Part One

INTRODUCTION AND LITERATURE REVIEW

CHAPTER I

INTRODUCTION

"*Considering the global distribution, incidence, human and veterinary health costs, and severe case-fatality ratio associated with the disease, rabies remains the most important viral zoonosis recognized today*" (Hanlon *et al*., 2001b).

1.1 Background and motivation

Rabies is viral encephalitis of mammals caused by the bullet-shaped lyssaviruses. The disease is almost invariably fatal once clinical symptoms appear, and only a single case of survival of an individual without history of pre- or post-exposure vaccination has been recorded (Willoughby *et al*., 2005). The rabies virus progressively infects the central nervous system of its victim, whereupon the infection manifests as symptoms reflecting cerebral and autonomic dysfunction. These characteristic, and often very dramatic, symptoms include hallucinations, paralysis, extreme salivation, furious behavior and hydrophobia. Rabies is a zoonotic disease and is invariably fatal in animals and humans once clinical symptoms appear. Additionally, rabies is recognized as the infectious disease with the highest case-fatality ratio when timely post-exposure prophylaxis is neglected (Hemachunda *et al*., 2002).

Rabies is currently considered a re-emerging disease and in addition new and novel lyssaviruses have been described in the recent past (Fauquet *et al*., 2005; World Health Expert Consultation on Rabies, 2005). Despite successful control and eradication efforts in much of the developed world, rabies remains endemic in wild and domestic animal populations in many regions worldwide (World Health Organization, 1998b). Most human deaths due to rabies, usually in the range of 30 to 60 000 per annum, are reported from these countries. Although effective pre- and post-exposure prophylaxis for rabies is available, various problems hamper the use thereof. These include the cost involved with the administration of the relatively expensive cell culture vaccines and anti-rabies virus immunoglobulins and the logistics involved in acquiring the treatment (Southern and Eastern Africa Rabies Group, 2003). Human exposures are still primarily

linked to dog exposures in many parts of the world. The most effective strategy for the control and prevention of rabies is the control of the disease in these dogs. Mass oral vaccination programs have time and again proven to be the most affordable and effective strategy to achieve control of the disease (Perry and Wandeler, 1993; World Health Organization, 1998a).

Despite being described as an "age old" disease, rabies remains a dynamic disease posing new challenges of control and prevention over time. Two of the currently prominent dilemmas facing the effective control of rabies were addressed in this study. Firstly the development of oral rabies vaccines for oral vaccination programs of domestic and feral dogs (and wildlife rabies vectors) remains a focal point of current research. Such a vaccine should infer adequate protection against infection, so that a single administration of the vaccine would be sufficient. Considering that oral vaccination programs involving domesticated dogs in urban and rural settings will bring the vaccine in closer proximity of humans, it must be exceptionally safe, not only for the target but for non-targets as well. Cost-effectiveness is also of primary importance and would be a deciding factor for the feasibility of such programs in poorer countries. Secondly, the lack of cross protection of current rabies biologics against some of the non-rabies lyssaviruses is appreciated. Rabies vaccines and immunoglobulins often only infer partial protection against infections of these viruses, and in the case of the newly described West Caucasian Bat virus, no cross neutralization is observed (Hanlon *et al*., 2005). Even though reports of non-rabies lyssavirus infections, particularly in humans, remain rare and consequently appear of minor importance from a public health point of view, the potential implications thereof should not be underestimated.

1.2 Objectives of the study

The aims of the study were defined as follows:

- Construct and analyze the efficacy of a recombinant Lumpy Skin Disease virus (Neethling vaccine strain) expressing a rabies virus glycoprotein in a mouse model, and

- Construct and analyze the efficacy of a recombinant Modified Vaccinia virus Ankara expressing a rabies virus glycoprotein in a mouse model and compare the responses with equivalent recombinant but replication competent Vaccinia viruses, and
- Investigate the cross-protective and cross-reactive immune responses of single or dual Lyssavirus glycoprotein gene expressing recombinant Vaccinia viruses in a mouse model.

1.3 Layout of the thesis

This writing constitutes five parts and eight chapters. Part one includes this introductory chapter as well as chapter II. The latter serves as a literature review addressing aspects of rabies virus virology and poxvirus vaccinology relevant to this particular study.

Parts two, three and four each address a particular objective of the study and are divided into two chapters. Part two and three are divided into two chapters each. The chapters discuss firstly the construction of the vaccine viruses and the subsequent chapter reports on immunization studies with the particular constructs. Part four, chapter VII focuses on the theme of vaccine cross-protection amongst the spectrum of Lyssaviruses. Part five, chapter VIII provides a discussion on the collective study.

CHAPTER II

LITERATURE REVIEW

2.1 Rabies – a topical overview

2.1.1 History of rabies: the development of prophylaxis and control strategies

Rabies is probably the oldest infectious disease recorded in man, and can be traced back further than any other disease of infectious nature in popular and medical literature (reviewed in Wiktor, 1980; Wilkinson, 1988; Steele and Fernandez, 1991, Neville, 2004). The first description of the disease dates back to the 23rd century BC with references to a rabid dog in legal documents from the ancient civilization of Mesopotamia. The first known description of a human case of rabies was only documented in the first century AD by Cornelius Celsus, who also coined the term *hydrophobia* to describe the characteristic fear of water associated with the disease. For many centuries popular belief had it that procedures such as corrosion, bloodletting, burning or application of caustic chemicals to a bite wound could prevent the disease from developing after being bitten by a rabid animal (Swabe, 2004). Some of these beliefs are still practiced in many countries of the developing world.

From the late 1700s through the 1800s various important scientific advances were made towards elucidating the nature of the disease and devising measures of control. In 1793, an Englishman, Samuel A. Bradsley noted that an outbreak of rabies could be controlled by placing rabid animals under quarantine. In this time it was also established that the disease could be transferred by inoculation with saliva of a rabid animal. The first serial transmission experiments were conducted by a German, Zinke in the early 1800s. In 1880, Louis Pasteur and his colleagues started their work on rabies which led to the generation of the first rabies virus vaccine. Pasteur's work established two fundamental facts, the neurotropic character of the virus and the concept of fixed or standardized virus. Pasteur prepared fixed virus by serial passage of a virus isolate in healthy canines and later in monkeys. This fixed virus was tested as a vaccine, but the

virus was not sufficiently attenuated and caused vaccine-induced rabies in a considerable number of inoculated dogs. Since serial passage did not yield virus with sufficient attenuation for vaccine purposes, Pasteur explored physical means of attenuating the virus. Pasteur and his workers attempted to attenuate (but rather inactivated) an isolate of rabies virus. This was achieved by desiccating strips of infected rabbit spinal cords over potassium hydroxide and administering suspensions of increasing virulence to patients, starting with material dried for 14 days and ending with material dried for two days. The vaccine was tested in dogs before the first human application. On 6 July 1885, the vaccine was administered to a human patient. A severely bitten nine year old boy, Joseph Meister, survived and rabies vaccination rapidly found widespread application. The first Pasteur Institute opened its doors in Paris, France in 1888. Soon thereafter Pasteur Institutes started opening up all around the world. Pasteur's inactivated vaccine was used in France and French colonies until the 1953. It is noteworthy that at the same time of Pasteur's breakthrough, in 1888, Hogyes devised a similar approach for preparing rabies vaccine (revieweved in Bunn, 1991). Hogyes diluted fresh spinal cords from rabid rabbits in a buffered salt solution and administered the preparation from the highest dilution to the lowest dilution. Protection from virulent rabies virus infection was also afforded by the vaccine preparation, and the vaccine had some success.

In the years to follow various modifications were made on Pasteur's inactivated neural tissue vaccine (reviewed in Wiktor, 1980; Wilkinson, 1988; Steele and Fernandez, 1991; Precausta and Soulebot, 1991). Research moved towards the improvement of safety and the efficacy of the nervous tissue vaccine. Attempts included serial dilution and inactivation of rabies virus with chemical agents (such as phenol and ether) or by physical means (such as ultraviolet radiation). Vaccines from neural tissue origin were the only available vaccine for almost 70 years but had doubtful immunogenicity and adverse reactions were noted in a considerable portion of human and animal vacinees. These adverse reactions were primarily associated with the myelin content of the nervous tissue vaccine preparation and therefore the preparation of vaccine from non-neural tissue was to be investigated.

During the 1950s there was a trend in the developed nations toward the replacement of dangerous nervous tissue vaccines with attenuated or modified live virus

vaccines, not only for human use but also for veterinary use (reviewed in Bunn, 1991; Dreesen, 1997). The Flury strain of rabies virus (named for its victim) was isolated from a young girl who died of rabies in 1939. The virus was passaged 136 times in one-day old chicks and then passaged a further two rounds in seven-day-old embryonated chicken eggs. The virus was further passaged and dubbed Flury Low-Egg-Passage (LEP) virus. Flury LEP could be used to immunize canines and offered protection for one or two years. In an attempt to further attenuate the virus (for safe use in cattle and felines) it was passaged a further 176 to 182 passages in embryonated chicken eggs. The latter virus was designated the transformed High-Egg-Passage (HEP) Flury virus. The virus was tested safe and immunogenic when inoculated intramuscularly in cats and cattle. Nevertheless, these vaccines caused anaphylactic reactions due considerable amount of chicken antigens in vaccine preparations, and were adapted to various other cell culture systems. Another street rabies virus strain, the Street Alabama Dufferin (SAD) strain was isolated from a rabid dog in 1935 and was passaged in different systems to yield the high-cell-passage attenuated vaccine strains, ERA (Evelyn- Rockitnicki- Abelseth) and Vnukovo-32. The SAD and ERA vaccines were extensively used in oral vaccination programs in Europe and the Vnukovo-32 strain recommended for use in dogs, cats and farm animals. The Kelev strain was isolated from a rabid dog in 1950 and was passaged in mice and 7-day-old embryonated chicken eggs. The attenuated virus was recommended for use in dogs and cattle. The Kissling strain (CVS-11) was developed by passage of the Challenge Virus Standard (CVS) in primary hamster kidney cells and licensed for use in dogs. A killed virus, suckling mouse brain vaccine, with lower myelin content, was also developed in the 1950s for veterinary and medical application. A trivalent, inactivated vaccine was extensively used in South America (Fuenzalida *et al*., 1964). The vaccine was prepared with three fixed stains of rabies virus, CVS, 51 and 91. The SADberne strain served as the parent strain for other live modified vaccines such as SAD-B19 and SAG-1. SAD-B19 vaccine virus could be used to orally vaccinate raccoons (*Procyon lotor*) and foxes (*Vulpes vulpes*), but it caused vaccine-induced rabies in skunks (*Mephitis mephitis*) (Rupprecht *et al*., 1989; Vos *et al*., 2002). SAG-1 was used for the oral vaccination of foxes in France, but was considered to be genetically unstable. SAG-2 offers improved genetic stability and is considered an effective vaccine

for the oral immunization of dogs, skunks and raccoons (Schumacher *et al*., 1993; Fekadu *et al*., 1996; Masson *et al*., 1996; Hanlon *et al*., 2002; Knobel *et al*., 2003). In 1956, an embryonated tissue vaccine, the inactivated lyophilized duck embryo vaccine was introduced for human application. The duck embryo vaccine was extensively used for over 25 years in the USA until it was discontinued in 1981. This vaccine produced a poor immune response in a substantial portion of the vaccinees and also frequently caused adverse reactions. A purified version of this vaccine has been developed since. Other attempts to prepare vaccine in cell culture included culturing in hamster kidney cells. This vaccine was extensively used in Russia. It became clear that in order to attempt to further minimize adverse reactions in human vaccinees the vaccines had to be propagated in human cell lines. This would assure that no protein unfamiliar to the human system would be included in the vaccine preparation. In 1961, Hayflick and Moorhead developed a diploid human cell line, WI-38 (Hayflick and Moorhead, 1961). The Pitman-Moore strain of rabies virus was subsequently adapted to growth in WI-38 cell culture and a vaccine developed in collaboration with the Wistar Institute, Philadelphia, USA (Wiktor *et al*., 1964; Wiktor and Koprowski, 1965). The human diploid cell culture vaccine consists of purified and concentrated inactivated rabies virus. It evokes a much higher level of immune response, as neutralizing antibodies appear earlier after exposure and in higher titers than with the previous vaccines. This vaccine has been used since 1976 and today still remains the benchmark for cell culture rabies vaccines and the preferred vaccine for human use. Other cell culture vaccines consisting of purified and inactivated virus grown in different cells, including primary chick or duck embryo and Vero cell culture are also recommended by the World Health Organization (WHO) (WHO Expert Consultation on Rabies, 2005). Post-exposure prophylaxis is also available for prevention of the development of rabies after an exposure to the virus. Rabies post-exposure protocol includes proper wound treatment, vaccination with cell culture vaccine and the administration of anti-rabies virus immunoglobulins (RIG) (WHO Expert Consultation on Rabies, 2005).

With the increased understanding of the molecular biology of the rabies virus and advances in the field of recombinant DNA technology the focus of vaccine development has shifted to that of recombinant subunit vaccines (Dietzschold, 1996). Most

importantly, a recombinant subunit vaccine for rabies was developed in the 1980s. A vaccinia virus recombinant expressing the glycoprotein of the ERA strain, known as V-RG, was generated and widely used as an oral vaccine (Kieny *et al*., 1984; Wiktor *et al*., 1984 and Rupprecht and Kieny, 1988). V-RG was widely used, with great success, in oral vaccination programs of wildlife in the USA and countries in Europe (Wandeler, 1991; Pastoret *et al*., 1992; Brochier *et al*., 1995).

Today, despite the number of rabies biologics already licensed for use around the globe, the development of new safer and better products continues. The development of customized control and prevention programs particularly in the developing countries is also a focal point of current research (Perry and Wandeler, 1993).

2.1.2 Etiology

Rabies is caused by members of the genus *Lyssavirus* of the *Rhabdoviridae* family (Fauquet *et al*., 2005). The *Rhabdoviridae* family constitutes three genera that affect mammals, the *Lyssavirus*, *Vesiculovirus* (prototype virus: *Vesiculovirus*) and *Ephemerovirus* (prototype virus: *Bovine Ephemeral Fever virus*). The genus Lyssavirus currently includes 7 species (or genotypes). These include genotype one, *Rabies virus*; genotype two, *Lagos Bat virus*; genotype three, *Mokola virus*; genotype four, *Duvenhage virus*; genotype five, *European Bat Lyssavirus 1*; genotype six, *European Bat Lyssavirus 2* and genotype seven, *Australian Bat Lyssavirus*. The prototype virus of this genus is represented by the genotype one rabies viruses. Viruses of the genotypes two to seven are collectively denoted as the rabies-related viruses or the non-rabies lyssaviruses. Several novel lyssaviruses have been described in the literature since 2003. These viruses were isolated from different bat species from central Asia and Russia and are called Aravan (in 1991), Khunjand (in 2001), Irkut and West Caucasian Bat viruses (both in 2002) (Arai *et al*., 2003; Botvinkin *et al*., 2003; Kuzmin *et al*., 2003). The latter is recognized as the most phylogenetically divergent virus when compared on nucleotide and amino acid levels to the genotype one rabies viruses, and in addition only exhibits limited relatedness to genotype two and three viruses (Botvinkin *et al*., 2003; Kuzmin *et al*., 2005). It has been suggested by the WHO Expert Consultation on Rabies that these

four viruses should be considered as separate genotypes and the International Committee on the Taxonomy of Viruses have already recognized these viruses as putative species of the genus (Fauquet *et al*., 2005; WHO Expert Consultation on Rabies, 2005).

The following sections are devoted to discussing characteristics which relate to the genotype one rabies virus (unless otherwise stated), but is also generally applicable to all the non-rabies lyssaviruses.

2.1.2.1 Morphology and protein functions of the rabies virus

The structure of rabies virus is reviewed in Tordo and Poch, 1988 and Wunner, 1991. The viruses of the *Rhabdoviridae* family have a characteristically bullet-shaped to cone-shaped virion and a non-segmented, negative-sense ribonucleic acid (RNA) genome (figure 2.1). Each virion carries a single molecule of genomic RNA (approximately 11 900 base pairs) and considering the genome's negative polarity, it is regarded as non-infectious (Tordo and Kouknetzoff, 1993). In addition, each virion comprises of five viral proteins. Three of the five proteins, are found in the infectious ribonucleocapsid. The nucleoprotein is associated with the RNA genome to form the helical capsid. The nucleocapsid-associated RNA-dependant RNA polymerase encodes the majority of viral enzymatic activity, including RNA synthesis; capping methylation; polyadenylation; and some phosphorylation reactions. The phosphoprotein is involved in regulatory functions. The other two proteins, the glycoprotein and matrix protein are associated with the lipid bilayer envelope.

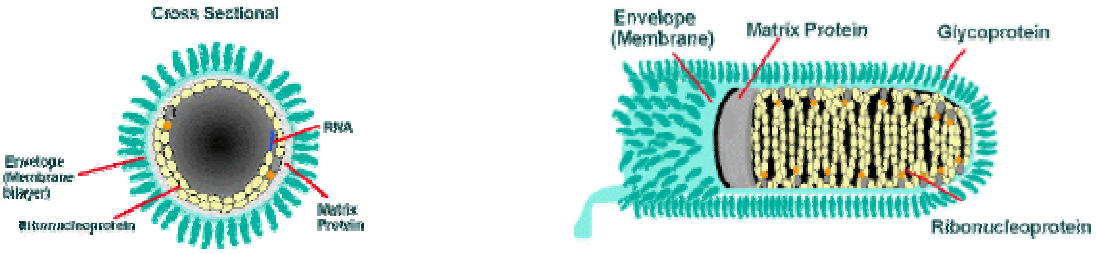

Figure 2.1: The morphology of the rabies virion. The rabies virion is approximately 75 nm in diameter and 100-300 nm (average of 180 nm) in length. The variation in length is attributed to the presence of defective interfering particles. The virion consists of two structural units, the ribonucleocapsid core and the surrounding host derived bilayer lipid envelope. The envelope is usually 8 nm wide and is covered with peplomers (usually 5 nm apart), except for the quasi-

(figure 2.1, continued) planar surface of the virion, which do not carry any projections. The peplomers are usually 10 nm in length and consists of three associated glycoprotein monomeric units. The helical nucleocapsid is 50 nm wide and 165 nm long with a period of approximately 4.5 nm and 30 to 35 coils. (Figures from www.cdc.org, internet reference 1).

2.1.2.2 The glycoprotein

The structural characteristics and functions of the rabies virus glycoprotein are reviewed in Wunner, 1991 and Coll, 1995.

The glycoprotein gene encompasses nucleotide position 3 291 to 4 964 (total of 1 675 nucleotide bases) on the Pasteur virus RNA genome. The gene is transcribed into a 18s monocistronic messenger RNA which contains a single long open reading frame, starting with an AUG initiator codon and terminating in an UGA stop codon and polyadenylation signal (Anilionis *et al*., 1981; Wunner, 1991). The messenger RNA is translated into a 524 amino acid residue glycoprotein precursor, which is glycosylated in the endoplasmic reticulum. The number of glycosylation sites of the rabies virus glycoprotein, varies from strain to strain and is possibly important for the antigenicity of the strain (Dietzschold *et al.*, 1984). A glycosylation site at amino acid residue position 319 is found in all the lyssavirus glycoproteins and also in the prototype rhabdovirus, vesicular stomatitis virus's glycoprotein (Rose *et al*., 1982; Wunner *et al*., 1985). After glycosylation the glycoprotein progresses to the golgi apparatus, where it is further modified by the covalent addition of fatty acid moieties. The protein is also acetylated within the transmembrane domain. A total of 19 carboxy-terminal amino acid residues are cleaved whilst nascent protein is vectorially translocated across the endoplasmic reticulum during post-translational modification. The mature glycoprotein therefore consists of only 505 amino acid residues.

Glycoprotein molecules are the only external protein of the rabies virion and form the spike-like peplomers found on the rabies virion surface (figure 2.1). The peplomers are homomers of three glycoprotein molecules, and each homomer is structurally divided in a knob-like head and stalk region (Gaudin *et al*., 1992). The rabies virus glycoprotein is a typical type one glycoprotein divided into three domains: the amino-terminal ectodomain (or antigenic domain), a transmembrane domain and a carboxy-terminal

endodomain (or cytoplasmic domain). The antigenic region of the glycoprotein, which constitutes the major segment of the protein, forms the head structure of the peplomers. The glycoprotein molecules are stably anchored in the virion membrane by a 22 amino acid hydrophobic region. The cytoplasmic domain has 44 charged and uncharged amino acid residues and projects to the interior of the virion, where it associates with the matrix, and possibly the nucleocapsid proteins.

The glycoprotein is involved in several viral functions including host cell receptor binding (Kawai and Morimoto, 1994). It is furthermore implicated in invasive events such as fusion of the viral and endosome membrane during viral uncoating and release, as well as envelope and virion formation during the budding of the virus (Kawai and Morimoto, 1994, Mebatsion *et al*., 1996). The glycoprotein also has implications for the pathogenicity of different rabies virus strains (Dietzschold *et al*., 1985; Seif *et al*., 1985; Takayama-Ito *et al*., 2004). It has been shown that an amino acid in antigenic site III, residue position 333 is a key determinant of neurovirulence (Tuffereau *et al*., 1989). Variations in amino acid sequence in antigenic site II also holds implications for the pathogenesis of the virus (Préhaud *et al*., 1988).

On immunological level, the glycoprotein is recognized as the major antigen of the rabies virion, since it stimulates the production of, and binds to virus neutralizing antibodies (VNAb) (Crick and Brown, 1969; Cox *et al.,* 1977). The VNAb response is the most important immune response for immunological protection against rabies virus infection (Perry and Lodmell, 1991; Hooper *et al*., 1998). Furthermore, the glycoprotein induces cytotoxic T cell responses (Celis *et al*., 1988). Because of the immunological importance of the glycoprotein, the expression of the glycoprotein gene in subunit vaccines has been extensively investigated and also applied successfully during oral vaccination of wildlife with a recombinant vaccinia virus vaccine (Kieny *et al*., 1984; Wiktor *et al*., 1984; Rupprecht and Kieny, 1988).

2.1.3 Immune responses against rabies viruses

During natural infection the rabies virus is able to evade the host immune responses in various ways (Lafon, 1991). Firstly, during the spread of the virus from the peripheral to the central nervous system, the virus is masked behind the blood-brain barrier. The neurotropism of the virus ensures a minimalist immune response attributed to the constraints of expression of elements of the immune system in nervous tissue.

Bahloul and Lafon (2003) showed the difference in immunological responses to the highly pathogenic CVS strain and the attenuated Pasteur virus strain. CVS evaded clearance by the immune response by triggering apoptosis of infiltrating T cells and limiting inflammation of the nervous system. In another study it has also been shown that the glycoprotein of attenuated rabies viruses such as the Pasteur virus plays an important role in the induction of apoptosis of infected cells and other essential anti-viral responses, although the mechanism whereby this is achieved is still unclear (Faber *et al*., 2002; Préhaud *et al*., 2003). In Wang *et al*., 2005, it was also suggested that pathogenic strains of rabies virus evade antiviral and specifically also innate immune responses, whilst attenuated strains activate these responses.

Immune responses can be manipulated by vaccination to afford immunological protection against rabies virus infection. The immune responses that play important roles in inferring immunological protection against rabies viruses infection is firstly and primarily, the VNAb response (Perry and Lodmell, 1991; Flamand *et al*., 1993, Hooper *et al*., 1998), and then to some extent the cytolytic T lymphocyte (CTL) response (MacFarlan, 1988; Nathanson and Gonzalez-Scarano, 1991; Perry and Lodmell, 1991; Hooper *et al*., 1998). The VNAb response clears virus from the site of infection before the virus can spread to the central nervous system and also neutralizes virus released from cells lysed by the cytolytic response (Shankar *et al*., 1991). The VNAb response also acts by binding to infectious virions, which triggers subsequent neutralization (or virolysis), and lysis of infected cells. The T lymphocyte response, on the other hand, has a dual role (Lafon, 1991). The T helper cells are essential for the induction of the antibody production whereas the cytotoxic T cells are involved in the lysis of infected cells, especially at the site of infection (Nathanson and Gonzalez-Scarano, 1991, Bahloul and

Lafon, 2003). The importance of inflammatory responses in the clearance of rabies virus from the central nervous system has also been indicated (Hooper *et al*., 1998).

2.1.3.1 The immunogenicity of rabies virus proteins

The antigenicity of the rabies virus is reviewed in Coulon *et al*., 1993. All of the viral proteins exhibit some level of antigenicity, but are not all equally important in eliciting responses that infer immunological protection. The glycoprotein is recognized as the most important antigenic entity for the induction of immunological protection (Lodmell *et al*., 1995). The glycoprotein is the most potent inducer of the production of protective, virus-neutralizing antibodies (Crick and Brown, 1969; Cox *et al*., 1977). At least eight antigenic sites, designated I - IV, "a" and G1 have been identified on the glycoprotein external domain (Lafon *et al*., 1983; Seif *et al*., 1985; Préhaud *et al*., 1988; Bunschoten *et al*., 1989). These antigenic sites do not contribute equally to the immunogenicity of the protein, with sites II and III recognized as the major epitopes. The glycoprotein epitopes are virus neutralizing and CTL epitopes (Dietschold *et al*., 1990; Benmansour *et al*., 1991). Expression of glycoprotein alone, from different recombinant subunit vaccines, including various viral vaccine vectors and plasmid DNA, protected laboratory animals against lethal rabies virus infection (Wunner *et al*., 1983; Wiktor *et al*., 1984; Xiang *et al*., 1995). The presentation and conformation of the glycoprotein is essential for its ability to induce immune responses (Perrin *et al*., 1985). This is shown in the poor immune responses elicited against secreted glycoprotein molecules (Dietzschold *et al*., 1982). These glycoprotein molecules lack the 58 amino acid residues from the carboxy-terminal end of the glycoprotein molecule, corresponding to the membrane-anchoring region. Although membrane-associated glycoprotein and the soluble glycoprotein are antigenically identical, the latter does not confer protective immunity *via* the induction of VNAbs. Even mild reduction of the glycoprotein structure abrogates antigenic activity (Wunner *et al*., 1983).

The immunogenicity of the rabies virus nucleoprotein is reviewed in Fu *et al*., 1991a. Various antigenic sites have been identified on the rabies virus nucleoprotein (Goto *et al*., 1995). The nucleoprotein carries various T helper and B cell epitopes (Ertl

et al., 1989 and 1991; Drings *et al*., 1999; da Cruz *et al*., 2002). Rabies virus nucleoprotein, in addition, has been implicated in the induction of non-neutralizing antibody responses (Fu *et al*., 1991; Sumner *et al*., 1991; da Cruz *et al*., 2002). Several investigations suggest that the nucleoprotein augments the VNAb response and antiviral cytokine production from $CD4^+$ T cells (Dietzschold *et al*., 1987b; Ertl *et al*., 1989; Fu *et al*., 1991b; Hooper *et al*., 1994, Drings *et al*., 1999). The immunological role of the nucleoprotein has been investigated in several recombinant subunit vaccine studies. Recombinant raccoon poxvirus expressing a rabies virus nucleoprotein gene offered some protection to laboratory mice upon lethal peripheral challenge, but not against lethal intracerebral challenge (Lodmell *et al*., 1991). In another study, ribonucleoprotein protected mice and raccoons against lethal peripheral challenge with rabies virus (Dietzschold *et al*., 1987b). Lodmell and coworkers (1993) also showed that anti-nucleoprotein sera inferred 56-80% protection (depending upon time of challenge) upon lethal peripheral challenge with rabies virus. Intradermal administration of recombinant vaccinia virus expressing rabies virus nucleoprotein protected mice against lethal peripheral challenge (Sumner *et al*., 1991). Postexposure administration of a recombinant vaccinia virus expressing a nucleoprotein gene did not confer any protection to mice (Fujii *et al*., 1994). The value of expressing the nucleoprotein antigen in subunit vaccines is still disputed (Drings *et al*., 1999). Firstly, it is that inclusion of the nucleoprotein in a subunit vaccine will enhance its cross-protection properties against lyssaviruses. Since the nucleoprotein gene is more conserved between rabies virus strains and the other lyssaviruses than the glycoprotein gene (Dietzschold *et al*., 1988), it is believed that including the antigen in subunit vaccines will broaden the cross-protection ability of such vaccines (Dietzschold *et al*., 1987a). Certain antigenic sites on the nucleoprotein are also found universally in lyssaviruses (Goto *et al*., 1995). Drings *et al*., 1999 have shown however, that protection against European Bat Lyssavirus-1 viruses, afforded when the nucleoprotein gene is included in a subunit vaccine, is not significantly higher than vaccination with a glycoprotein antigen only. Expression of a Mokola virus nucleoprotein and glycoprotein gene in a DNA vaccine model did not expand the cross protection capacity of the vaccine (Nel *et al*., 2003).

T cell epitopes have been mapped to the rabies virus phosphoprotein (Larson *et al*., 1991). The phosphoprotein, however, plays a minor role in conferring immunological protection against rabies virus infection. Immunization studies with a recombinant vaccinia virus expressing the phosphoprotein failed to protect mice when challenged with a lethal dose of street virus (Larson *et al*., 1992). The phoshoprotein elicits a cytolytic T cell response but negligible T helper and B cell responses (Larson *et al*., 1992). Phosphoprotein gene knockout rabies virus mutants have been used as attenuated vaccines and induced high level of protective antibody responses (Shoji *et al*., 2004).

Little is known about the immunogenicity of the rabies virus matrix protein. The value of co-expressing this protein with other rabies virus proteins in subunit vaccines is being investigated (Personal communication, Nobantu Phalatsi).

2.1.3.2 Expression of rabies virus glycoprotein in different systems

Rabies virus glycoprotein has been expressed in various prokaryotic and eukaryotic expression systems (Dietzschold, 1996). The glycoprotein expressed in *Escherichia coli* failed to elicit protective immune responses (Yelverton *et al*., 1983). This result can be primarily attributed to improper folding and lack of post translational modification of the native protein. The expression of rabies virus glycoprotein genes in eukaryotic expression systems are summarized in table 2.1. From the table it is clear that most of the eukaryotic expression systems expressing the rabies virus glycoprotein were immunogenic in animal studies. Furthermore, glycoprotein expressed in genetically engineered plants was immunogenic in mice upon intraperitoneal or oral administration (Modelska *et al*., 1998; Yusibov *et al*., 2002).

Table 2.1: Expression of rabies virus glycoprotein genes in eukaryotic expression systems.

EXPRESSION SYSTEM	CONCLUSIONS AND REMARKS	REFERENCES
Yeast	Different forms of the rabies virus glycoprotein expressed by *Saccharomyces cerevisae* were not significantly immunogenic[1, 2].	Klepfer *et al*., 1993[1]; Sakamoto *et al*., 1999[2]
Baculovirus	Differentially glycosylated forms of the glycoprotein (rabies and Mokola virus) were produced in the baculovirus sytem[3, 4]. Rabies virus or Mokola virus glycoprotein expressed in this system proved immunogenic and protective responses in mice[4, 5]. Protein expressed by *Autographa californica* nuclear polyhedrosis virus in *Spodoptera frugiperda* cell culture (Sf9) was expressed in an immunogenic manner and protected raccoons from lethal rabies challenge after oral administration[6].	Tuchiya *et al*., 1992[3] Tordo *et al*., 1993[4] Préhaud *et al*., 1989[5] Fu *et al*., 1993[6]
Adenovirus	Replication defective human adenoviruses expressing a rabies virus glycoprotein elicited protective immune responses in mice upon different routes of administration (including topical) [7, 8, 9], and boosted immune responses in previously vaccinated dogs[8]. Modified adenovirus vectors could be used to immunize orally[10]. Antibody response to the antigen expressed by recombinant adenovirus was up to ten times superior to that expressed by an equivalent recombinant vaccinia virus[11].	Lees *et al*., 2002[7]; Tims *et al*., 2000[8]; Xiang *et al*., 1996[9]; Yarosh *et al*., 1996[10] Xiang and Ertl, 1999[11]
Poxvirus (amongst others various strains of vaccinia virus, raccoon poxvirus, canarypoxvirus, fowlpoxvirus, lumpy skin disease virus)	The rabies virus glycoprotein gene has been expressed in virtually every available poxvirus vaccine model. Some, but not all, of these are highlighted here. Raccoonpoxvirus expressing rabies virus glycoprotein proved immunogenic upon oral administration in raccoons[12], and subcutaneous administration in cats[13]. Recombinant canarypoxvirus (also known as ALVAC) expressing the glycoprotein antigen induced protective immunity[14]. It also proved safe and elicited long lasting and potent immune responses in humans[15, 16]. Recombinant fowl poxvirus expressing rabies virus glycoprotein gene protected mice, cats and dogs against lethal rabies virus infection[17]. Cattle sero-converted and achieved protective levels of antibodies after intramuscular inoculation with recombinant lumpy skin disease virus expressing the rabies virus glycoprotein[18]. These animals also exhibited cytotoxic T cell responses. The same recombinants were used in a mouse model. Although seroconversion could not be shown in these animals, cytotoxic T cell responses were indicated[19]. Rabies glycoprotein has also been expressed from vaccinia virus, and is in use as a commercial vaccine in oral vaccination programs[20].	Esposito *et al*., 1988[12]; Hu *et al*., 1997[13]; Taylor *et al*., 1990[14]; Cadoz *et al*., 1992[15]; Fries *et al*., 1996[16]; Taylor *et al*., 1988[17]; Aspden *et al*., 2002[18]; Aspden *et al*., 2003[19]; Wiktor *et al*., 1984[20]
Herpes virus	Rabies virus glycoprotein was expressed immunogenically by recombinant canine herpes virus, and elicited antibody response greater than for commercial rabies vaccines and lasted for at least 6 months after vaccination[21].	Xuan *et al*., 1998[21]
Nucleic acid vaccines	The rabies glycoprotein gene expressed under regulation of the SV40 promoter or the cytomegalovirus immediate early promoter induced both humoral and cellular immune responses to the same extent. In contradiction with previous studies, a secretory form of glycoprotein was found to be immunogenic and induced protective responses in mice[22].	Xiang *et al*., 1995[22]

2.1.4 The control and prevention of rabies

Rabies is a zoonotic disease and rabies viruses are believed to have the potential to infect all mammalian species. Effective measures and strategies are available to control the disease in domestic and wild animals, and prevent the disease in humans (Beran, 1991; Wandeler, 1988 and 1991; Fishbein, 1991). The control of rabies is ultimately aimed towards the protection of human and animal life and minimizing economic losses related to the disease. The transmission of rabies viruses is discussed as a preamble to the discussion of the control and prevention of the disease.

2.1.4.1 Transmission of rabies viruses

The rabies virus is excreted in the saliva of the infected and is transmitted to humans and animals through bites and scratches. Although the presence of virus is reportedly detectable by polymerase chain reaction in other bodily fluids such as the urine of a rabid subject, the infectivity of these excreta is doubtful (Sitprija *et al*., 2003). The factors that assure successful transmission of the rabies virus include the dose, route of inoculation, and the strain of the virus. The severity, location and multiplicity of inflicted wounds on the victim also influence the outcome of an exposure (WHO Expert Consultation on Rabies, 2005). Bites to the head and neck are generally associated with shorter incubation periods and higher mortality rates. Although rabies viruses can infect possibly all mammals, a hierarchy of susceptibility exists (Baer *et al*., 1990, Rupprecht *et al*., 2002). The susceptibility of the host to the rabies virus plays a role in the successful transmission of the virus. Susceptibility of different host species for the rabies virus has not been experimentally tested, but estimates are based on cumulative epidemiological data. Foxes, coyotes and jackals have been rated as extremely susceptible whilst skunks, raccoons, cats, cattle, mongooses and most rodents are deemed highly susceptible. Dogs, sheep, goats, horses and primates (including humans) are described as moderately susceptible and opossums are considered as low susceptible hosts. Accordingly certain groups of animals serve as reservoirs of the virus in nature whereas others only serve as vectors or dead end hosts of the disease.

Non-bite transmissions are a rarity and a summary of citations is provided in Swanepoel, 2004. Most prominently, several transmissions of rabies virus through organ transplants in the USA and Germany have been reported (Morbidity and Mortality Weekly Report, 2004; Srinivasan *et al*., 2005). The virus has been reportedly transmitted through transplantation of corneas, liver, kidneys, pancreas and arterial grafts (Doe, 1999; Srinivasan *et al*., 2005).

2.1.4.2 Control and prevention of urban rabies

In urban centers of developing coutries, rabies is maintained primarily by dogs (Fekadu, 1993). For the most part dogs remain the most important vector associated with transmission of the rabies virus to humans. In North America, some South American and European countries, where urban rabies control was and is very successful, less than 30% of fatal human cases are associated with a dog vector (WHO, 1998b).

Control programs for urban rabies primarily consist of three elements: the vaccination of domestic pets; the restriction of the movement of pets in and out of regions; and the removal of unrestricted animals (or strays) (WHO Expert Consultation on Rabies, 2005). Appropriate legislation, surveillance and the support of adequate diagnostic laboratory and veterinary services are some of the important components for a successful urban rabies control program (Beran, 1991; Swanepoel, 2004). The destruction of strays alone is not very successful in the control of rabies. Taking in account factors such as dog population turnover rate, 50 to 80 percent of the population has to be removed annually to have the desired effect in a given region (Swanepoel, 2004). Mass immunization of domestic dogs remains the most effective control strategy for urban rabies (Beran, 1991; Estrada *et al*., 2001; Perry *et al*., 1995; Cleaveland *et al*., 2003). Dog immunization, nevertheless, is compulsory in only 52 % of African countries and 30 % of Asian countries, the countries presently most affected by urban dog rabies. In addition, some of these countries still rely on the use of unsafe nerve tissue vaccines for protection against rabies (WHO, 1998b). The urban rabies problem in some poorer, developing countries is further compounded by social structures that allows for a large number of free-roaming dogs (owned or strays) in communities (Southern and Eastern

Africa Rabies Group, 2003). These dogs are often not approachable for parental vaccination and strategies involving vaccines presented as baits may be more effective (Linhart, 1993; Perry and Wandeler, 1993; Southern and Eastern Africa Rabies Group, 2003). The use of the rabies virus mutant, SAG-2, for the purpose of oral vaccination of dogs in South Africa has been investigated. Despite the vaccine's proven efficacy and safety in canines (and some non-target recipients) it proves to be relatively unstable when exposed to the elements and repeated vaccination may be required to attain protective immunity (Fekadu *et al*., 1996, Masson *et al*., 1996; Bingham *et al*. 1999; Lambot *et al*.., 2001; Knobel *et al*., 2003). This renders the vaccine less reliable for use in bait vaccination programs.

2.1.4.3 Control and prevention of sylvatic rabies

Various wildlife vectors, varying geographically, maintain sylvatic rabies in countries around the globe. In countries where urban rabies has been brought under control, the focus is shifting to control of wildlife rabies (Rupprecht *et al*., 1995; Finnegan *et al*., 2002). According to WHO surveys, 84 % of European and 44 % of American rabies cases are reported to include wildlife involvement (WHO, 1998b). Increasingly more new and emerging wildlife vectors are being recognized (Morimoto *et al*., 1996). Apart from public and veterinary health concerns, rabies is also considered as a wildlife conservation threat to some endangered mammals (Macdonald, 1993; Piper *et al*., 2000).

Various methods have been attempted to control rabies in wildlife (Winkler and Bögel, 1992; Hanlon *et al*., 1999). The earliest measures involved the indiscriminate destruction of animals, based on the principle that a threshold population density is required to sustain the transmission of rabies virus (Debbie, 1991). This approach has questionable efficacy and raises wildlife conservation concerns. Another early approach is one of habitat modification and entails the alteration of potential habitats of wildlife vectors to decrease the possibility of interaction with humans. Examples of such modifications include the regular removal of garbage and screening of doors and windows. Vaccination of wildlife is a more popular option, and is achieved in various

ways. Trap-vaccinate-release programs have been used (Rosatte *et al*., 1992). These programs involve the trapping, immunization and subsequent release of the animals. Despite the success of this approach, it is not popular since it is highly impractical and costly. Another approach involves different self-vaccination mechanisms that are designed to inject vaccine upon triggering. This method, however, poses some impractical difficulties in execution. Oral vaccination of wildlife is currently recognized as the strategy of choice in many control programs (Rupprecht *et al*., 2004). The concept of oral vaccination of animals was born after studies showed the susceptibility of laboratory animals to oral infection with the rabies virus (Baer *et al*., 1971). The first successful oral vaccination program was conducted in Switzerland towards the end of 1978 (Steck *et al*., 1982; Wandeler, 1988; Winkler and Bögel, 1992). Chicken heads filled with SAD virus vaccine packets were used in a program to attempt to halt an ensuing epizootic of rabies in red foxes (*Vulpes vulpes*). With the success of this trial, more oral vaccination programs were conducted in different Swiss valleys. In 1999, Switzerland was declared free from rabies due to the implementation of oral vaccination programs. Several other European countries have also been declared rabies free due to such programs. These include the Netherlands (1991); France (2000); Belgium and Luxembourg (2001) and the Czech Republic (2004) (Fooks, 2005). The approach also proves successful with the oral vaccination of foxes in the USA with the ERA derivative of the SAD virus strain and the recombinant virus vaccine, V-RG (Wandeler, 1991; Pastoret *et al*., 1992; Brochier *et al*., 1995). Oral vaccination might also be the only feasible way of targeting animals such as bats, which are not approachable by convenient means (Aguilar-Setien *et al*., 2002, Almeida *et al*., 2005).

2.1.4.4 Prevention in humans

In 1998, 33 373 human rabies deaths were confirmed with 99 % of the cases reported in the developing countries of Africa, Asia and the Americas (WHO, 1998b). It is widely accepted that the incidence of rabies cases is grossly underestimated. The underestimation may be ascribed to underreporting due to the state of public health infrastructure but also due to social customs and cultural beliefs in these countries

(Southern and Eastern Africa Rabies Group, 2003; WHO Expert Consultation on Rabies, 2005). In an isolated study it was shown that the number of human cases of rabies may be underestimated up to hundred-fold (Cleaveland *et al*., 2002). At the present time, rabies is not a curable disease, but prevention of the disease is possible through pre- and post exposure strategies (Rupprecht and Gibbons, 2004). Rabies in humans is prevented on two levels. Firstly, reducing the probability of exposure to the virus prevents the disease. Humans are exposed to the virus mainly from domestic animals, particularly dogs (WHO, 1998b). But exposures to the virus from wild animals are also reported (WHO, 1998b). The risk of human exposure to the virus is minimized by controlling the disease in wild as well as domestic animals. Secondly, post-exposure prophylaxis (PEP) is available in the case of suspect exposures. Pre-exposure vaccination is also available for individuals at high risk of exposure to the rabies virus. Pre-exposure treatment involves active immunization with several doses of human rabies vaccine. Patients at continuous risk of exposure to the virus may also receive periodic boosters of human rabies vaccine. The Human diploid, purified Vero and Chick embryo cell culture vaccines are preferred for human application (Sureau, 1992; WHO Expert Consultation on Rabies, 2005).

PEP is administered to persons that with suspected exposure to the rabies virus (Piper and Xenakis, 1998; Rupprecht and Gibbons, 2004). Although the treatment recommendations vary from country to country, proper PEP is threefold. Local wound cleansing serves to mechanically dilute or remove the virus from the site of infection. The latter entails prompt first aid and copious washing of the infected wound with soap and water. The use of agents such as benzalkonium chloride and other agents such as ethyl alcohol and iodine-based solutions have also been described. Although very simple, proper wound care has been shown to decrease incidence of rabies after exposure in 90 % of cases (Dean *et al*., 1963). Active immunization follows with several doses of vaccine and passive immunization with anti-rabies serum. Pre-exposure treatment does not totally alleviate the need for post-exposure prophylaxis. In the event of exposure the vaccinated patient will still receive two doses of vaccine, but the application of RIG is eliminated. In the case of PEP failure, incomplete schedules, while delayed treatment or insufficient treatment of wounds and multiple severe wounds to the head are among the

primary reasons for failure (Haupt, 1999; Macgregor and Mehta, 2001; Southern and Eastern Africa Rabies Group, 2003; Sriaroon *et al*., 2005). Rabies biologics have been shown to fail in human immunodeficiency virus (HIV) infected patients in advanced stages of AIDS (Thisyakorn *et al*., 2000; Pancharoen *et al*., 2001; Thisyakorn *et al*., 2001). The level of immune response induced after rabies vaccination in HIV-infected patients is coupled to the level of CD4+ T cells and therefore the progression of AIDS (Jaijaroensup *et al*., 1999; Tantawichien *et al*., 2001). The WHO Expert Consultation on Rabies (2005) recommends that special care should be taken in proper wound care and infiltration of the wound with RIG, in exposed HIV-infected individuals.

2.1.5 Epidemiology of rabies viruses

Rabies is currently reported endemic in wild and domestic animal populations in about 100 countries (WHO, 1998b). The epidemiology of rabies viruses is characterized by geographical host distribution and host susceptibility to the virus. Countries and regions reportedly free of genotype one rabies viruses are often islands and peninsulas, where a physical barrier is present to restrict the movement of wildlife vectors and thereby the spread of the virus. Countries reportedly free of classic rabies, amongst others, include Australia; Finland; Greece; Italy; Japan; Norway; the United Kingdom of Great Britain; and Sweden (WHO, 1998b). These countries occasionally report imported rabies cases. Bat-associated lyssaviruses are, however, endemic in the United Kingdom and Australia (Schneider and Cox, 1994; Fraser *et al*., 1996; Hooper *et al*., 1997; Gould *et al*., 1998; Brookes *et al*., 2005a). In 1998, dog rabies accounted for 66%, wildlife for 28% and bat rabies for 6% in the 71 countries that reported to the WHO (WHO, 1998b). As described earlier in this chapter, urban rabies has been brought under control in some countries, and sylvatic rabies now often plays a more important role (Finnegan *et al*., 2002). Relatively little information is available on the epidemiology of rabies on the African continent. Studies have however shown that rabies is endemic in Sudan with the dog as principle vector (Johnson *et al*., 2004b). In southern Africa, two distinct groups of rabies viruses are found (von Teichman *et al*., 1995). The groups of viruses are adapted to host species of the family *Herpestidae* and *Canidae* (King *et al*., 1993; von Teichman

et al., 1995; Nel *et al*., 1997; Sabeta *et al*., 2003; Johnson *et al*., 2004a; Nel *et al*., 2005; Mansfield *et al*., 2006). A seemingly unique epizootic in Kudu (*Tragelaphus strepsiceros*) have been reported in Namibia (Mansfield *et al*., 2006).

2.1.6 Rabies in the developing world: rationale of control and elimination programs

Rabies is often perceived as a minor problem, especially in developing countries plagued by many hardships (Fooks, 2005). Rabies competes with countless other human and animal health problems for both economic and human resources. Despite being considered as the infectious disease with the highest case fatality ratio (if timely post-exposure prophylaxis is neglected) and being rated as the 10th most common lethal infectious disease, rabies contributes to not even 1 % of the global mortality caused by infectious agents, which accounts largely for its inconsequential status (Martinez, 2000; Rupprecht *et al*., 2002). It is noteworthy to mention that the rabies problem may however be more substantial than currently anticipated because of severe underreporting and lack of proper surveillance programs in most developing countries as portrayed in some case studies (Cleaveland *et al*., 2002).

Rabies is frequently considered only an animal health problem. Although rabies is most definitely a sizeable animal health problem, it poses a public health burden. In most developed countries where dog rabies is still rampant human cases are also reported. The control of the disease in these animals, that are more often than not free roaming, are by no means an easily feat. Prevention in humans is complicated by scarcity of biologics such as RIG and the exorbitant prices of cell culture vaccines. For reasons such as these, PEP regimens are often not completed and may lead to preventable deaths. In countries where urban rabies has been brought under control, wildlife rabies now often poses a threat. Control strategies of rabies in wildlife are by no means less complicated. For example, there are no strategies available for the sustainable control of rabies in bat species. Rabies also poses an economic burden. This burden is composite of the loss of livestock and other animals and the cost of vaccination and continued control programs, which requires the public health infrastructure and diagnostic services. The cost

associated with PEP and pre-exposure vaccination for humans are also considerable with millions of doses of PEP administered annually (WHO, 1998b).

An insightful way of looking at the burden of rabies is considering disability-adjusted life year (DALY) scores which are estimated by taking factors such as those discussed above into consideration (WHO Expert Consultation on Rabies, 2005). DALY scores revealed that the public health burden due to rabies is higher than of important communicable diseases such as chagas disease and dengue (Coleman *et al*., 2004; Knobel *et al*., 2005).

The control of sylvatic and urban rabies is nevertheless a realistic foresight as shown by the successful control campaigns in many developed countries. Oral vaccination of dogs is considered an important factor in the elimination program of rabies in developing countries (WHO, 1998a). The development of a safe and effective oral vaccine that could be distributed in bait form is essential to such a vaccination program. The continued development of control and prevention strategies for rabies is therefore justified by the reduction of human and animal mortality and the moderation of costs involved in continued control efforts (Meslin *et al*., 1994).

2.2 Vaccines and vaccination – an overview

2.2.1 Concise account of the history of vaccinology

Vaccination is viewed as the most important and cost effective approach to prevent infectious disease. In modern medicine, vaccines are also explored for therapeutic means against infectious diseases and cancers and are called pharmacines or theraccines (Bonnet *et al*., 2000; Moingeon *et al*., 2003). An example of a vaccine that is also applied as a therapeutic is the rabies vaccine in PEP. Nevertheless, the earliest reports of the practice of administering an antigenic agent to prime a specific immune response dates back to 1000 AD. Chinese people practiced a form of variolation that involved intranasal administration of concoctions containing ground smallpox scabs which offered protection during the scourging smallpox epidemics (Fenner *et al*., 1988). In 1798, Edward Jenner applied material derived from vaccinia pustules of cows to

protect against smallpox in humans. He published his findings, and is widely considered the father of vaccination. Smallpox has since become the first, and so far the only, infectious disease that has been declared eradicated. The effective vaccine was a key tool in this feat (Fenner *et al*., 1988).

Early vaccines consisted of attenuated pathogens (Levine and Lagos, 1997). The bacteria was attenuated by altering culture conditions particularly temperature. Examples of these vaccines include Louis Pasteur's fowl cholera vaccine which consisted of temperature attenuated *Pasteurella septica* and an anthrax vaccine that consisted of temperature attenuated *Bacillus anthraxis.* Inactivated bacterial vaccines followed. These vaccines consisted of pure bacterial cultures that were inactivated by heat or chemicals. In 1885, Louis Pasteur prepared inactivated rabies virus vaccine by drying infected rabbit spinal cord over sodium potash. During the late 1800s the first toxoid based vaccines was prepared by using sterile filtrates from bacterial cultures.

With advances in molecular science and better understanding of disease pathogenesis and immunology, more targeted approaches for the design of vaccines are now explored (Liljeqvist and Ståhl, 1999; Green and Baker, 2002; Wack and Rappuoli, 2005). Since the 1980s the investigations into the development of recombinant subunit vaccines have soared. The first recombinant subunit vaccine, a yeast produced Hepatitis B surface antigen vaccine, was licensed in 1986 (Valenzuela *et al*., 1982). Various types of expression systems are available for subunit vaccines and depending on the antigens involved and the particular immune responses required for protective efficacy, the most suitable system should be sought (Hansson *et al*., 2000). Another increasingly popular approach for vaccine design is the use of reverse genetics. Reverse genetics (also referred to as the genome-based approach) involves the mining of sequence information for putative targets for vaccine development (Mora *et al*., 2003; Schnell *et al*., 2005; Wack and Rappuolli, 2005). This technology allows for the generation of novel attenuated strains which can be investigated as vaccines.

2.2.2 Types of vaccines

Vaccines are prepared in different ways and concurrently display certain universal characteristics. Killed or inactivated vaccines are prepared by eliminating the infectivity of the pathogen through heat or chemical treatment or radiation. These vaccines may need multiple doses for sustained protection and elicit mainly humoral responses. There is also the possibility of residual infectivity of vaccine organisms on batch to batch level. Attenuated vaccines are live vaccines consisting of the pathogen in a less virulent form. The pathogen is rendered less pathogenic through passage in cell culture or animals. During passage genes are lost or truncated and alters not only the genotype but also the phenotype of the organism. Modern technologies now also allows for site directed mutagenesis for a more targeted approach. Live, attenuated vaccines pose the potential danger of reversion to virulence and the risk of transmission from the vaccinated to the unvaccinated (Traavik, 2002). Nevertheless, live attenuated vaccines and killed or inactivated vaccines are still being successfully applied for various infectious diseases (Hansson *et al*., 2000).

On the other hand, subunit vaccines comprises of one or more disease antigens that are presented to the immune system by recombinant bacteria, viruses or nucleic acid carriers or as protein immunogens (Hansson *et al*., 2000). These vaccines are considered safer since there is no possibility of reversion to pathogenicity and the vaccines only consist of the antigenic portions of the disease agent. The main drawbacks of subunit vaccines are that multiple doses may be required for long-term protection depending on the system and antigen used. In the following section the use of poxviruses as vaccine vehicles for the development of subunit vaccines will be discussed.

2.2.3 Poxviruses as vaccine carriers

2.2.3.1 Characteristics of the poxvirus group of viruses

The poxviruses represent a large group of viruses that infect both vertebrates and invertebrates. Poxviruses of vertebrate species belong to the family *Poxviridae*,

subfamily *Chordopoxvirinae* (Fauquet *et al*., 2005). The subfamily is subdivided into eight genera, *Avipoxvirus*, *Capripoxvirus*, *Orthopoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, *Suipoxvirus* and *Yatapoxvirus*. The orthopoxviruses and particularly the Vaccinia virus, which is considered a species of the genus, represent the most studied group of poxviruses. The poxviruses have large, complex brick-shaped virions of 200-400 nm in length (figure 2.2). The poxvirus genome is a single linear double stranded deoxyribonucleic acid (DNA) molecule with hairpin loops at the ends. The genome of vaccinia viruses is roughly 200 000 base pairs in size (encoding almost 200 genes), but genome size varies from approximately 130 000 base pairs for the Orf virus (parapoxvirus) to up to 375 000 base pairs for some of the avipoxviruses.

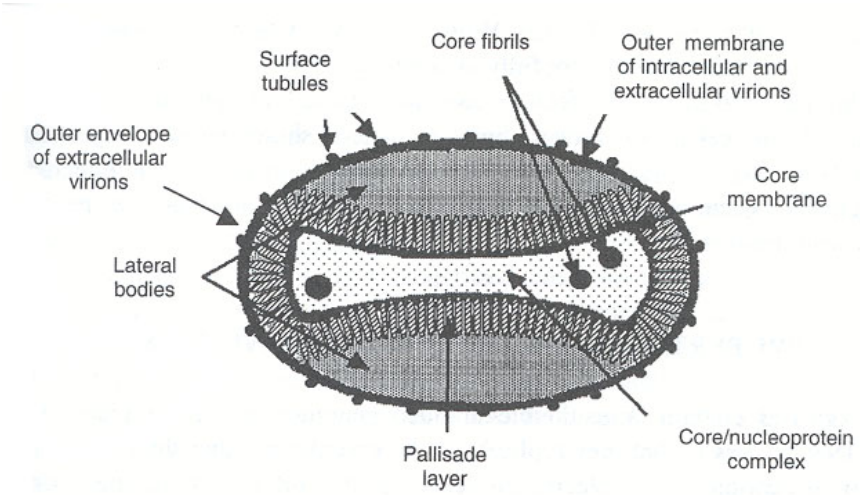

Figure 2.2: Structure of the poxvirus virion. The virion compromises of a core and host derived lipid bilayer envelope. Another characteristic feature of poxviruses is the presence of lateral bodies (Internet reference 2).

2.2.3.2 Development of poxviruses as vaccine carriers

In 1980, the assembly of the WHO declared the eradication of smallpox and recommended the discontinuation of vaccination against the disease. Smallpox was caused by the *Variola virus* (*Poxviridae*, genus *Orthopoxvirus*). It is regarded as one of the most dreaded diseases known to mankind. It is estimated that more than 300 million human lives were lost to the disease and those that survived infection were marked with

pitted scars and blindness (Fenner *et al*., 1988). One of the major contributing factors to the success of the Global Smallpox Eradication Effort (1967-1980) is recognized as the highly effective vaccine (Fenner *et al*., 1988). Various strains of Vaccinia virus (*Poxviridae*, genus *Orthopoxvirus*) were used as prophylactic, attenuated, live-virus vaccines because of immunological cross protection amongst the orthopoxviruses. The vaccine proved highly efficacious, in that it elicits long-term protective immunity after a single immunization (Fenner *et al*., 1988; Fenner, 1989).

The successful application of the vaccinia virus during the smallpox eradication campaign and the novel properties of poxviruses fuelled the study of these viruses for different molecular biology applications (Smith and Mackett, 1992; Carroll and Moss, 1997b). Firstly, the poxvirus system presents the feasibility of expressing in the same vector, diverse and non-related antigens and can tolerate insertions of over 30 000 base pairs of foreign DNA into the virus genome without loss of infectivity (Smith and Moss, 1983). Multiple foreign genes have therefore been simultaneously expressed in the host cytoplasm (Perkus *et al*., 1985). The relative ease with what these vaccines can now be constructed is also encouraging, also taking in account the uninfectious nature of the virus genomic DNA. Recombinant poxviruses generally induce relatively potent and long-lasting immunity, and because the vaccines are live viral vaccines, both humoral and cellular responses are elicited (Zavala *et al*., 2001; Andrew *et al*., 1992). In addition there is the lack of persistence of the vaccine virus in the recipient. There is also no available evidence to suggest integration of the virus genomic material on the host genome. Apart from being used in vaccine applications, poxvirus expression systems are also extensively used in molecular biology for the study of protein structure and function relationships, protein processing and intracellular trafficking, antigen presentation and the determinants of cellular and humoral immunity (Carroll and Moss, 1997b).

As result of the continued need for new and novel vaccines and because of its known efficacy, the use of poxvirus-based vectors for vaccines was and still is extensively evaluated (Pastoret and Vanderplasschen, 2003). Recombinant gene expression by a vaccinia virus was achieved in 1982 and the generation and selection of recombinant vaccinia viruses has since become standard laboratory procedure (Mackett *et al*., 1982; Panicalli and Paoletti, 1982; Mackett *et al*., 1984; Smith and Mackett, 1992).

The recombinant vaccine potential of the vaccinia virus is realized in the form of the recombinant vaccine for rabies, V-RG (Pastoret *et al*., 1992). V-RG was constructed by the insertion of rabies virus glycoprotein cDNA (ERA strain) into the thymidine kinase (TK) locus of the Copenhagen strain of vaccinia virus (Kieny *et al*., 1984). Because of the relative thermostable nature and oral innocuity of the virus in target species, it could be distributed in baits for wildlife immunization purposes (Pastoret *et al*., 1992). Another success story is that of an attenuated fowlpoxvirus which serves as a vector in a recombinant vaccine expressing Newcastle disease virus antigens and which has commercial licensure in the USA (Moss, 1996; Paoletti ,1996). A number of other poxvirus vaccines for human and veterinary applications are also in vaccine trials (Moss, 1996, Paoletti, 1996).

However, there are also factors discouraging the use of potent and replication-efficient vaccine vectors such as the vaccinia virus, especially in a population with a high incidence of acquired immunodeficiencies. Vaccinia virus has a very wide host range and is capable of infecting animals of many different species, in addition to the target species of the vaccine. Therefore the use of vaccinia virus may lead to the widespread dissemination of the virus. During the smallpox eradication program severe complications were associated with 1/500 000 immunocompetent vaccinees, and much higher values in the immunocompromised (Fenner *et al*., 1988; Fenner *et al*., 1989). Even successful vaccinations often caused pitted lesions at the site of inoculation and constitutional disturbance in the vaccinees. Vaccinia virus vaccination has also led to the death of an immunocompromised vaccinee and, recently, safety concerns where raised concerning the recombinant rabies vaccine, V-RG, after exposure of a human subject in the United States (Redfield *et al*., 1987; Rupprecht *et al*., 2001). Laboratory accidents with vaccinia virus have also been reported and in one particular case a worker suffered from progressive vaccinia at the site of infection (Moussatché *et al*., 2003).

These factors prompt the investigation of safer vaccinia virus derivatives and study of the attenuated poxviruses as vaccine vectors. Such a vaccine vector should be unable to replicate in mammalian cells or be naturally host range restricted and produce diminished cytopathic effects in target and non-target organisms. It should retain the capacity for high-level gene expression and immunogenicity while promising exceptional

safety for laboratory workers and potential vaccine recipients. The poxviruses investigated towards this goal are dubbed the *new generation poxvirus vectors* (Moss *et al*., 1996; Pastoret and Vanderplasschen, 2003). Some of these vectors include naturally host restricted viruses. Examples include the use of attenuated fowlpoxvirus and canarypoxvirus in non-avian species and attenuated lumpy skin disease virus in non-ruminant species (Taylor *et al*., 1988; Fries *et al*., 1996; Aspden *et al*., 2003). Strains have also been attenuated by serial passage or site directed mutagenesis. Examples include the NYVAC strain that was prepared by deletion of 18 open reading frames that are involved in host range and virulence (Tartaglia *et al*., 1992). NYVAC delivers antigens immunogenically in the absence of complete replication (Tartaglia *et al*., 1992; Brockmeier *et al*., 1993).

The poxvirus vectors of interest in this study are discussed in the following sections 2.2.3.3 and 2.2.3.4.

2.2.3.3 Modified Vaccinia virus Ankara

During the 1970s German researcher Anton Mayr and co-workers developed and characterized a highly attenuated and severely host restricted strain of vaccinia virus, Modified Vaccinia virus Ankara (MVA) (reviewed in Sutter and Staib, 2003). Material isolated from a pox-like lesion on a horse in Ankara, Turkey was serially passaged more than 570 times, through primary chick embryo fibroblasts (CEFs). During passage the virus adapted to growth in the avian cells that resulted in six major genomic deletions of 31 000 base pairs (or 15%) of the wild type vaccinia virus Ankara genome (Meyer *et al*., 1991, Antione *et al*., 1998). The virus proves to be stable after the 570 passages and multiple gene defects must be corrected for reversion to wild type (Antione *et al*., 1998). The deleted and mutated genes are primarily genes involved in host range of the virus and host immuno-regulators (Meyer *et al*., 1991; Antione *et al*., 1998; Blanchard *et al*., 1998).

Further factors encouraging the use of MVA in vaccines, is previous field experience during which 120,000 patients from South Germany and Turkey were vaccinated with MVA towards the end of the smallpox eradication campaign. No

complications or side effects were recorded despite the deliberate vaccination of high-risk groups such as young, elderly and eczematous patients (Fenner *et al*., 1988). These reports were substantiated in clinical trails with MVA recombinants (Hanke *et al*., 2002; Moorthy *et al*., 2003; Van Rompay *et al*., 2003). The inability of the virus to productively replicate in human cell lines (and most mammalian cell lines) makes it exceptionally safe for use in human vaccines (Carroll and Moss, 1997a; Blanchard *et al*., 1998; Drexler *et al*., 1998). The block in replication in non-permissive cells occurs late during the replication cycle - during viral morphogenesis when the proteolytic processing of the viral structural proteins is interrupted (Sutter and Moss, 1992). Even though the replication of MVA in mammalian cells is not complete, recombinant genes under early or late promoters are still however efficiently expressed (Sutter and Moss, 1992). Since the phenotype of MVA is the combined result of the truncation or deletion of several genes, reversion to more virulent or wild type is highly unlikely (Wyatt *et al*., 1998). The safety of MVA in the immunocompromised has also been established (Stittelaar *et al*., 2001). Studies have shown that MVA can deliver antigens in a highly immunogenic way, to stimulate both cellular and humoral responses (Ramirez *et al*., 2000).

Immunization studies with MVA recombinants and MVA as an immunogen

The efficacy of MVA as a vaccine vector has been evaluated in a myriad of infectious disease and tumor models. Studies with recombinant MVA expressing the relevant antigenic proteins have indicated protection against lethal doses of:

- Influenza virus in mice (Sutter *et al*., 1994, Bender *et al*., 1996)
- Parainfluenza virus type 3 in cotton rats and rhesus monkeys respectively (Wyatt *et al*., 1996; Durbin *et al*., 1998)
- Simian Immunodefiency virus in macaques (Hirsch *et al*., 1996, Ourmanov *et al*., 2000a and b, Nilsson *et al*., 2001, Nilsson *et al*., 2002)
- Dengue type 2 virus in rhesus monkeys (Men *et al*., 2000)
- Measles virus in macaques, mice and cotton rats (Stittelaar *et al*., 2000; Weidinger *et al*., 2001)

- *Mycobacterium tuberculosis* in a murine model (Feng *et al*., 2001)
- Japanese Encephalitis virus in swine (Nam *et al*., 2002)
- Human cytomegalovirus in mice (Wang *et al*., 2004)
- Severe acute respiratory syndrome coronavirus in mice (Bisht *et al*., 2004).

The use of MVA in cancer vaccines and therapy is suggested in Minev *et al*., 1999 and Bonnet *et al*., 2000. In Carroll *et al.,* 1997, the efficacy of MVA expressing a tumor associated antigen in the protection and active treatment of established pulmonary metastases, expressing the same antigen, is indicated in a murine model. MVA is also extensively evaluated as the vector for a prophylactic vaccine for Human Immunodeficiency virus type 1, and is currently in clinical trials (Hanke *et al*., 1998; Hanke *et al*., 2002; Gomez *et al*., 2002). Recombinant MVA expressing pre-erythocytic *Plasmodium berghei* antigens is also envisioned as part of vaccination regime against malaria and is also currently being evaluated in clinical trials (Schneider *et al*., 1998).

Recombinant MVA viruses have proven to be excellent boosters of vaccine-induced immunity. This is especially true when DNA vaccine prime and recombinant MVA boost regimes are employed, but also when attenuated live vaccines are used as primary vaccine (Dégano *et al*., 2000; González-Aseguinolaza *et al*., 2003; Goonetilleke *et al*., 2003; Bertley *et al*., 2004; Ramsburg *et al*., 2004). This phenomenon has been repeatedly shown in numerous studies (Ramshaw and Ramsay, 2000; Woodland, 2004). The booster responses are mainly enhanced T-cell responses characterized by the induction of interferon secreting, antigen specific T cell responses, which are up to a 100 fold compared to DNA or MVA vaccines, administered alone (McConkey *et al*., 2003). The use of recombinant MVA as primary vaccine and booster vaccination with recombinant fowlpoxvirus elicited even stronger T cell responses than DNA prime and MVA boost regimes (Vázquez-Blomquist *et al*., 2004).

Vigorous and long-lasting protective immune responses are associated with vaccinia virus immunization, so that after a single administration virus-specific CD4+ and CD8+ cells are generated (Demkowicz *et al*., 1996; Dorrel *et al*., 2001). The possible impact of severe attenuation, as is the case with MVA, especially with the loss of many host immunoregulatory factors, is apparent. Comparative studies on the efficacy

of recombinant MVA and vaccinia virus proved evidence for the contrary. The biology and immunology of MVA is investigated in Blanchard *et al*., 1998 and Ramirez *et al*., 2000. The ability of MVA to reach target tissues in non-avian species is indicated in Ramirez *et al*., 2000. MVA was found in the same target tissues as the replication competent Western Reserve strain. In the same study it was shown that MVA-driven protein expression peaked earlier and lasted for a shorted period than with the Western Reserve strain. The latter phenomenon is attributed to the limited spread of MVA from the initially infected cells. In Ramirez *et al*., 2000, the nature of the immune response against MVA in mice model is extensively investigated at humoral and cellular levels. The level of cellular immune response to the foreign antigen in recombinant MVA infected mice was similar or higher than with recombinant Western Reserve strain recombinant virus. The immune response (both humoral and cellular) against vector antigens was substantial lower for MVA than with the vaccinia virus recombinants. The latter holds implications for the clearing of the recombinant virus due to pre-existing immunity to vaccinia antigens (Perkus *et al*., 1985; Kündig *et al*., 1993).

2.2.3.4 Lumpy Skin Disease virus vaccine strains

Lumpy Skin Disease virus (family *Poxviridae*, genus *Capripoxvirus*) causes acute or sub-acute disease in cattle in sub-Saharan Africa, Madagascar and Israel (reviewed in Barnard *et al*., 1994; Hunter and Wallace, 2001). The disease is most probably insect-borne and affects cattle (Weiss *et al*., 1968). In addition to generalized symptoms such as fever and loss of appetite, lumpy skin disease virus (LSDV) infection manifests as multiple, firm and circumscribed skin nodules. The disease does not lead to a high level of mortality but is of economic importance because of the prolonged debilitating effect it induces on cattle. The disease also causes emaciation, temporary or permanent cessation of milk production, infertility in bulls and cows, abortion and permanent damage to hides. Current control efforts include vaccination with live attenuated vaccines. The Neethling vaccine strain (LSDV-SA) is used in southern Africa and was prepared by passage of a virulent field isolate in 61 passages on lamb kidney cell monolayers, 20 passages in

chorioallantoic membranes of embryonated chicken eggs, and a final 3 passages on lamb kidney cell culture (Van Rooyen *et al*., 1969).

Because of the severe host restriction and the apathogenicity of the LSDV to humans and other animals, the virus is considered as a possible vector candidate for new generation poxvirus-based vaccines of veterinary importance. Furthermore the LSDV system also exhibits all the advantages of a poxvirus-based system. The LSDV virions are thermostable, and vaccine preparations remained stable during and after long-term storage (Ngichabe *et al*., 2002). The latter is advantageous in the under developed countries where maintenance of a cold chain for vaccine distribution is often difficult. Furthermore, LSDV is indigenous virus to Africa, which means that use as vaccine here would not entail the introduction of foreign agents in the environment. An additional advantage to the use of the LSDV system is attributed to the immunological cross protection among the capripoxviruses (Kitching *et al*., 1987). An attenuated LSDV that expresses a foreign disease antigen can therefore be utilized as a dual vaccine, against the heterologous infection as well as capripoxvirus infections (Romero *et al*., 1993; Romero *et al*., 1994a, b and c; Berhe *et al*., 2003).

Immunization studies with LSDV recombinants

The LSDV system has been extensively investigated in the development of a rinderpest vaccine for cattle. Romero *et al*., 1994a showed that a recombinant LSDV (Kenya-1 sheep strain) expressing the rinderpest fusion protein gene fully protected cattle against lethal challenge with virulent rinderpest and LSDV viruses (Romero *et al*., 1993). It was also found that a lower dose of the recombinant LSDV was required for protection against lethal rinderpest virus challenge than for the equivalent vaccinia virus recombinant in a ruminant host (Romero *et al*., 1994c). A recombinant LSDV expressing the rinderpest virus hemagluttinin protein was used to achieve 90% protection in cattle at even lower doses than needed with the LSDV expressing the fusion protein of the rinderpest virus (Romero *et al*., 1994a). Once again, higher doses of recombinant vaccinia virus expressing the rinderpest fusion protein gene were required to achieve complete protection against virulent rinderpest virus challenge. Protection against LSDV

was afforded at doses as low as 1.5 x 10^4 foci forming units. The LSDV recombinants expressing the rinderpest virus fusion and hemaglutinin genes were used determine the cross protection that these antigens would afford against peste-des-petits-ruminants in goats (Romero *et al*., 1994b). Recombinant LSDV expressing peste-des-PETITSits-ruminants antigens protected goats against lethal challenge (Berhe *et al*., 2003). In another study, a vaccine cocktail consisting of one half-part recombinant LSDV expressing the hemagluttinin gene and one half-part recombinant LSDV expressing the fusion protein of the rinderpest virus was evaluated for immunological efficacy (Ngichabe *et al*., 1997, Ngichabe *et al*., 2002). In this study, partial long-term protection was achieved against both virulent rinderpest and LSD virus challenge. Vaccination did not induce any adverse clinical responses and no transmission of the vaccine virus to non-vaccinated, contact control animals could be indicated. In addition, a recombinant LSDV expressing bluetongue virus major core structural protein, VP7, offered partial protection of sheep against bluetongue virus challenge has also been constructed (Wade-Evans *et al*., 1996).

In Aspden *et al.,* 2002, a recombinant LSDV (LSDV-SA strain) expressing the rabies virus glycoprotein protein was investigated as a dual vaccine against rabies and lumpy skin disease in cattle. All the immunized cattle sero-converted after primary inoculation and an enzyme-linked immunosorbant assay were used to measure the induction of anti-rabies glycoprotein antibodies. It was also indicated that the recombinant virus elicited a T-cell proliferative response but the value of this observation was not indicated by challenge studies to show protection of the vaccinated animals. LSDV-SA recombinants expressing bovine ephemeral fever glycoprotein elicited both humoral and cellular responses in cattle although it did not protect the animals against lethal challenge (Wallace and Viljoen, 2005). In Aspden *et al*., 2003 the use of recombinant LSDV-SA was explored in a non-ruminant model. The recombinant virus expresses the foreign antigen despite not fully replicating in the non-permissive host.