CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

The concept of viruses as a natural phenomenon separate from other infectious organisms is less than 100 years old, and their nature began to be understood less than 50 years ago. The realization that many diseases of different organisms such as plants, animals and humans can be attributed to this newly recognized type of agent came even more recently, and the appreciation that practically all living species may have viruses associated with them defines the widespread prevalence of these agents and their potential importance in nature.

The concept of a biological disease agent, or pathogen, has its origins in the nineteenth century. The nature and history of viruses and virus diseases are reviewed in Levy *et al.*, 1994. Until about 1850, the intrinsic differences between various harmful agents, some of which were called by the Latin word for poison, *virus*, were poorly understood. The development of the concept of the virus as an agent entirely distinct from cellular disease agents such as bacteria and fungi was a slow process fraught with difficulties since viruses challenged the known principles and facts associated with bacteria and fungi. The first documented cases of viruses recognized as the disease-causing agents were in 1898 and involved tobacco mosaic disease of plants and foot-and-mouth disease of animals. The researchers in both cases implicated an infectious agent that was small enough to pass through the pores of filters and was thus much smaller than the smallest bacteria known. The agents were referred to as "filterable viruses", and it was understood that these "filterable viruses" were not only smaller than, but also inherently different from micro-organisms. At the beginning of the twentieth century, researchers in England showed that the human wart agent was also filterable, and there followed many other demonstrations of filterable viruses.

Today viruses are well known and can probably be best described as infectious agents and obligate intracellular parasites that use the genetic and metabolic apparatus in the cells of their hosts to reproduce themselves (Levy *et al.*, 1994). Viruses are immensely diverse, but each represents a definitive entity with shared properties and concepts different from all other forms in nature (Levy *et al.*, 1994). Viruses can be pathogenic and thus harmful to their hosts, while other viruses are symbiotic and in some cases infection with specific viruses can even provide an advantage to the host.

Viruses are however of interest not only as agents of disease but also as tools for investigating the cellular and molecular biology of the host organism. The study of viruses, or virology, plays an

important role in the field of molecular biology that can best be described as an amalgamation of scientific approaches involving the multidisciplinary effort of biochemists, physicists, chemists, geneticists, virologists, microbiologists and others (Levy *et al.*, 1994).

In this review of the literature, the present knowledge of the molecular nature of the orbiviruses, mainly BTV and AHSV, will be discussed. The focus will be on NS3, the non-structural protein encoded by segment 10 of the viral genome.

1.2. THE *ORBIVIRUS* GENUS OF THE *REOVIRIDAE* FAMILY

Viruses can be broadly classified based on the type of genomic nucleic acid and the types of molecules transcribed from the genomic nucleic acid as the virus directs the synthesis of messenger RNAs and new genomic nucleic acid (Levy *et al.*, 1994).

The genus *Orbivirus* in the family *Reoviridae* consists of viruses with similar morphological and physiochemical properties. The genus name, *Orbivirus*, was derived from the Latin *orbis*, meaning ring or circle (Borden *et al.*, 1971). Orbiviruses include pathogenic agents of man (e.g. Colorado tick fever), domestic animals (e.g. bluetongue of sheep and African horsesickness), native animals (e.g. epizootic haemorrhagic disease of deer) and many other viruses not yet linked with disease (e.g. St. Croix River virus) (Gorman, 1979).

The genus is divided into serogroups based on cross reactivities in complement fixation tests (Borden *et al.*, 1971) and serotypes within a serogroup are recognized by specific reactions in serum-neutralization tests (Gorman, 1979; Gorman and Taylor, 1985). Reassortment, a natural occurrence in the case of viruses with segmented genomes, has been shown to occur between some member viruses of a serogroup, but not between members from different serogroups (Holmes *et al.*, 1995). Examples for the AHSV serogroup include studies by Martin *et al.* (1998) and O'Hara *et al.* (1998); examples of studies where reassortment was observed for BTV S10/NS3 include Sugiyama *et al.* (1981), Collison and Roy (1983) and Pierce *et al.* (1998).

At present there are 11 genera in the family *Reoviridae*, and within the *Orbivirus* genus there are 19 serogroups of orbiviruses, each with a number of serotypes, as well as a group containing a number of unclassified viruses (Mertens *et al.*, 2000). Bluetongue virus is the prototype virus for this group (Murphy *et al.*, 1971). The present classification and nomenclature of the family *Reoviridae* are shown in Table 1.1.

Table 1.1. Classification and nomenclature of the *Reoviridae* family of viruses (adapted from Mertens *et al.*, 2000)

Family	*Reoviridae*	
Genera	*Orthoreovirus*	
	Orbivirus	
	Rotavirus	
	Coltivirus	
	Seadornavirus	
	Aquareovirus	
	Cypovirus	
	Entomoreovirus	
	Fijivirus	
	Phytoreovirus	
	Oryzavirus	
Serogroups in *Orbivirus* genus	African horsesickness group (AHSV-1 to 9)	*Culicoides*
	Bluetongue virus group (BTV-1 to 24)	*Culicoides*
	Changuinola virus group	phlebotomines
	Chenuda virus group	ticks
	Chobar Gorge virus group	ticks
	Corriparta virus group	*Culicine* mosquitoes
	Epizootic haemorrhagic disease virus group (EHDV-1 to 8)	*Culicoides*
	Equine encephalosis virus group (EEV-1 to 7)	*Culicoides*
	Eubenangee virus group	*Culicoides*, anopheline and *Culicine* mosquitoes
	Ieri virus group	mosquitoes
	Great Island virus group (e.g. Broadhaven virus)	*Argas*, *Ornithodoros*, *Ixodes* ticks
	Lebombo virus group	*Culicine* mosquitoes
	Orungo virus group	*Culicine* mosquitoes
	Palyam virus group (e.g. Chuzan virus)	*Culicoides*, *Culicine* mosquitoes
	Umatilla virus group	*Culicine* mosquitoes
	Wad Medani virus group	*Boophilus*, *Rhipicephalus*, *Hyaloma*, *Argas* ticks
	Wallal virus group	*Culicoides*
	Warrego virus group	*Culicoides*, anopheline and *Culicine* mosquitoes
	Wongor virus group	*Culicoides*, mosquitoes
	Unassigned virus group (e.g. St Croix River virus)	

The first EEV serotype was isolated in 1967, with the subsequent identification of another 3 serotypes in March 1971, December 1973 and April/May 1976 (Gorman and Taylor, 1985). Another serotype was also identified in 1993 (Gerdes and Pieterse, 1993). To date, seven serotypes namely Kyalami, Bryanston, Cascara, Gamil, Kaalplaas, Langeberg/Langebaan and Potchefstroom have been identified in southern Africa (Gerdes and Pieterse, 1993). Cross complement fixation tests have failed to demonstrate a serological relationship or relatedness between any of the EEV serotypes and other orbiviruses such as BTV, AHSV, EHDV or Corriparta virus (Erasmus *et al.*, 1970; Gorman and Taylor, 1985; Viljoen and Huismans, 1989).

The seven EEV serotypes are not equally prevalent under field conditions, probably indicating that some serotypes have adapted better to the specific vector and host environments than other serotypes (Barnard and Paweska, 1993; Venter *et al.*, 1999; Howell *et al.*, 2002; P. G. Howell, personal communication). Some of the seven identified serotypes, however, need to be validated (Venter *et al.*, 1999). In this regard samples of virus, representing what has been referred to as the Langeberg/Langebaan EEV serotype, have been investigated and have been shown to be AHSV-5 (Howell *et al.*, 2002). The same group however identified another isolate, E21/20, that was subsequently shown to be a previously undescribed member of the EEV serogroup, so that the number of known EEV serotypes remains at seven (Howell *et al.*, 2002). Howell *et al.* (2002) therefore concluded that to date seven valid serotypes of EEV have been identified which they arranged into alphabetical order, corresponding with a numerical serotype designation, in order to avoid confusion in the nomenclature (Table 1.2.).

Table 1.2. All known EEV serotypes with a proposed numerical serotype designation (Howell *et al.*, 2002)

Name of virus	Numerical serotype designation of virus
Bryanston	EEV-1
Cascara	EEV-2
Gamil	EEV-3
Kaalplaas	EEV-4
Kyalami	EEV-5
Potchefstroom	EEV-6
E21/20	EEV-7

Cascara (EEV-2) was the first serotype to be isolated (Erasmus *et al.*, 1970) and as such was viewed as the representative of the EEV group. Bryanston (EEV-1) has however superseded Cascara to become the dominant serotype and thus the present representative serotype of the EEV serogroup (Paweska, 1999; P.G. Howell, personal communication). For the purposes of this thesis, the EEV

serotypes will be referred to using the numerical serotype designation proposed by Howell *et al.* in 2002.

The diseases caused by some members of the *Orbivirus* genus, notably bluetongue virus (BTV) and African horsesickness virus (AHSV) are of great economical importance due to the livestock that are affected and the trade of these livestock. Trade often involves the import and export of the animals or products of the animals such as genetic material (semen), and there are many strict regulations regarding trade of the livestock or their products. AHSV is especially important, as it affects horses and thus plays an important role in the strictly regulated industries of horse racing and show jumping. In the case of EEV, which also infects horses, the importance of the disease lies not in the losses caused by it, as it is in many cases a subclinical infection, but rather in the nature of the affected animal and the potential impact it can have on the strictly regulated industry of which the animals are an integral part. The limited knowledge on the epidemiology, pathogenicity, and viral characteristics of EEV is seen as a potential barrier to the international movement of horses.

1.3. EPIDEMIOLOGY OF A NUMBER OF ORBIVIRUSES

1.3.1. Geographic distribution

Bluetongue seems to have originated on the African continent and for many decades it was believed to be confined to Africa. The first confirmed outbreak of the disease outside Africa occurred in Cyprus (Roy, 1989), subsequently several outbreaks of the disease have been reported in different parts of the world such as the Middle East (Erasmus, 1985; Roy, 1989), the United States of America, Portugal and in Spain. It is now known to occur in a broad belt around the world, encompassing the tropical and temperate zones in which a large percentage of the world ruminant population is also present (Ozawa, 1985). EHDV has also been isolated in countries in the same regions (Parsonson and Snowdon, 1985).

African horsesickness is endemic in eastern and central Africa, although AHS is also widely distributed in sub-Saharan Africa. Occasional outbreaks have occurred in North Africa, West Africa, the Middle East, parts of Asia and in Southern European countries (such as Spain and Portugal), but the virus has not been able to maintain itself outside the sub-Saharan enzootic zones (Williams *et al.*, 1993; Zientara *et al.*, 1993).

Equine encephalosis is endemic at least in southern Africa, including South Africa, Zimbabwe, Botswana and Kenya (Barnard, 1997; Venter *et al.*, 1999). EEV is found more widely throughout southern Africa than AHSV which is restricted to certain areas, this suggests an epidemiological pattern and vectors unlike that of AHSV (Barnard, 1997). Evidence of EEV infection has however been found in other regions of the world such as Australia (P.G. Howell, personal communication).

1.3.2. Transmission by insect vectors

The first breakthrough in identifying insect vectors of BTV came in 1943 when Du Toit (1944) succeeded in transmitting bluetongue to sheep with *Culicoides imicola* (*pallidipennis*) which had fed on a viraemic sheep 10 days earlier. Subsequently, *Culicoides variipennis* was incriminated as a vector of BTV in the US (Erasmus, 1985) and different species of *Culicoides* midges have been incriminated as vectors of BTV and other orbiviruses in various countries e.g. *Culicoides brevitarsis*, *Culicoides fulvus*, *Culicoides wadai*, *Culicoides acetoni* in Australia (Parsonson and Snowdon, 1985; St. George, 1985), and *Culicoides variipennis* (now known as *Culicoides sonorensis*) and *Culicoides insignis* in the US (Rao and Roy, 1983; Callis, 1985; Greiner *et al.*, 1985; Bonneau *et al.*, 2001). EHDV is also mainly transmitted by *Culicoides* species, e.g. *Culicoides brevitarsis* in Australia (Parsonson and Snowdon, 1985).

AHSV is also mainly transmitted by *Culicoides* species, specifically *Culicoides imicola* (Du Toit, 1944). In some cases, AHSV is also transmitted by mosquitoes of the *Aedes*, *Anopheles* and *Culex* species, as well as the brown dog tick *Rhipicephalus sanguines* (Lubroth, 1992; Tomori *et al.*, 1992).

EEV is also transmitted by *Culicoides* species, and specifically *Culicoides imicola* (Theodoridis *et al.*, 1979; Nevill *et al.*, 1992; Paweska, 1999; Venter *et al.*, 1999). The first isolation of a virus from the EEV serogroup from *Culicoides* species in South Africa was done by Theodoridis *et al.* (1979) during an unsuccessful attempt to isolate bovine ephemeral fever virus. Subsequently, *Culicoides bolitinos* has also been implicated in EEV transmission (Paweska, 1999).

Due to the nature of the insect vectors involved in the transmission of bluetongue, African horsesickness and equine encephalosis, the diseases all occur seasonally, and outbreaks (usually in the late summer and autumn) are influenced by conditions that favour the occurrence, breeding and spread of the vector (Theodoridis *et al.*, 1979; Venter *et al.*, 1999).

Culicoides imicola is widely distributed in sub-Saharan Africa, parts of North America and southern Europe, and southern Asia (Venter *et al.*, 1999). *Culicoides imicola* is a common vector for BTV, AHSV and EEV, and the potential thus exists for all these diseases to spread to these regions when conditions are favourable and even to persist within these regions since a suitable insect vector that can sustain the virus is present. Outbreaks in countries where AHSV and BTV are not endemic have occurred in the past due to the dissemination of the insect vectors (through the movement of the infected midges by wind, or climatic conditions that favour their growth) or the introduction of equidae or other livestock carrying the viruses. In this regard, the national and international movement of game or wild animals e.g. zebras, is an important factor relating to the spread and persistence of the diseases. Zebras can introduce disease into previously disease free regions, as zebras are potential

reservoirs of both AHSV and EEV. The status of previously disease free regions can therefore change permanently due to the introduction of livestock or game, as well as climate changes, that will allow the establishment and sustained growth of the vector species.

1.3.3. Host range

Bluetongue has traditionally been regarded as a disease of sheep and more particularly of European breeds. However, other domestic ruminants have also been shown to be susceptible, e.g. calves, cattle, goats, water buffaloes and camels (Ozawa, 1985; Roy, 1989). BTV and EHDV also share common hosts such as cattle, buffaloes, deer, antelope, white-tailed deer, mule deer, bighorn sheep, and in some cases EHDV can also infect zoological animals (Callis, 1985; Jessup, 1985; Parsonson and Snowdon, 1985).

In the case of AHSV, the host range may be limited mainly to equine animals such as horses, mules, donkeys and zebras (Barnard, 1993, 1997). The infection of man by AHSV has however also been reported. Humans exposed to freeze dried vaccine preparations of AHSV neurotropic strains 1 and 6 were shown to develop encephalitis and uveochorioretinitis (Swanepoel *et al.*, 1992).

EEV, like AHSV, infects horses and antibodies against EEV have also been found in zebras (Burchel's zebra and the Cape mountain zebra), in donkeys (Gerdes and Pieterse, 1993; Williams *et al.*, 1993) and African elephants (Barnard, 1997). It has been speculated that zebras function as a possible reservoir of the virus (Barnard and Paweska, 1993; Williams *et al.*, 1993; Barnard, 1997; Paweska, 1999).

AHSV and EEV thus share vectors and hosts as both infect equids. It has been found that the serum of a single animal can show antibodies to both viruses, this creates the possibility that an animal can be simultaneously infected with both viruses or rather that a single animal can be infected by both viruses in the same season, possibly even at the same time leading to confusion especially regarding symptoms of either disease (Williams *et al.*, 1993). This is further illustrated by the fact that during a recent outbreak of AHS in the Western Cape, EEV Bryanston (EEV-1) was isolated from horses showing disease symptoms (P.G. Howell, personal communication), confirming that EEV can indeed co-circulate with AHSV during the same season.

1.3.4. Epidemiology, clinical symptoms and pathogenesis of EEV

The history of the first known cases of EEV as well as the symptoms of the disease are discussed by Erasmus *et al.* (1970). EEV was first isolated in 1967 from a 13-year old thoroughbred mare (Cascara) at Mauritzfontein stud farm in the Kimberley district in South Africa. The horse first showed

symptoms 24 hours before death. The symptoms included listlessness and an anxious facial expression with tightening of the face, mouth and ocular commissures, and clinical examination revealed a slightly elevated temperature of 39.5°C and a pulse rate of 44 per minute. The autopsy revealed marked venous congestion particularly of the liver, kidneys and subcutaneous tissues. The liver showed signs of fatty degeneration and sharply demarcated areas of catarrhal enteritis were observed in the distal half of the small intestine. The brain was congested and oedematous with an excessive amount of cerebrospinal fluid.

In the next few days, two other adult mares became ill. One of them died the same day, whereas the other one recovered after about 14 days. Fatal cases occurred amongst horses at the Police College in Pretoria and were also reported from other parts of the country. All these animals showed essentially the same symptoms and lesions. It is interesting to note that all the clinical and fatal cases were invariably seen in animals older than seven years. Two young horses experimentally infected with liver and spleen suspensions from the horse Cascara, showed marked febrile responses that commenced 3 days p.i. and persisted for 4 days, attaining a maximum of 41°C, after which the horses recovered fully. Apart from the febrile responses, the horses showed no other clinical symptoms. This suggests that age seems to play a role in the pathogenesis of the disease.

Virus was isolated from organ specimens collected from case 1 (Cascara) as well as from other fatal cases, and from the blood of horses, which showed fever only. All these virus isolates appeared identical and consequently the Cascara isolate was selected as prototype strain for further characterization.

After isolation of the Cascara serotype, a serological survey was also conducted. The results indicated a widespread occurrence of infection with EEV during the summer (first three months) of 1967. An interesting point was that in the collected serum samples, unstabled horses were more likely to show antibodies against EEV than stabled horses, this suggested nocturnal biting insects possibly played a role in the transmission of the virus. This was confirmed by the first isolation of a virus from the EEV group from *Culicoides* species by Theodoridis *et al.* in 1979. Another interesting point was that antibodies against EEV were also found in horses that had shown no clinical signs, suggesting that the great majority of infections were subclinical in nature and that EEV exhibits a low pathogenicity for horses in endemic areas where annual exposure occurs (Gerdes and Pieterse, 1993). EEV thus causes a disease characterized by a high morbidity (60% to 70%) but a low mortality (5%) (Bremer and Viljoen, 1998). During this serological study, twenty serum samples collected during the ten years prior to 1967 were also examined and none of these were found to possess antibodies to EEV. This suggested the very recent appearance of the virus in South Africa.

Since 1967, equine encephalosis has taken on epidemic proportions in some years with more than 75% of horses tested having antibodies against EEV (e.g. 1994 to 1997) and 85% of donkeys tested (1995) (Venter *et al.*, 1999). Since the first outbreak in 1967, except for localized epizootics in 1976, 1978 and 1990 only sporadic isolations of the virus have been made (about 60 since 1983). A very high EEV seroprevalence in horses and donkeys in South Africa, but only a limited number of isolates made, thus again suggests that most EEV infections are subclinical in nature (Paweska, 1999). It has also been found that there is a continuous circulation of the virus between its host and vector in the case of zebras, which function as a possible reservoir of the virus and which ensures the persistence of the infection (Barnard and Paweska, 1993).

In a recent study, Howell *et al.* (2002), used serum samples from a ten year period (1990 to 2000) and found that 56.9% were positive for neutralizing antibody against one or more serotypes of the virus. The antibodies found were not necessarily against one type of virus, because it has been found that the host can be infected by more than one serotype, thus showing antibodies to more than one serotype at one point in time (Howell *et al.*, 2002). This allowed them to conclude that EE is a vector-borne infection of horses of a mild to subclinical character, which, when the diagnosis is confirmed, would in the vast majority of cases, closely resemble a mild attack of AHS (Howell *et al.*, 2002). In addition, it has been found that EEV Bryanston (EEV-1) can co-circulate with AHSV, this was illustrated in the Western Cape during a recent outbreak of AHS (P.G. Howell, personal communication).

In the Western Cape, a demarcated area of an estimated extent of 9 000 km^2, has been set aside as a surveillance zone to monitor the possible introduction or occurrence of AHS which would compromise the integrity of pre-export quarantine facilities for horses in Cape Town. Within this area serum samples were collected in May 1998 that showed the presence of antibodies against EEV. Subsequently, over a period of 18 months, serum samples were collected and assayed for neutralizing antibody against the serotypes of EEV. The results of these tests indicated the presence of EEV serotypes in the region, and it appeared that prior to the implementation of the surveillance program an outbreak of EE had taken place in the region. At the commencement of the survey, Bryanston was the predominant serotype, 18 months later Bryanston was still the most prominent serotype, followed by Potchefstroom and E20/21 (Howell *et al.*, 2002). The fact that EEV is present in this region is obviously of great concern and can have grave consequences for the export of horses.

1.3.5. Epidemiology of other orbiviruses

Other orbiviruses mentioned in this thesis include Orungo, Lebombo, Chuzan, Broadhaven and St. Croix River virus. Orungo and Lebombo viruses have both been isolated in Africa. Orungo virus has been isolated from *Aedes*, *Anopheles* and *Culex* mosquitoes, as well as from humans, where it has

been associated with febrile illness. Antibodies to this virus have been detected in sera from camels, cattle, goats, humans, monkeys and sheep. Lebombo virus has been isolated from *Aedes* and *Mansonia* mosquitoes, human plasma and rodents (Brown *et al.*, 1991). Chuzan virus, a member of the Palyam virus serogroup, was isolated from the biting midge *Culicoides oxystoma* and sentinel calves in Japan in 1985. The virus was subsequently implicated in an epizootic of congenital abnormalities with hydraencephaly-cerebellar hypoplasia syndrome of calves. Palyam serogroup viruses are usually associated with a variety of haematophagous arthropod vectors and large mammals, principally cattle, in many parts of the world including Asia, Australia and Africa. Although the Palyam serogroup of viruses seem to be involved in producing abortion and congenital malformations in cattle, the pathogenic importance of most of them remains unknown (Yamakawa *et al.*, 1999). Broadhaven virus is a member of the Great Island virus group, which is currently classified as a serogroup in the *Orbivirus* genus. This virus is transmitted by ticks and was first isolated from *Ixodes uriae* (Moss *et al.*, 1992; Attoui *et al.*, 2001). St. Croix River virus was isolated from a tick cell line established prior to 1994 from eggs of *Ixodes scapularis*. The eggs were collected from a tick obtained from a hunter-killed white-tailed deer in western Wisconsin, near the St. Croix River. This is a new orbivirus that has not been studied to a great extent, and although its genome has been sequenced, nothing is presently known about its replication characteristics and pathogenicity (Attoui *et al.*, 2001).

1.4. THE STRUCTURAL CHARACTERISTICS OF ORBIVIRUSES

Most of the work done on the viruses of the *Orbivirus* genus has concentrated on the prototype member, namely BTV. In recent years information has also become available regarding AHSV and EHDV. Very little is however known at this stage about the structural or functional characteristics of EEV.

1.4.1. Viral morphology and chemical composition

The prototype orbivirus, BTV, has a regular structure, is non-enveloped and the size of the virion is approximately 54 mμ. BTV consists of a dsRNA genome enclosed by a capsid of complex nature containing a single layer of 32 well-defined protein capsomeres with a hexamer-pentamer clustering of units arranged in accordance with icosahedral symmetry (5:3:2). The capsomeres are relatively widely spaced and have a flattened, hollow cylindrical or prismatic shape (Els and Verwoerd, 1969). This nucleocapsid structure (core particle) consists of 5 types of protein; two major (VP3 and VP7) and three minor components (VP1, VP4 and VP6) (Verwoerd *et al.*, 1970; Els, 1973; Huismans and Howell, 1973; Bremer, 1976; Mertens *et al.*, 1984; Roy, 1989; Huismans and Van Dijk, 1990; Roy, 1996). This icosahedral core particle is surrounded by an easily removed outer capsid structure (outer shell) composed of two proteins (VP2 and VP5). This outer capsid lacks clearly visible morphologic

subunits. None of the proteins is glycosylated or, as far as been determined, otherwise modified. (Roy, 1989). The core particle and outer capsid together is referred to as the virion. In protein synthesis studies at least 10 virus-induced polypeptides have been identified in infected cells. Therefore in addition to the seven structural proteins, there are at least three non-structural proteins, i.e. NS1 (P5a), NS2 (P6a) and NS3 (P8). Each of the major structural and non-structural proteins is encoded on a different genome segment (Mertens *et al.*, 1984; Pedley *et al.*, 1988; Van Dijk and Huismans, 1988; French *et al.*, 1989).

AHSV has a similar regular structure, also consisting of a protein capsid which consists of a single layer of 32 capsomeres arranged in a regular icosahedral symmetry to form a particle 55 nm in diameter (Verwoerd and Huismans, 1969).

EEV in turn possesses a similar structure to that seen for both BTV and AHSV. The virus particles are 73 nm in diameter (60 nm when negatively stained) and the particles do not appear to possess an envelope (Theodoridis *et al.*, 1979). Experimental observations suggested that the capsid is composed of 32 hexamer-pentamer morphological subunits with icosahedral symmetry or a dimer or trimer arrangement of the capsomeres (Erasmus *et al.*, 1970; Lecatsas *et al.*, 1973) and the capsid shell of the virion is composed of two layers (Holmes *et al.*, 1995).

Electron microscopic studies have shown that negatively stained particles of BTV, AHSV and EEV are indistinguishable, and as representative example, negatively stained BTV particles are shown in Fig. 1.1.

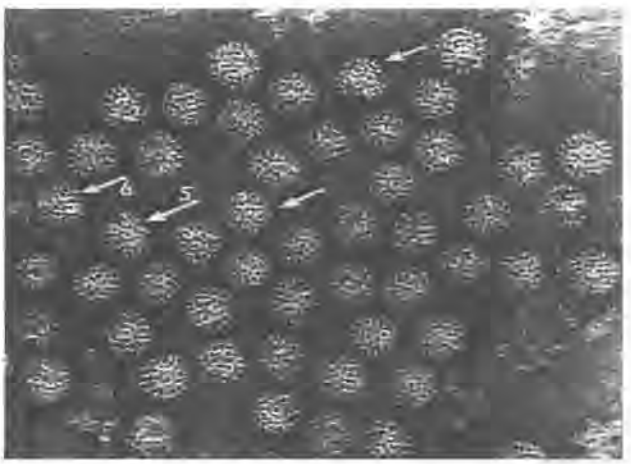

Figure 1.1. Negatively stained bluetongue virus particles. X 156 000. Particles are indicated by arrows (adapted from Els and Verwoerd, 1969).

Orbiviruses such as AHSV and BTV also share many similarities regarding their physical, chemical and biological properties. This includes their pH sensitivity, stability towards organic solvents and transmission by *Culicoides* species. They are thus mainly distinguished on serological grounds and because of their different host specificities (Verwoerd and Huismans, 1969).

BTV and AHSV, depending on the experimental conditions, can be either slightly sensitive or resistant to ether, chloroform and deoxycholate (Bowne and Ritchie, 1970; Borden *et al.*, 1971; Bremer, 1994). AHSV is resistant to the action of trypsin (Bremer, 1994), BTV is labile at pH 3.0 (Borden *et al.* 1971), while AHSV can be inactivated within 15 min at pH below 5.6 and above 10.9 (Bremer, 1994).

EEV, depending on the experimental conditions, can be either resistant or sensitive to chloroform, relatively sensitive to sodium deoxycholate and sensitive to trypsin. The virus is labile at pH 3.0 and 5.0, as well as temperature sensitive as it is inactivated at 56°C (stable at temperatures lower than 56°C). The virus is also susceptible to actinomycin D (Erasmus *et al.*, 1970; Theodoridis *et al.*, 1979).

1.4.2. The viral genome

Orbiviruses such as BTV, AHSV and EHDV, contain segmented genomes composed of 10 dsRNA segments ranging in size from 0.5 to 2.7 x 10^6 Daltons. A double layer protein coat surrounds these 10 segments (Bremer, 1976; Van Dijk and Huismans, 1988). The segments are numbered 1 to 10 in order of their migration in an agarose gel (Huismans and Van Dijk, 1990) and are divided into three size groups of segments (Els and Verwoerd, 1969; Verwoerd, 1969; Bowne and Ritchie, 1970; Verwoerd *et al.*, 1970), large segments (L, 1 to 3), medium segments (M, 4 to 6) and small segments (S, 7 to 10) (Roy, 1989). Each segment probably contains the information for the synthesis of one virus specific polypeptide; this is supported by a study that indicated that no secondary proteolytic cleavage of virus-induced polypeptides was observed to occur (Gorman *et al.*, 1981). As an example, the 10 dsRNA genome segments of AHSV encode 7 structural proteins (VP1 to VP7) and at least three distinct non-structural proteins (NS1 to NS3) (Bremer, 1976; Grubman and Lewis, 1992). Each segment codes for only one protein except the smallest segment, S10, which codes for two proteins, NS3 and NS3A, presumably via different in-frame translation initiation sites (Van Staden and Huismans, 1991; Van Staden *et al.*, 1991).

AHSV, similar to BTV, also contains double-stranded ribonucleic acid as its genetic material and can be fractionated into at least 10 components of similar but not identical size distribution after isolation (Verwoerd, 1969; Verwoerd and Huismans, 1969, Verwoerd *et al.*, 1970; Borden *et al.*, 1971; Bremer, 1976; Gorman *et al.*, 1981; Knudson *et al.*, 1982). A small and rather variable amount of hybridization has been found between bluetongue and African horsesickness viruses (Verwoerd and Huismans, 1969), which indicates that the genomic segments of AHSV and BTV show some similarity.

EEV, similar to both BTV and AHSV, contain RNA as its nucleic acid (Erasmus *et al.* 1970; Theodoridis *et al.* 1979) in the form of 10 segments of linear dsRNA (Viljoen and Huismans, 1989; Bremer *et al.*, 1990). The sizes of the ten genome segments for EEV Cascara (EEV-2) have been estimated by electrophoresis on a 1% agarose gel using BTV segments as a standard (Viljoen and Huismans, 1989) (Table 1.3.).

Table 1.3. EEV Cascara (EEV-2) genome segments and their estimated sizes (Viljoen and Huismans, 1989) compared to the estimated sizes of AHSV-3 (Bremer *et al.*, 1990) and BTV-10 (Roy, 1989)

Genome segment	EEV Cascara (EEV-2)	AHSV-3	BTV-10
	Segment length (bp)	Segment length (bp)	Segment length (bp)
1	3 900	3 314	3 954
2	3 220	3 038	2 926
3	2 750	2 663	2 772
4	2 020	2 033	2 011
5	1 750	1 894	1 638
6	1 570	1 639	1 769
7	1 080	1 137	1 156
8	1 080	1 137	1 124
9	1 080	1 137	1 046
10	710	693	822

Table 1.4. The conserved terminal sequences of the genome segments 10 of a number of different orbiviruses

Virus	Conserved terminal sequence at 5' end		Conserved terminal sequence at 3'end	Reference
	Position	Sequence[a]	Sequence	
AHSV	1-6 4	5' **GUU**UAA 3' A, U, or C	5' ACU**UAC** 3'	Mertens and Sangar, 1985; Van Staden and Huismans, 1991
BTV	1-6	5' **GUU**AAA 3'	5' ACU**UAC** 3'	Rao *et al.*, 1983; Mertens and Sangar, 1985; Gould, 1988; Roy, 1989; Van Staden and Huismans, 1991; De Mattos *et al.*, 1992b; Huismans *et al.*, 1992
BRDV	1-6	5' **GUA**AAA 3'	5' GGA**UAC** 3'	Moss *et al.*, 1992
EEV-1 and EEV-5	1-6	5' **GUU**WAD 3'	5' HSU**UAC** 3'	Potgieter *et al.*, 2002
Palyam virus	1-6	5' **GUU**AAA 3'	NA[b]	Van Staden and Huismans, 1991; Huismans *et al.*, 1992
St. Croix River virus	1-5	5' **GUA**AU 3'	5' CA**UAC** 3'	Attoui *et al.*, 2001

[a] Refer to Appendix A for description of single-letter codes

[b] Information not available is indicated by NA

Viruses belonging to the *Reoviridae* family have the interesting feature that all ten genomic dsRNA segments possess 5' and 3' terminal sequences which are conserved and genus specific. The

conserved terminal sequences of a number of different orbivirus genome segments 10 are shown in Table 1.4. The consensus terminal sequences of EEV Bryanston (EEV-1) and EEV Kyalami (EEV-5) have only recently been determined (Potgieter *et al.*, 2002). Orbiviruses additionally feature an inverted repeat which differs in sequence for each segment and which is present next to the conserved termini (Rao *et al.*, 1983; Roy, 1989; Nel *et al.*, 1990; Moss *et al.*, 1992). In AHSV it is a 7 bp inverted repeat adjacent to the terminal sequences (Van Staden and Huismans, 1991; Huismans *et al.*, 1992). No inverted repeats were however identified for EEV S10, although repeats have been identified in the genes that encode VP6 and VP7 (Potgieter *et al.*, 2002, refer to Table 1.5.). The conserved terminal features of the RNA segments of the viruses in the *Reoviridae* family are thought to play a role in transcription initiation, RNA replication, ribosome binding and translation of the mRNAs (Rao *et al.*, 1983), as well as in the determining of mRNA secondary structure, which could be important in the sorting and assembly of the genome during viral replication (Anzola *et al.*, 1987; Huismans *et al.*, 1992).

Table 1.5. Inverted terminal repeats of the cloned EEV Bryanston (EEV-1) gene encoding VP6 and EEV Kyalami (EEV-5) gene encoding VP7 (adapted from Potgieter *et al.*, 2002)

Gene	Inverted repeat	Position (bp)
VP6	AAATAACGTTC	4 and 1065
VP7	TTTGGCC	7 and 1161

1.4.3. The viral proteins

As seen in the previous section, each dsRNA segment encodes at least one viral polypeptide, with the exception of genome segment 10, which encodes two polypeptides. In this section the various viral proteins, structural proteins, as well as the non-structural proteins, will be discussed. The focus will however be on the non-structural proteins, specifically NS3.

In Table 1.6. the general coding assignment for the genome segments of BTV, as well as other information such as number of amino acids and predicted size of the encoded proteins, are indicated to serve as a framework for the discussion that will follow. The coding assignment of EEV has recently been studied (Potgieter *et al.*, 2002) and found to correspond to that of AHSV and BTV except for the fact that segments 7 and 8 are reversed encoding NS2 and VP7 respectively. The NS2 gene (segment 7) is also slightly larger than the VP7 gene (segment 8) (Potgieter *et al.*, 2002). In analogy to BTV, the protein(s) encoded by EEV genome segment 10 will be referred to as NS3/NS3A.

Table 1.6. Generalized coding assignments for the genome segments of BTV determined using 1% agarose gels. The number of amino acids, the predicted size (M_r) and function are as given by Roy, 1996.

Genome segment	Viral protein	Number of amino acids	Predicted size (Mr)	Type	Morphology/function
1	VP1	1302	149 588	Structural core protein	Part of core; RNA polymerase
2	VP2	956	111 112	Structural outer capsid protein	Part of outer capsid; serotype-specific antigen
3	VP3	901	103 344	Structural core protein	Part of core; structural protein
4	VP4	654	76 433	Structural core protein	Part of core; capping enzyme: guanyltransferase
5	NS1	552	64 445	Non-structural protein	Forms tubules
6	VP5	526	59 163	Structural outer capsid protein	Part of outer capsid; structural protein
7	VP7	349	38 548	Structural core protein	Part of core; group-specific structural protein
8	NS2	357	40 999	Non-structural protein	Phosphoprotein; binds ssRNA
9	VP6	328	35 750	Structural core protein	Part of core; binds ssRNA, dsRNA
10	NS3 NS3A	229	25 572	Non-structural protein	Glycoprotein; aids virus release

1.4.3.1. Structural proteins

Cryo-electron microscopy and computer image reconstruction techniques have been used as tools in the study of the architecturally complex virus particle of BTV and resulted in the proposal of a three dimensional model for BTV (reviewed by Roy, 1992). These studies indicated icosahedral symmetry with a triangulation number of 13 for the core. The core structure was shown to be divided into two concentric layers of protein, which enclose the inner core. The first layer, forming the outer surface of the core is made up of five or six-membered rings or clusters of VP7 trimers. The VP7 trimers form knob-like protrusions and are located at all the local and strict threefold axes. In between the VP7 structures are aqueous channels of which some penetrate the inner VP7 layer. The inner layer makes a smooth bed upon which VP7 trimers are located. The VP7 structures are the points for the deposition of the two surface proteins of this particle, VP2 and VP5. Under VP7 is a shell of VP3 molecules, which are arranged as 12 pentamers. The two layers enclose the inner core, which contains the three minor proteins, VP1, VP4 and VP6 and the viral genome.

Similar studies revealed that the structure of the outer capsid of the virus is unlike the fuzzy appearance revealed by earlier electron microscopic analysis of negatively stained virus particles, and that the proteins contained in the outer capsid have distinctive shapes. The VP5 protein, existing as a globular and almost spherical structure, sits in the channels formed by each of the six-membered rings of VP7 trimers. The VP2 protein exists as sail-shaped spikes, which project beyond the globular proteins and are located above the VP7 trimers. A cross-sectional diagram of the complete, assembled bluetongue virus particle, with the positions of the various structural proteins within the particle, is shown in Fig. 1.2.

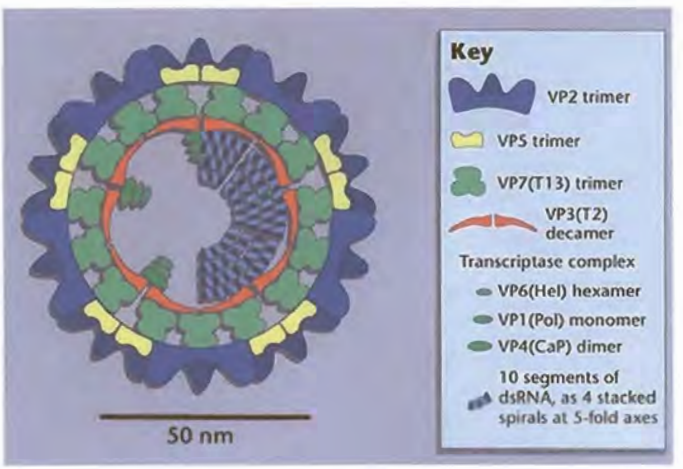

Figure 1.2. Cross-sectional diagram of the bluetongue virus particle showing the positions of the various proteins within the virus particle (Mertens, 2001)

The minor core proteins may be involved in virus replication and transcription processes. The largest of these proteins, VP1, is present in a low molar ratio in the core. Based on its size, and the presence of a motif common to all polymerases in the gene encoding this protein, it is the prime candidate for the virion RNA polymerase (Mertens *et al.*, 1984; Roy *et al.*, 1988; Huismans and Van Dijk, 1990; Roy, 1992; Vreede and Huismans, 1998). VP4 binds GTP and may act as a guanylyltransferase which catalyses the capping and methylation of the 5' end of viral mRNA (Roy, 1992; Roy *et al.*, 1994). The genome segment 4 of 5 different US BTVs have been analysed and a potential leucine zipper motif has been observed near the carboxy terminus of the deduced VP4 amino acid sequences (Huang *et al.*, 1993). A co-operative enzymatic function between VP1 and VP4 has been postulated involving RNA transcription and/or RNA replication (Huismans and Van Dijk, 1990). VP6, a highly basic protein and the smallest structural protein, is closely associated with the virus genome. This is based on the finding that it has a strong binding affinity for ss- and dsRNA species (Roy *et al.*, 1994). VP6 also binds ATP and exhibits RNA dependent ATPase activity and a helicase activity that catalyses the unwinding of dsRNA substrates prior to the synthesis of the mRNA (Roy, 1992). AHSV VP6 has a conserved motif in the primary sequence of the gene that is associated with a helicase (Turnbull *et al.*, 1996). VP6 may also aid or be responsible for the encapsidation of RNA (Roy, 1992).

VP3 and VP7 are the major constituents of the core particle which are associated in a highly ordered, three-dimensional manner resulting in icosahedral symmetry. Sequence data previously obtained for the VP3 gene, and deduced protein of BTV, AHSV and EHDV, demonstrates that of the four major capsid proteins, VP3 is the most conserved with between 57% to 58% amino acid identity (Iwata *et al.*, 1992). This high level of sequence conservation in the VP3 gene is probably due to the fact that the VP3 scaffold plays a major role in the structural integrity of the virus core. VP3 is hydrophobic and its size, as well as amino acid content, is similar for different orbiviruses e.g. AHSV-4, BTV-10 and EHDV (Iwata *et al.*, 1992). VP3 is also a group-specific antigen (Iwata *et al.*, 1992). VP3 has another function besides its structural function; it has been shown that it can bind ssRNA in a non-sequence specific manner, probably during the virus assembly process (Loudon and Roy, 1992).

The main component of the core, VP7, is highly conserved among serotypes and serogroups (Bremer *et al.*, 1990; Iwata *et al.*, 1992) and is the group specific antigen (Mertens *et al.*, 1984; Oldfield *et al.*, 1990; Chuma *et al.*, 1992). VP7 has been found to have limited exposure on the surface of the intact virion since a portion of the VP7 molecule is exposed to the surface (Roy, 1992). VP7 has at least two epitopes exposed on the virus surface (Lewis and Grubman, 1990) and Eaton *et al.* (1991) reported that the BTV serogroup reactive epitope in the amino terminal half of VP7 is accessible on the surface of BTV. VP7 exists as trimers in the virion, which appears as tripod-like structures with two distinct domains (Basak *et al.*, 1992; Grimes *et al.*, 1995, Basak *et al.*, 1996).

VP2 is the most surface exposed capsid protein of the orbiviruses and has been identified as the major serotype-specific neutralizing antigen (Huismans and Howell, 1973; Vreede and Huismans, 1994). VP2 is the most variable protein (Roy *et al.*, 1990) and as such exhibits low conservation between serotypes and serogroups (Iwata *et al.*, 1992; Vreede and Huismans, 1994). Regions involved in eliciting protective immune response and determination of specificity have not yet been fully identified. Studies with BTV have shown that VP2 is a haemagglutinin and is directly involved in attachment of virus to cells (Eaton and Crameri, 1989; Huismans and Van Dijk, 1990). There is also evidence that VP2 plays a role in the virulence of certain virus strains (Huismans and Howell, 1973; O'Hara *et al.*, 1998).

Little is known about VP5 relative to the other major capsid proteins. VP5 is more conserved than VP2 among serotypes, but not as conserved as the core proteins (Oldfield *et al.*, 1991). VP5 is also more conserved among serogroups including AHSV, BTV and EHDV, than VP2. It shows approximately the same level of conservation as the group specific antigen VP7 when compared between orbivirus serogroups (Iwata *et al.*, 1992). Although VP5 is located in the outer capsid, it is mostly unexposed or only partially exposed on the surface of the virion (Lewis and Grubman, 1990; Hewat *et al.*, 1992), and it does not appear to have any direct role in binding neutralizing antibodies. However, studies with BTV have demonstrated that VP5 enhances neutralization and protective immune response (Roy *et al*, 1990). The protein however, may also make some contribution to

virulence as was found in one case where the most obvious difference between a virulent and avirulent strain of BTV was in the mobility of the VP5 genome segment (Huismans and Howell, 1973). More recently, reassortment studies with BTV attributed increased neuro-invasiveness to VP5, an obvious virulence determinant (Carr *et al.*, 1994). Possibly an important role for the VP5 protein is to facilitate the interaction between the highly variable VP2 protein with the more highly constrained structure of the virus core, probably by imposing conformational constraints on VP2 (Cowley and Gorman, 1989).

The assembly of all seven structural proteins of BTV into virus-like particles has been demonstrated in the absence of viral genome or non-structural proteins, indicating that assembly is spontaneous requiring neither the genome nor the non-structural proteins (for review see Roy, 1992).

The structural proteins of EEV Cascara (EEV-2) have been characterized by comparison with BTV and AHSV using SDS-PAGE (Viljoen and Huismans, 1989). They identified seven structural proteins (VP1 to VP7 in analogy with BTV and AHSV) that ranged in molecular weight from 36 000 to 120 000. The protein fractionation pattern of EEV on polyacrylamide gels closely resembled that of AHSV and BTV, and showed a typical orbivirus profile with four major and three minor proteins. VP2 and VP5 of EEV was presumed to be the proteins in the outer capsid as they were lost or reduced in relative amount after centrifugation on caesium chloride density gradients, VP2 was also implicated as the possible serotype-specific antigen (Viljoen and Huismans, 1989). In a recent study by Potgieter *et al.* (2002) involving EEV Bryanston (EEV-1) and EEV Kyalami (EEV-5) the sizes of the genes encoding VP6 and VP7 were determined to be 1080 bp and 1175 bp respectively. This group also expressed proteins of EEV Kyalami (EEV-5) that were equivalent to the four major structural proteins of BTV namely VP2, VP3, VP5 and VP7, using the baculovirus expression system. Co-expression of the EEV Kyalami (EEV-5) VP3 and VP7 proteins resulted in the intracellular accumulation of particles they concluded to be EEV CLPs, since their size and morphology resembled that of native EEV cores and they had capsomeres typical of orbiviruses.

1.4.3.2. Non-structural proteins

At least three non-structural proteins (NS1 to NS3) have been identified (Gorman *et al.*, 1981; Mertens *et al.*, 1984). Van Dijk and Huismans (1988) and French *et al.* (1989) however reported that the NS3-encoding genome segment not only encodes NS3, but also a second, closely related polypeptide designated NS3A. Non-structural viral proteins are known to be involved in the replication and morphogenesis of viruses (specifically assembly and release) and are frequently responsible for the formation of characteristic structures in virus-infected cells. One of the non-structural proteins, NS3, has also been shown to influence virulence (O'Hara *et al.*, 1998). For BTV, the amino acid sequences of each of these NS proteins are highly conserved among different serotypes with an average homology of 96% (Huismans and Cloete, 1987; Huismans *et al.*, 1987a; Roy *et al.*, 1990).

Non-structural protein NS1:

Large quantities of NS1 are synthesized in orbivirus-infected (AHSV and BTV) cells, constituting approximately one quarter of the total virus-specified protein complement. Accumulation of NS1 in infected cells results in the formation of unique non-specific tubular structures (Mertens *et al.*, 1984; Nel *et al.*, 1990). The BTV NS1 protein is cysteine rich (Nel *et al.*, 1990) and NS1 has been found to be highly conserved within serogroups with strong regional conservation between serogroups (Huismans and Cloete, 1987; Nel and Huismans, 1991). Little is known about the function of NS1 tubules in BTV replication, it has been suggested that the tubules may be a repository of NS1 used in a prior stage of virus morphogenesis, and that NS1 may be involved in the translocation of virus particles to the host cell membrane (Hyatt *et al.*, 1993). This was supported by the demonstration of the association of NS1 with distinct areas of the VIBs especially at the periphery. These areas correlated with the presence of virus particles of all described size dimensions (Brookes *et al.*, 1993). This suggested that NS1 is involved in the early stages of BTV morphogenesis.

Non-structural protein NS2:

NS2 is also synthesized in large quantities and is responsible for the formation of virus inclusion bodies (VIBs) (Thomas *et al.*, 1990; Brookes *et al.*, 1993). NS2 has been shown to exist as multimers (Uitenweerde *et al.*, 1995) with ssRNA binding properties (Huismans *et al.*, 1987b; Theron *et al.*, 1994). This led to the proposal that NS2 may be involved in the selection and condensation of the RNA segments for encapsidation. The N-terminal half of NS2 is more conserved among BTV, AHSV and EHDV than the C-terminal half which has a hydrophilic character and is predicted to have a high content of α-helix conformation (Van Staden *et al.*, 1991). The N-terminal half of the three orbiviruses, as well as the ssRNA-binding proteins σ_{NS} of three reovirus serotypes and NS34 of bovine and simian rotavirus, contain a conserved 9 amino acid region. This, together with experiments where deletions of the N-terminal portion of the protein where found to abolish RNA binding activity (Zhao *et al.*, 1994), point to the importance of the N-terminus for NS2 function. NS2 has the unique property of being the only virus-specific phosphoprotein in BTV and AHSV-infected cells (Huismans *et al.*, 1987b; Theron *et al.*, 1994) The functional significance of NS2 phosphorylation is not yet known. Theron *et al.* (1994) reported that phosphorylation of NS2 of BTV, EHDV and AHSV affects ssRNA binding and may involve a ubiquitous cellular kinase.

Non-structural protein NS3/NS3A:

Unlike NS1 and NS2, the two closely related non-structural proteins NS3 and NS3A are synthesized in small amounts in orbivirus-infected cells (Huismans *et al.*, 1979, Van Dijk and Huismans, 1988; French *et al.*, 1989, Van Staden *et al.*, 1995). Both these proteins are encoded by genome segment 10. The two related protein products have been shown to be synthesized from two alternative in-phase initiation codons in the same open reading frame (Mertens *et al.*, 1984; Lee and Roy, 1986; Gould, 1988; Van Dijk and Huismans, 1988; French *et al*, 1989; Van Staden and Huismans, 1991;

Hwang *et al.*, 1992). The BTV counterparts have subsequently been identified in AHSV-infected cells; these proteins were designated NS3 and NS3A in accordance with BTV nomenclature by Van Staden and Huismans (1991), but they have also been described as NS4 and NS4a (Grubman and Lewis, 1992), and P21 and P20 (Laviada *et al.*, 1993). The nucleotide and amino acid sequences of the NS3 genes of all 9 AHSV serotypes have been determined (Sailleau *et al.*, 1997), following initial work by Van Staden and Huismans (1991) on S10 of AHSV-3 and AHSV-9, and De Sá *et al.* (1994) on S10 of AHSV-1, AHSV-4 and AHSV-8. Sequence data on the NS3 genes of a number of AHSV serotypes indicate that these genes are not as conserved as the cognate genes within the BTV serogroup (Hwang *et al.*, 1992; De Sá *et al.*, 1994). However, in all the BTV and AHSV NS3 genes investigated to date, two in-phase initiation codons are conserved in a suboptimal context for translation initiation, possibly explaining the low levels of expression of NS3 and NS3A (Gould, 1988; Van Staden *et al.*, 1995).

NS3 and NS3A proteins have characteristic features that are conserved amongst most orbiviruses (Lee and Roy 1986; Gould, 1988, Van Staden and Huismans, 1991; Hwang *et al.*, 1992; Moss *et al.*, 1992, Jensen *et al.*, 1994; Van Staden *et al.*, 1995; Yamakawa *et al.*, 1999). Analysis of the predicted amino acid sequence of the NS3 gene product of AHSV revealed two conserved hydrophobic regions in the C-terminal half of the proteins (residues 116 to 137 and 154 to 170), which may serve as transmembrane domains. Predicted protein sequence analysis of the NS3 gene products of the different BTV and AHSV serotypes has also revealed two N-linked glycosylation sites and a cluster of proline residues. In the case of AHSV, a cluster of five prolines is observed within the 13 amino acid region between residues 22 to 34, and in the case of BTV a cluster of six prolines within the 15 amino acid region between residues 36 to 50 has been observed. Alignment of the predicted amino acid sequences of NS3 of five different orbiviruses, BTV, AHSV, Palyam virus, Broadhaven virus and EHDV also revealed a 50 amino acid conserved region in the N-terminal half of the proteins between residues 43 to 92.

Models have been suggested for the membrane topology of a variety of orbivirus NS3 proteins. Van Staden *et al.* (1995) suggested a model for the membrane topology of AHSV NS3 that predicts that each of the two hydrophobic regions (HDI and HDII) of the protein spans the host cell membrane, resulting in the localization of both the N- and C-termini of NS3 on the cytoplasmic side of the membrane (Fig. 1.3.). This leaves a large part of the protein available for interactions with components of virus particles and the N-terminal domain may mediate the transport and egress of the virion from infected cells in the final stages of virus morphogenesis (Yamakawa *et al.*, 1999). Similar membrane topology models have also been predicted for the NS3 proteins of BTV, Chuzan virus, Palyam virus and Broadhaven virus (Van Staden and Huismans, 1991; Jensen and Wilson, 1995; Bansal *et al.*, 1998; Yamakawa *et al.*, 1999) confirming a conserved role for this protein amongst all orbiviruses.

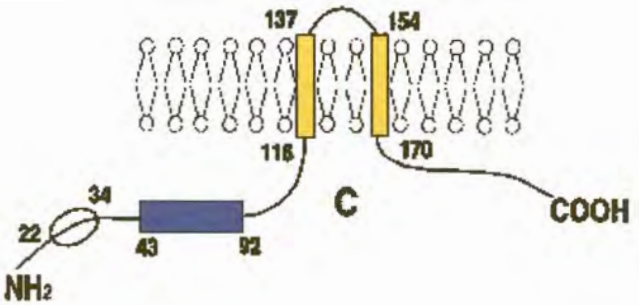

Figure 1.3. Model proposed for the membrane-associated topology of the AHSV NS3 protein. The yellow boxes represent membrane-spanning regions while the blue box depicts the 50 amino acid region, which is conserved amongst the different orbiviruses. The oval area illustrates a cluster of conserved proline residues (C = cytoplasm). The numbers refer to amino acid residues in AHSV-3 NS3 (adapted from Van Staden *et al.*, 1995)

A recent study by Beaton *et al.* (2002) has confirmed the role played by NS3 to mediate viral release. This group showed that NS3 interacts with a cellular protein p11, which is the light chain component of the annexin II complex, in which two heavy chains (p36) and two light chains (p11) interact to form the functional tetramer that is important for many cellular processes, including Ca^{2+}-dependant exocytosis and the correct trafficking of proteins out of the cell. They mapped the NS3 region of interaction with p11 to a 13-residue peptide found at the N-terminus of the protein and showed that it effectively competes with p36 for p11 ligand binding. This 13-residue peptide (the 13 residues between the initiation codon of NS3 and the initiation codon of NS3A) contains a putative amphiphatic α-helix and has been found to be highly conserved amongst various BTV serotypes, indicating its importance in the virus life cycle. They further showed that the C-terminal domain of NS3 interacts with VP2, the outermost protein of the fully assembled virus particle, thus suggesting that NS3 forms a bridging molecule that draws assembled virus into contact with the cellular export machinery, allowing orbivirus egress from infected cells. The exact method of virus release has however not been elucidated, but it is possible that the link between p11 and virus, via NS3, allows active transport of virus across the membrane. This suggests that NS3 may be analogous to rotavirus NSP4, which has been shown to bind cores and transport them across internal membranes to form mature enveloped virions (refer to section 1.5.7.). It can also be speculated, due to the fact that the region involved in the binding of p11, is found in NS3 and not in NS3A, that the synthesis of both types of proteins could be a form of regulation of the process of viral release.

A different model to that shown in Fig. 1.3. has however been suggested for the membrane topology of EHDV NS3. The first helix at position 115 to 135 (which is homologous to the 116 to 137 region of NS3 of AHSV) of the NS3 protein of EHDV-1 is predicted to be hydrophilic on one side and hydrophobic on the other, and would be expected to lie on the surface of the membrane, with the hydrophobic side touching the membrane. The second helix at position 158 to 178 is also predicted to be hydrophilic on the one side, but less so than the first, and there is a greater likelihood that this helix

inserts itself across the membrane rather than residing on top of it. This second helix was also predicted to be multimeric, i.e. associated with another transmembrane segment through use of a single cysteine residue at position 168 that forms a disulphide bridge with the cysteine residue of another transmembrane segment to form either a pair or a bundle. This would result in the combined helices forming a channel, stabilized by the disulphide bridge, where the non-polar sides were used for mutual association of the helices to each other (Jensen and Wilson, 1995). There is however no biochemical evidence for the dimerization of the NS3 protein of EHDV-1. The first membrane associated segment of EHDV-1 NS3 is consistent with the consensus sequence for a signal peptide. It has a highly positive charge (lysine, arginine, arginine, arginine) in the four residues preceding the hydrophobic region, followed by a slightly long hydrophobic region of 16 residues, and a strongly polar stretch of seven residues distally (serine, alanine, serine, threonine, leucine, threonine, serine). The first membrane associated region, although not likely to be stably integrated into the membrane, may function as an insertion signal peptide that is translocated into the ER lumen and lies buried in the lipid bilayer surface, while the second hydrophobic domain functions as a transmembrane segment, which then anchors NS3 as an integral membrane protein. Insertion signal peptides also often have a cleavage site for removal of the signal peptide after insertion of the protein in the membrane, the consensus sequence for a cleavage site was however not seen in the EHDV-1 NS3 sequence (Jensen and Wilson, 1995).

When a polypeptide is inserted into a membrane, either the amino or carboxy terminus is retained on the cytoplasmic side of the membrane. One indicator for determining this orientation is the presence of glycosylated residues, which are found on the luminal side of membranes. EHDV-1 NS3 has potential O- and N-linked glycosylation sites along its amino terminal end, this implies that the glycosylated amino terminus of EHDV-1 resides on the luminal side of the membrane while the carboxy terminus is free on the cytoplasmic side, having a complex extended-turn-helix conformation (Jensen and Wilson, 1995). All of the above information is used to predict the model of the membrane topology of EHDV-1 NS3 which is shown in Fig.1.4.

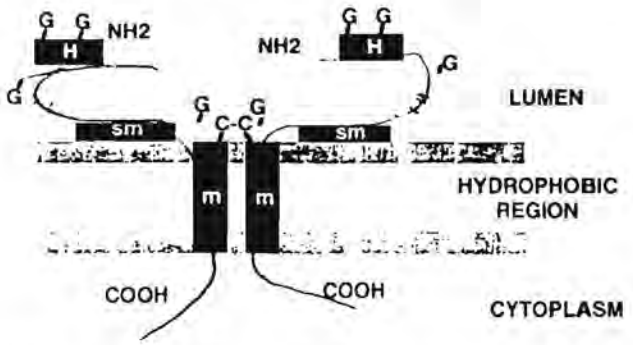

Figure 1.4. Model of the EHDV-1 NS3 protein inserted across the membrane. m = transmembrane segment; sm = surface membrane segment; H = hydrophobic region; G = potential glycosylation sites; C = cysteine residues; NH2 = amino terminus of protein; COOH = carboxy terminus of protein (adapted from Jensen and Wilson, 1995).

The 116 to 137 region in AHSV NS3 showed only 45% identity between the 9 AHSV serotypes (Sailleau *et al.*, 1997). The degree of variation in this region could be explained by the fact that this region would not be a complete transmembranous helix, as predicted by Van Staden *et al.* (1995), but would interact with the cellular membrane according to the membrane topology of the NS3 protein of EHDV (Wilson and Jensen, 1995; Sailleau *et al.*, 1997). Van Niekerk *et al.* (2001a) did not find any evidence to exclude any one of the two possible membrane topology models, it was however evident that only a very specific membrane-associated conformation of AHSV NS3, dependent on the structure of both hydrophobic domains, makes NS3 cytotoxic to host cells.

In BTV infected cells, the NS3 protein was found to be associated with intracellular smooth-surfaced vesicles (ER and Golgi) as well as with areas of the plasma membrane that had been disrupted by the egress of BTV (Hyatt *et al.*, 1989; Hyatt *et al.*, 1991). Similar results were obtained with a vaccinia virus expressing NS3, indicating that the localization of NS3 does not depend on the presence of BTV particles (Hyatt *et al.*, 1991). The exact role of NS3 and NS3A during virus replication is not clear, but co-expression of BTV structural proteins and NS3 by recombinant baculoviruses resulted in the release of BTV-like particles from infected insect cells (Hyatt *et al.*, 1993). These findings seem to indicate a role for BTV NS3 in the final stages of viral morphogenesis by enabling the release of progeny viruses from infected cells. Less information is available on the role of AHSV NS3 in the viral replication cycle, although results indicate a function similar to that of BTV NS3. AHSV NS3 has been shown to be membrane-associated and to be associated with areas of virus release involving both budding and extrusion (Van Staden *et al.*, 1995; Stoltz *et al.*, 1996). This membrane association of AHSV NS3 could result in a possible alteration of membrane permeability, caused by the insertion of the protein in the membrane, leading to osmotic disregulation and the eventual death and disruption of cells (Van Staden *et al.*, 1995; Stoltz *et al.*, 1996). This membrane destabilizing effect has also been detected in the disrupted plasma membranes of cells infected with an NS3 recombinant baculovirus (Stoltz *et al.*, 1996). The regulation of membrane permeability by NS3 could also lead to the control of the biochemical homeostasis of infected cells. NS3 may thus play a key role in both the viral pathogenesis and the viral release processes that regulate the disease progression of AHS (Van Niekerk *et al.*, 2001a).

The synthesis of AHSV NS3 in infected cells has been studied (Van Staden *et al.*, 1995). When the time and level of NS3 and NS3A expression in AHSV-3 infected Vero cells are monitored, both NS3 and NS3A are synthesized from 13 hours p.i. onward, and are present in approximately equimolar amounts, differing from the results obtained for BTV in which NS3 is the predominant protein product with nearly no NS3A being synthesized. This apparent aberration may find an explanation in the study of mRNA secondary structure, since two stable stem-loop structures, which can be formed on either side of the BTV NS3A initiation codon, may lead to disproportional amounts of NS3 and NS3A being expressed in BTV infected cells (Hwang *et al.*, 1992). The absence of similar structures in AHSV

mRNA possibly results in an equivalent frequency of initiation at either of the two AUGs, thereby producing equimolar amounts of NS3 and NS3A (Van Staden *et al.*, 1995).

When the AHSV NS3 gene is expressed using a recombinant baculovirus, low levels of a single product (NS3) of approximately 25 kDa are detected. No NS3A (24 kDa) is synthesized from the second initiation codon (Van Staden *et al.*, 1995) and it has been found in other studies that some membrane proteins are, for unknown reasons, not expressed to high levels by baculoviruses (King and Possee, 1992). It was further found that NS3 mRNA and protein were only synthesized during a relatively short period, after which time a drastic decline in the number of viable cells occurred (Van Staden *et al.*, 1995). Thus, the low levels of protein expression are due primarily to an apparent cytotoxic effect on the insect cells, and not because of a continued low level of expression during the viral infection cycle, although this may be a contributing factor. Electron microscopic examination of the morphology of cells expressing NS3 at late stages of infection revealed these cells to be completely permeabilized or disrupted (Van Staden *et al.*, 1995; Stoltz *et al.*, 1996), while immunofluorescence indicated a possible membrane association for NS3.

Immunofluorescence studies with a baculovirus-expressed BTV NS3 protein indicated that BTV NS3 and NS3A exist as N-linked glycoproteins and that they are transported to the cell membrane of infected cells via the endoplasmic reticulum (ER) and Golgi apparatus (Wu *et al.*, 1992). In another study, glycosylation was shown to be necessary for the correct processing of NS3 (Bansal *et al.*, 1998). NS3 and NS3A of six BTV serotypes all have two conserved glycosylation sites (Hwang *et al.*, 1992), while in contrast NS3 and NS3A of AHSV serotypes 4, 5, 6 and 7 (Sailleau *et al.*, 1997) have one potential glycosylation site but not in a conserved position, and the AHSV-3 NS3 protein does not contain such a site. Furthermore, no experimental evidence for the glycosylation of AHSV-3, 4 or AHSV-9 NS3 has been found (Grubman and Lewis, 1992; Van Staden *et al.*, 1995). The differences in the glycosylation of BTV and AHSV NS3 would not necessarily influence their predicted similar function in viral morphogenesis, as the requirement for carbohydrates for the transport of membrane proteins is not universal, and is protein specific (Doms *et al.*, 1993).

1.5. STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF MEMBRANE PROTEINS

Studies have indicated that BTV NS3, AHSV NS3 and Chuzan virus NS3 are all integral membrane proteins (Wu *et al.*, 1992; Yamakawa *et al.*, 1999; Van Niekerk *et al.*, 2001a). These proteins all have certain structural features in common, for e.g. two hydrophobic regions that are predicted to form transmembrane helices. For this reason, a brief discussion of membrane proteins, specifically integral membrane proteins, and their structures and functions will follow. The information in sections 1.5.1., 1.5.2. and 1.5.3. was obtained from Lodish *et al.* (1995); the information in subsequent sections was obtained from the references as indicated.

Membrane proteins are proteins that are associated with, or incorporated into, biological membranes. The ensemble of proteins associated with a membrane is a function of the cell and membrane type involved and the subcellular localization of the protein itself, which is determined by its function. Membrane proteins are usually found in only small amounts in cells, which makes the study of their structure and membrane interaction difficult. New technology and tools, notably bioinformatics, has made it possible to predict the structure of a protein from its amino acid sequence, which allows the prediction of its function, including its interaction with the membrane.

Membrane proteins can interact with membranes in various ways and can be classified into two broad categories based on the type of interaction: integral membrane proteins or peripheral membrane proteins.

1.5.1. Integral membrane proteins

Integral membrane proteins require the disruption of the lipid bilayer for release from the membrane. They contain one or more peptide segments that are embedded in the phospholipid bilayer itself, most integral proteins also contain residues with hydrophobic side chains that allow them to interact with the fatty acyl groups of the membrane phospholipids, thus effectively anchoring the protein within the membrane bilayer. Virtually all the integral membrane proteins characterized thus far have been transmembrane proteins, which span the phospholipid bilayer. These proteins may contain one or more membrane spanning regions (membrane-spanning α-helices), from a few residues up to several hundred residues in length, the bulk of which may extend into the aqueous medium bordering each side of the membrane. In some cases these multiple helices can interact with each other to form a coiled-coil structure stabilized by specific interactions between the amino acid side chains present at the interface between the two helices (Cohen and Parry, 1986). In contrast, some integral membrane proteins are anchored in only one of the two layers of the lipid bilayer, these proteins contain covalently bound hydrocarbon chains that function to anchor them in the bilayer. These membrane proteins are grouped into three classes according to the type of attached hydrocarbon chain. The first class is the glycosylphosphatidylinositol-anchored proteins, the second class is the farnesyl-anchored proteins and the third is the myristate-anchored proteins. The proteins of the third class are found within the cytoplasm, anchored to the cytosolic face of the plasma membrane by myristic acid, a 14-carbon saturated fatty acid. Myristate is always bound by an amide linkage to the glycine residue found at the N-terminus of such proteins.

1.5.2. Glycosylation

Carbohydrates are found associated with many of the known membrane proteins and the carbohydrate components of integral membrane proteins are almost always linked to the exoplasmic

domains of these proteins. When carbohydrates are covalently bound to proteins, the proteins become part of a class of proteins termed glycoproteins, which are especially abundant in the eukaryotic cell membrane and have also been adopted by virus systems to enhance their interaction with these cells. Carbohydrates have the function of increasing the hydrophilic character of proteins thereby increasing their solubility in aqueous medium, and may assist in the stabilization of protein conformations necessary for membrane association, protein folding and activity. The carbohydrate component of proteins are also necessary to ensure the correct integration of the protein in the membrane, as each type of membrane protein has a single, specific orientation with respect to the cytosolic and exoplasmic surfaces of the cell and is orientated accordingly during its synthesis.

Carbohydrate residues present in glycoproteins are linked to two classes of amino acid residues; sugars are O-linked if they are bound to the hydroxyl oxygen atom of serine, threonine or hydroxylysine, and N-linked when attached to the amide nitrogen of asparagine. The structures of O-linked and N-linked oligosaccharides are very different and most often contain different classes of sugars. N-linked oligosaccharides are generally longer and less varied than the O-linked components and often contain more sugar residues than is the case with the O-linked structures. N-linked glycans are often crucial to ensure the correct export of proteins from the endoplasmic reticulum and many proteins that are secreted from the cell are also glycoproteins. It is interesting to note that even oligosaccharides found at the same locus in a single protein type may frequently differ and the requirement for carbohydrates in the transport of membrane or secretory proteins is not universal and is highly protein specific.

1.5.3. Protein targeting and sorting

Protein targeting and sorting is of critical importance in determining the correct site of protein activity and function. Integral membrane proteins are part of the secretory pathway.

In order to be incorporated into a membraneous structure, the relevant proteins must be translated on membrane-bound ribosomes attached to the rough endoplasmic reticulum. Sequences up to 25 amino acids in length contained within the membrane-targeted protein called topogenic sequences, function to ensure that each transmembrane protein acquires its proper orientation during its insertion into the ER membrane during translation. These proteins then move, via transport vesicles, from the ER to their respective final destinations, which may be as diverse as the plasma membrane or the membranes of the lysosomal or Golgi apparatus. It is important to note that the subcellular targeting of viral (and cellular) membrane proteins are not exclusively determined by the sequences of the transmembrane regions. Retention sequences or transport sequences in the luminal and/or cytoplasmic domains of the protein, including glycosylation, myristylation or multimerization may further influence targeting. Specific mechanisms also operate to recognize and retain incorrectly folded proteins in the ER (Doms *et al.*, 1993).

It has also been found that plasma membrane glycoproteins follow the same maturation pathway as secretory proteins and it has been established that the newly synthesized glycoproteins are inserted into the rough ER membrane usually by way of a signal peptide translated early in the life of the targeted protein. The signal peptide is usually located at the N-terminal of the protein; it is 20 to 40 amino acids in length and contains a stretch of 9 hydrophobic amino acids flanked by basic residues on the N-terminal side. This signal peptide is recognized by a signal recognition particle, which directs the nascent protein to the rough ER. These proteins then move to the Golgi vesicles, targeting them to the plasma membrane. In addition to the signal peptide, an additional peptide signal consisting of a membrane spanning hydrophobic peptide is also needed to target a protein to the plasma membrane.

1.5.4. Integral viral membrane proteins

Virus particles enveloped in a membraneous structure usually contain one or more types of integral membrane proteins. These proteins have functions as diverse as recognizing receptors on the host cell plasma membrane, mediating penetration of host cells, co-ordinating viral morphogenesis at the budding site and serving as receptor-destroying enzymes necessary for viroid release. The majority of these proteins are also important antigens for the induction of neutralizing antibodies (Doms *et al.*, 1993).

Numerous viral integral membrane proteins have a single membrane-spanning domain. The largest parts of these proteins are usually found on the outside of the viral envelope. The cytoplasmic domains are usually less elaborate, but are nonetheless important in interactions with internal viral components. Nearly all viral membrane proteins examined to date have been found to be oligomeric structures, with some being homo-oligomers containing two to four identical subunits, while others are hetero-oligomers containing two or more different types of subunits. Usually, discrete genes encode the latter subunits, with each subunit consisting of either a single or double polypeptide chain, these being stabilized by non-covalent interactions or disulphide bonds. Interactions between adjacent subunits are extensive and extend throughout the length of the ectodomains (Doms *et al.*, 1993). The majority of these viral integral membrane proteins are also (just as is the case with normal cellular transmembrane proteins) post-translationally modified by the addition of N- or O-linked carbohydrate chains. The majority of the previously mentioned hydrophobic and disulphide bonds are generally hidden inside the folded subunits or subunit interfaces, while the N-linked oligosaccharides are found on the external surfaces (Doms *et al.*, 1993).

1.5.5. Folding of integral viral membrane proteins

Like most cellular glycoproteins, viral membrane proteins are typically translated on ribosomes bound to the membrane of the ER. These proteins are inserted co-translationally into the ER in an unfolded

form. Due to their being transmembrane proteins, their folding must also occur in three distinct environments: the ER lumen, the ER membrane and the cytosol. The large ectodomain which usually carries most of the protein's mass and all of the carbohydrate moieties and disulphide bonds, undergoes folding in the lumen of the ER, with the mechanisms resembling those associated with the secretory proteins and membrane glycoproteins of the cell. The transmembrane area(s) most likely adopt a α-helical structure, thereby anchoring the protein into the hydrophobic interior of the bilayer. The area, which is to become the cytoplasmic domain, generally follows the rules that apply for folding of cytosolic proteins (Doms *et al.*, 1993).

Folding of viral membrane proteins within the ER is not a spontaneous process, but is dependent on the presence of chaperone molecules and folding enzymes which are necessary to prevent the aggregation of proteins in the process of being translated, and to catalyse rate limiting steps such as the formation of disulphide bonds. This greatly increases the efficiency of the folding process and is especially important for the large, complex molecules, which sometimes constitute viral membrane proteins. However, the most important principle regarding protein folding is that the information required for a protein to attain its final three-dimensional structure resides in its primary amino acid sequence (Doms *et al.*, 1993). It is thus clear that folding of viral transmembrane proteins is a dynamic process involving a host of cellular folding enzymes and molecular chaperones, much the same as for cellular membrane proteins.

1.5.6. Integral viral membrane protein structures and functions

Various virus-encoded proteins have been identified which have transmembrane structures similar to those in eukaryotic transmembrane proteins. While showing structural similarity in the form of transmembrane spanning helices, coiled-coil structures and other elements found in membrane proteins of higher organisms, many of the models elucidated in the past few years indicate specific roles for these proteins in promoting successful virus attachment, disassembly, morphogenesis and release of virus particles. One of the more extensively characterized examples will be discussed, namely rotavirus NS28/NSP4 non-structural protein, with which BTV NS3 also share a number of similarities.

1.5.7. Rotavirus non-structural glycoprotein NSP4

Rotaviruses are non-enveloped and mature by budding through the rough (R) ER of infected cells prior to the final assembly of the outer capsid (Estes and Cohen, 1989). Subviral single-shelled particles (SSP) assemble in viral inclusion bodies bordering the RER membrane and subsequently bud into the lumen of the RER (Bellamy and Both, 1990). This event is mediated by the binding of VP6, a protein present on the SSPs, with a virus-encoded receptor, which is translocated prior to the

budding event to the RER membrane. Au *et al.* (1989) and Meyer *et al.* (1989) have shown that this receptor is the rotavirus non-structural protein NS28, which has subsequently been reclassified as non-structural protein NSP4.

NSP4 is a 28 K non-structural glycoprotein, classified as an integral membrane protein (specifically a transmembrane protein) in the ER membrane (Chan *et al.*, 1988; Au *et al.*, 1989; Estes and Cohen, 1989). This protein is encoded by genomic segment 10, is 175 amino acids in length (Chan *et al.*, 1988; Au *et al.*, 1989; Meyer *et al.*, 1989) and has three internal hydrophobic domains (H1, H2 and H3). The membrane topology of simian rotavirus non-structural glycoprotein NS28 (NSP4) has been determined by Chan *et al.* (1988) and is shown in Fig. 1.5. The amino terminus spans the membrane or is located on its lumenal side while the hydrophilic carboxyl half is exposed on the cytoplasmic side of the ER membrane. The hydrophobic region between position 7 to 21 (H1) was not predicted to be membrane-associated, the hydrophobic region between position 28 to 47 (H2) was suggested to be monomeric and a transmembrane region, and the third hydrophobic domain position 71 to 81 (H3) was predicted to be multimeric and a transmembrane region.

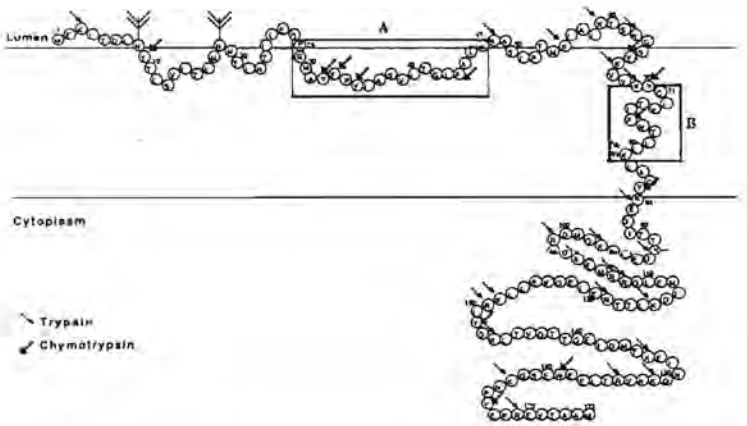

Figure 1.5. Proposed topology of NS28 (NSP4) in the ER membrane. Each amino acid is represented by one circle and the one-letter code. The carbohydrates on amino acids 8 and 18 are represented by Y. The small arrows pointing to the right indicate the possible trypsin cleavage sites, whereas the large arrows pointing to the left indicate the chymotrypsin cleavage sites. The boxed areas A and B show the regions predicted by analysis of hydrophobic moment plots to be transmembrane or membrane associated (adapted from Chan *et al.*, 1988).

Subsequent studies proposed that the second hydrophobic domain is the membrane spanning domain that anchors the protein, instead of the third domain as previously proposed (Estes and Cohen, 1989), a larger cytoplasmic domain is also suggested by this subsequent model. This means that the protein is orientated in the RER membrane with the amino terminus on the luminal side and approximately 131 amino acids accessible from the cytoplasmic side (Meyer *et al.*, 1989).

The three hydrophobic domains have specific functions within this membrane topology, based on their positions. The H2 domain contains a signal peptide and acts as the transmembrane domain leaving the C-terminal 131 residues exposed on the cytoplasmic side of the membrane and available to perform the receptor function which involves the binding of SSPs (Au *et al.*, 1989). H3 remains exposed in the C-terminal 131 residues on the cytoplasmic side of the membrane and allows the binding of the structural protein on the SSPs by orientating a C-terminal residue which is required to bind the structural protein (Taylor *et al.*, 1992). H1 is luminally orientated and includes two glycosylation sites at asparagine residues at amino acid positions 8 and 18 near the amino terminus, which are glycosylated with high mannose sugars (Chan *et al.*, 1988; Estes and Cohen, 1989).

The location of the glycosylation sites suggests that the signal peptide is not cleaved (Chan *et al.*, 1988). This NSP4 receptor protein is a homotetramer, its oligomerization is mediated by a coiled-coil domain that has been identified in the cytoplasmic tail, immediately adjacent to its membrane spanning region (Taylor *et al.*, 1996) and modification of its C-terminus has been shown to abolish SSP-binding (Au *et al.*, 1989; Taylor *et al.*, 1992). The budding event results in the inclusion of the virus in a transient envelope, but the mechanism whereby this is accomplished is poorly understood. Following translocation to the cell membrane, the membrane envelope containing NSP4 is removed and two structural proteins, VP4 and VP7, interact to form the outer virus capsid (Poruchynsky and Atkinson, 1991). It is not understood how the membrane envelope containing NSP4 is removed. However, it is likely that NSP4 itself is also involved in this process, and it has been shown that glycosylation of the protein is required for removal of the transient envelope from budding particles (Estes and Cohen, 1989). Non-enveloped particles are subsequently released from infected cells by cell lysis and cell death (Poruchynsky and Atkinson, 1991).

The region 114 to 135 of NSP4 has been predicted to fold as an amphipathic helix, to localize in the cytoplasmic domain and to be able to mobilize intracellular calcium in eukaryotic cells. A tyrosine residue at position 131 was subsequently shown to be important in the functionality of the protein (Ball *et al.*, 1996). This tyrosine-based motif in the cytoplasmic domain of membrane associated glycoproteins appears to be of critical value for other virus systems as well, such as the HIV-1 Env protein, as well as the alphavirus envelope protein (Zhao *et al.*, 1994). The 114 to 135 region also proved to be antigenic, NSP4 antibodies directed against the region showed the potential to block rotavirus-induced disease (Ball *et al.*, 1996).

Other studies indicated that NSP4 is cytotoxic to insect cells and it is hypothesized that the cytotoxic effect of this protein might be due to the sudden increase of intracellular calcium levels, leading to membrane destabilization and altered membrane permeability which causes the rupture of the cells and the subsequent release of rotavirus particles from the cell during late stages of the viral replication cycle (Tian *et al.*, 1994; Van Niekerk *et al.*, 2001a).

More recently, Newton *et al.* (1997) have delivered proof which indicates that NSP4 alters membrane permeability in cultured mammalian epithelial cells when expressed in a dual recombinant vaccinia virus system which mimics the approximate level of NSP4 synthesis as found in rotavirus-infected cells. Paradoxically, their work shows that the region of NSP4 necessary to effect this cytotoxic effect is not the region corresponding to residues 114 to 135 of the protein as was speculated by Ball *et al.* (1996) and Tian *et al.* (1996). Rather, they have shown by mutational analysis that it is an N-terminal region between amino acids 54 and 75 which causes the membrane destabilization activity as described. This region is predicted to form a protease accessible loop, which has the ability to form a helical structure with a pronounced amphipathic character. Cationic amphipathic helices are distinct motifs in lytic viral proteins, and cause permeabilization of cellular membranes. After having modified two clones with mutations overlapping the 54 to 75 residue sequence, Newton *et al.* (1997) found a significant reduction in the intrinsic membrane destabilizing activity of these mutants, indicating that this amphipathic α-helix represents a critical functional domain.

Zhang *et al.* (1998) recently sequenced two pairs of virulent and attenuated porcine rotaviruses. Comparisons made between the two pairs revealed that structural changes within a 10 amino acid region (amino acids 131 to 140) were important for pathogenesis. Their studies indicated that the 131 to 140 amino acid region, like the amino acid 54 to 75 region mentioned above, is also a domain critical to the functioning of NSP4.

1.6. AIMS OF THIS STUDY

From the review of the literature, it is clear that a vast amount of information has been gathered about the *Orbivirus* genus in the family *Reoviridae*. Most of the information however pertains to BTV and AHSV, while less is known about other viruses such as EHDV. Many of the details regarding structural characteristics, the process of viral replication, morphogenesis and assembly still need to be elucidated for other members of the family and this specifically includes the role of the viral non-structural proteins.

Over the past few years, equine encephalosis has emerged as an important disease of horses and other equids; a recent study by Lord *et al.* (2002) has indicated that in most parts of South Africa EEV has a higher transmission rate than AHSV (at least in donkeys), and from the literature review it is clear that little is known about the structural characteristics of EEV, specifically regarding its structural and non-structural proteins. To date, only two studies have been carried out regarding the molecular characterization of EEV. The first dates back to 1989 and involved the Cascara serotype (EEV-2) (Viljoen and Huismans, 1989). At that time Cascara (EEV-2) was still regarded as the predominant serotype and thus the representative of the EEV group; at present, the Bryanston serotype (EEV-1) has replaced Cascara as both the dominant serotype and as the representative of the group. The

second study is a very recent study and involves EEV Bryanston (EEV-1) and EEV Kyalami (EEV-5). Information regarding serotypes other than Cascara (EEV-2), EEV Bryanston (EEV-1) and EEV Kyalami (EEV-5), and information specifically regarding genome segment 10 and the gene product(s) it encodes, is lacking.

For these reasons it has become important to increase our knowledge of the virus. Investigations, such as that by Van Niekerk *et al.* (2001b), have shown that AHSV NS3, due to the large variation found in the protein, has potential for use as an epidemiological marker to subtype AHSV populations within an outbreak of AHS in order to trace the origin of the virus involved in the outbreak. NS3 of AHSV, BTV and EHDV has also been shown to play an important role in the release of virus particles from a cell and it also may be involved in virulence. Many other studies on the NS3 gene and its gene product(s) have also been undertaken in the case of BTV, AHSV, EHDV, and Palyam virus.

The long-term aim regarding these studies is to elucidate the structure/function relationship of NS3 and specifically its role in viral infection and morphogenesis, with emphasis on the mechanism responsible for viral virulence. Within that context, the aim of this study was to characterize the EEV NS3 gene and gene product(s) and to compare them to their orbivirus counterparts in an attempt to identify conserved and/or unique characteristics.

In order to achieve these aims, the following short-term aims were identified as part of this study:

1. To determine the NS3 sequences of the different EEV serotypes in order to identify unique structural features, and to compare the observed structural features and genetic variation within this serogroup to that observed for other orbiviruses (Chapter 2)

2. To determine if the expression of the EEV NS3 gene results in two overlapping gene products (NS3 and NS3A) and to establish a system to express NS3 of EEV in sufficient quantities for future functional studies (Chapter 3)