the first and the second challenge, respectively) is consistent with previous results (Bragg, 2002a) and confirms the association of the C-3 serovars with most severe outbreaks in South Africa. The lack or poor virulence of South African serovar B has also been recorded previously (Bragg, 2002a). Good levels of protection were, however, recorded for the C-3 serovar for all the vaccine formulations, at both challenges, which strengthen the value of vaccination for the control of IC in South Africa.

One of the main objectives of the present study was to investigate the duration of protection provided by the different vaccines. In order to do this, the chickens were kept until they were older than 50 weeks. Vaccinated birds were then challenged with the same challenge strains as those used at approximately 30 weeks of age. The results of the second challenge with serovar C-3 in unvaccinated birds showed an increase of the mean disease score from 1.01 in the first challenge to 1.65 in the second challenge, indicating a possible worsening in bird susceptibility

to the bacterium associated with increasing age. A similar increase in clinical signs as the birds aged was also recorded with the NAD-independent C-3 strain, while the opposite was observed with the C-2 and the A-1 strains. Further work is required to confirm these observations. Differences of occurrence of IC in different age groups have been reported elsewhere with variations between different countries and production systems. Blackall (1999) reported on a study of village chickens in Thailand where it was reported that IC was the most common cause of death in chickens less than 2 months old and in those over 6 months of age.

While the protection level with the ComA vaccine decreased from 81.1% to 64% from the first to the second challenge, the OBP CoryzaPlus vaccine generated an unchanged level of protection (85.1% for the first challenge and 85.2% in the second challenge). The protection levels were found to increase to 90% when the Montanide™ ISA 70 VG and Montanide™ ISA 206 VG formulations were used. The Montanide™ ISA 70 VG vaccine generated the best protection overall to all challenge strains, closely followed by the OBP vaccine. This can be attributed to the similarity in their formulation, water-in-oil and the inclusion of the NAD-independent C-3 strain. Montanide™ ISA 70 VG also showed the best safety results. Larger field experiments are required to confirm the findings of the present study.

During the first round of challenge experiments, the clinical signs associated with IC in the unvaccinated control birds challenged with the Tongaat strain (C-3) were very severe. The levels of protection obtained when the different vaccinated groups of birds were challenged with serovar C-3 ranged from 64.4% (Montanide™ ISA 206 VG) to 87.1% for the Montanide™ ISA 70 VG formulation. The clinical signs obtained when unvaccinated birds were challenged with the serovar C-2 strain were less severe than those obtained when the unvaccinated birds were challenged with serovar C-3. These results confirm previous findings by Bragg (2002a). The levels of protection obtained by the different vaccines against the serovar C-2 challenge ranged from 50.0% for the OBP vaccine to 100% for the Montanide™ ISA 70 VG formulation.

When unvaccinated birds were challenged with strain 0083 (serovar A-1), the clinical signs were less severe than those obtained in previous challenge experiments. The levels of protection

obtained against challenge with serovar A-1 ranged from between 52.7% for Montanide™ ISA 70 VG and 69.1% obtained with the Montanide™ ISA 206 VG formulation. When unvaccinated birds were challenged with serogroup B, no clinical signs were seen. Previous work has demonstrated that the South African serogroup B strains are of very low virulence (Bragg, 2002b), but in those experiments, some clinical signs were recorded. The fact that no clinical signs were seen in the unvaccinated birds makes any comparison of the protection impossible.

The birds were also challenged with a NAD-independent strain of *A. paragallinarum*. In previous experiments, the virulence of both naturally occurring NAD-independent strains (Bragg, 2002b) and experimentally produced NAD-independent strains (Taole *et al.*, 2002) were found to be of low virulence. It has also been demonstrated that the levels of protection obtained when vaccinated birds were challenged with the NAD-independent strains were very low (Bragg, 2004). In the present study the prevalence of clinical signs noted in the unvaccinated birds was indeed very low: a mean disease score of only 0.15 was obtained in the unvaccinated birds challenged with the NAD-independent serovar C-3 strain, as compared to a mean disease score of 1.01 obtained with the NAD-dependent serovar C-3 strain of *A. paragallinarum.*

The need for the inclusion of a NAD-independent strain in the vaccine was shown by the low protection level afforded by the commercial vaccine ComA, which lacks such a strain. The protection afforded by the commercial vaccine to a challenge with the NAD-independent strain improved in late challenge, possibly as a result of an improved cross-protection over time with the other strains in the vaccine. These findings are in contrast to those of Jacobs *et al.* (2000) who demonstrated that the Nobilis Coryza vaccine (Intervet International BV) provided protection against the NAD-independent strains in 9 week-old chickens vaccinated 2 weeks earlier, despite the fact that it does not include an NAD-independent vaccine antigen. Bragg (2004), on the other hand, demonstrated that there was evidence of immune evasion by the NAD-independent strains when an experimental vaccine only containing NAD-dependent strains was used to vaccinate the birds. The difference between the finding of Jacobs *et al*. (2000) and Bragg (2004) could be attributed to the different challenge models used. Bragg (2002b) demonstrated that the naturally occurring NAD-independent strains are of low virulence. Taole

et al. (2002) showed that there is a substantial decrease in virulence when NAD-dependent strains of *A. paragallinarum* are experimentally converted to NAD-independent strains. This low virulence level could account for the perceived efficacy of a vaccine against the NAD-independent strains, as reported by Jacobs *et al.* (2000). Given the high prevalence of NAD-independent serovars in South Africa, their involvement in a number of outbreaks throughout the country, and previous observation of the limited ability of the NAD-dependent vaccine strain to protect birds against them, it is critical that vaccines in use in the country contain NAD-independent strains.

The two experimental vaccines, as well as the OBP CoryzaPlus vaccine contained an NAD-independent strain of *A. paragallinarum*, while the ComA vaccine did not. Protection levels of only 16.7% were recorded with ComA vaccine, while it was between 46.7% (Montanide™ ISA 70 VG) and 73.3% (Montanide™ ISA 206 VG and OBP) for the vaccines containing an NAD-independent strain. These data clearly indicate the ability of a vaccine containing an NAD-independent strain to protect against these strains. In a previous study conducted in South Africa, it was demonstrated that a commercial vaccine without an NAD-independent strain generated good protection against the local NAD-independent C-3 strain (Jacobs *et al.*, 2000). The fact that the chickens used in the experiment were vaccinated at 3 and 7 weeks of age and challenged 2 weeks later could explain the good level of protection recorded. In the present study the chickens were vaccinated and challenged much later (vaccination at 17 and 20 weeks, challenge at 33 or 55 weeks of age), in order to evaluate the level of protection at different stages of the productive life of layers. The results of this study are certainly much closer to the layer production system and suggest the need for the use of a vaccine that includes NAD-independent strains in areas where NAD-independent variants are known to occur. The low virulence recorded for the NAD-independent strains discussed above could also contribute to this discrepancy.

The results obtained in the present study confirm the variation in the virulence of different serovars occurring in South Africa, with serovar C-3 being the most virulent and serovar B having almost no virulence. The results indicate an age-related increase in susceptibility to infection, as illustrated in the increased disease scores. The study highlights the importance of a

suitable vaccine formulation that generates protection throughout the productive life of chickens and the need for incorporation of local dominant strains in the vaccine, including NAD-independent strains.

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CHAPTER FIVE

CONCLUDING REMARKS

The role of adjuvants in modulating and improving the protective characteristics of vaccines was recognized and exploited from the time inactivated and toxoid vaccines were developed at Onderstepoort in the middle of the 20th century. The Onderstepoort oil-adjuvanted vaccines have since been using the Freund's formulation, with very few modifications over the following decades. A better understanding of immunology and immunity involved in protection has led to tremendous evolution and improvement in the field of oil adjuvants and their use in veterinary vaccines. It has therefore become imperative for Onderstepoort Biological Products (OBP) to re-evaluate most of its adjuvanted vaccines, not only as a way of being current but also to (i) improve the safety profile of some of the vaccines; (ii) improve the efficacy of the vaccines; and (iii) comply with regulatory requirements for the registration of veterinary vaccines. The present studies were thus designed to review the impact of certain adjuvants, more specifically oil adjuvants, and eventually improve the efficacy and safety of OBP vaccines formulated with these adjuvants. Four oil-adjuvanted OBP vaccines were evaluated during the course of this study, *i.e.* the *E. coli*, the enterotoxaemia or Pulpy kidney, the *Vibrio* or *Campylobacter fetus* and the Infectious Coryza vaccines.

The key components of an oil vaccine emulsion are white oils, surfactants and the water phase, which includes the vaccine antigen. Since OBP is a commercial company, financial considerations, together with ease of sourcing, are critical in the selection of raw materials used in different vaccines, including adjuvants. Evaluation of the oil adjuvant thus focused on the assessment of the white oils, as well as the evaluation of different surfactants. One of the critical questions to be answered in the present study was whether to select commercially available pre-made emulsions, such as Montanide™ ISA 206 VG and Montanide™ ISA 70 VG, or in-house formulations where different components are procured separately and blended in-house. Further to laboratory and scientific evaluation, financial and costs analyses are important factors in the selection of the most suitable formulation and therefore should also be carefully assessed. However, financial evaluation was beyond the scope of the present studies.

The evaluation of white oils was considered to be an appropriate starting point (Chapter 2). Three different liquid paraffin mineral oils were evaluated in order to determine their suitability in the formulation of oil-based vaccines. Two of the oils, PFP C8 and PFP C14, were identified as alternatives to replace the classical Marcol 52 oil. The selection of PFP C8 and PFP C14 was based on their similarity to Marcol 52, their local availability and their low costs. The results of the different laboratory analyses, including safety evaluations in laboratory animals, indicated that the PFP C8 and PFP C14 oils could be used as alternatives to Marcol 52. The PFP C8 oil was considered as preferable due to its viscosity value, which was lower than that of PFP C14 and Marcol 52, thus allowing for better injectability. Consequently, PFP C8 was therefore used in further evaluations of emulsifiers.

The study on emulsifiers focused on the non-ionic surfactants used in the oil phase of the vaccines under evaluation (Chapter 3). Two non-ionic surfactants or emulsifiers are generally included in the formulation of oil vaccine emulsions, *i.e.* a water-phase and an oil-phase surfactant. The present study focused on evaluating possible alternative surfactants to Cirrasol EN-MP®, which is used in the oil phase. Three surfactants were thus evaluated, namely Simulsol-P2®, Arlacel C and Montanide 103™. Initially, different incorporation rates of Simulsol-P2® were evaluated *in vitro* in order to determine the stability of the resulting emulsions. The Pulpy kidney antigen was used for these experiments. It was established that Simulsol-P2® could not be used due to an inability to form the desired emulsion when generating different hydrophilic-lipophilic balances (HLBs). Subsequently, Arlacel C and Montanide 103™ were compared to Cirrasol EN-MP® in the formulation of the *E. coli*, Pulpy kidney and *Vibrio* vaccines. The Arlacel C and Montanide 103™ formulations of all the vaccines passed all the registered quality control tests. In certain cases the alternative formulations were much more effective than the current formulation that contained Cirrasol EN-MP®. Indeed, the Arlacel C formulations showed good safety characteristics and, in certain instances, a better immunogenicity compared to the other two formulations. The Montanide 103™ formulations showed also very good physical and immunological characteristics when formulated with the three vaccines under evaluation. However, in the case of the *E. coli* vaccine, there were persistent local reactions at the injection site of the vaccinated cattle. Although requiring further

investigation, these results, together with the high cost of Montanide 103™, compared to Arlacel C, pointed towards the latter as the better alternative for further field evaluation of the vaccines.

The evaluation of the OBP quadrivalent Infectious Coryza vaccine provided an opportunity to assess the Arlacel C formulation in comparison to two commercial oil adjuvants, namely Montanide™ ISA 70 VG and Montanide™ ISA 206 VG (Chapter 4). Although the Montanide™ ISA formulations showed the least number of local post-vaccination reactions, the mean overall protection level of the OBP vaccine was indeed comparable to those obtained when the Montanide™ ISA vaccine formulations were used. The study also highlighted the differences in the virulence of different *Avibacterium paragallinarum* serovars and emphasized the need for incorporation of NAD-independent strains into the vaccine. Based on the obtained results, the Arlacel C formulation was preferred to the Montanide™ ISA formulations, mainly due to the final cost of the vaccine since the poultry market is very price-sensitive.

Overall, the results obtained during the course of this study raised a number of important points that can be summarized as follow:

- The re-assessment and subsequent adjustment of the vaccine formulation was a very effective way of improving the safety and efficacy characteristics of the vaccines under study, thus extending product life span.
- Such studies may also provide an opportunity to better understand the protection mechanisms of specific vaccines. Using more appropriate immunological and molecular evaluation tools, it would be possible in future to evaluate very specific subsets of the immune system and the relevant mechanisms of protection.
- The increased immunogenicity observed with the Arlacel C vaccine formulations require further evaluation in large-scale field trials, using additional immunological assays and the benefit of larger numbers in the analysis of the results. If the results that were obtained during the course of this investigation could be confirmed, they will have an extremely positive impact on the protection of livestock against the relevant pathogens, *i.e. E. coli* and *Campylobacter fetus*.